ADHESION, GROWTH AND OSTEOGENIC DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS ON POSITIVELY AND NEGATIVELY CHARGED AND UNCHARGED FERROELECTRIC CRYSTAL SURFACES

Marta Vandrovcova[1], Lucie Bacakova[1]*, Premysl Vanek[2], Jan Petzelt[2]

[1] Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, CZ-14220 Prague, Czech Republic
[2] Institute of Physics of the Czech Academy of Sciences, Na Slovance 2, CZ-18221 Prague, Czech Republic
*e-mail: Lucie.Bacakova@fgu.cas.cz

Abstract

The cell-material interaction is significantly influenced by the physicochemical properties of the material surface, including its electrical charge. In this study, the effect of the surface polarity of ferroelectric $LiNbO_3$ single crystals on the adhesion, growth and osteogenic differentiation of human bone marrow mesenchymal stem cells was investigated. The cells were cultured on the normal-to-plane poled and in-plane poled plates resulting in positive, negative and zero surface charge. The number of initially adhering cells on day 1 after seeding, their spreading, shape, and their metabolic activity, production of type I collagen, activity of alkaline phosphatase and mineralization in the following days of cultivation (days 6 and 20) were comparable on all three tested surfaces. However, significant differences were found in the expression of mRNA for type I collagen, alkaline phosphatase and osteocalcin, i.e. an early, medium-term and late markers of osteogenic cell differentiation, respectively. On day 20, the expression of type I collagen was significantly lower in cells on negatively-charged than on non-charged surfaces. Moreover, the expression of alkaline phosphatase and osteocalcin was higher in cells on positively-charged than on negatively-charged surfaces. These differences were generally more pronounced in standard cell culture medium than in osteogenic medium, which could, at least partly, mask the influence of the material surface properties on the cell behaviour. Thus, positively-charged $LiNbO_3$ surfaces seemed to be more suitable for the osteogenic differentiation of bone marrow mesenchymal stem cells than the negatively-charged surfaces.

Keywords: *electroactive ceramics, surface charge, cell number, resazurin, type I collagen, alkaline phosphatase, osteocalcin, bone matrix mineralization*

[Engineering of Biomaterials 135 (2016) 2-7]

Introduction

Cell-biomaterial interaction strongly depends on the physical and chemical properties of the material surface, such as its polarity, wettability, roughness and topography, rigidity and deformability, pH, electrical charge and conductivity (for a review, see [1]). In order to modulate the physicochemical properties of the material surface, a wide range of the material surface modifications have been developed, including irradiation with ions or ultraviolet light, plasma treatment, etching in acids or alkalis, grinding, polishing, electric discharge machining, shot peening, and particularly coating with various films based on oxides, nitrides, carbon materials or ceramics (for a review, see [1,2]).

In this study, we concentrated on the adhesion, growth and osteogenic differentiation of human bone marrow mesenchymal stem cells on electrically-charged surfaces with different polarization – positive or negative. There is a controversy among the studies dealing with the influence of the positive or negative charge on the cell behaviour. One group of studies reported that the positive charge was more advantageous for the cell adhesion, growth and differentiation. For example, the attachment and spreading of osteoblasts and fibroblasts on positively charged 2-hydroxytheythylmethacrylate and poly(ethylene glycol) hydrogels were higher than on the negatively charged and electroneutral surfaces [3]. Similarly, the positive charge on hydroxyapatite/TiO_2 hybrid surfaces increased the number of rat bone marrow-derived osteoblast-like cells cultured on these surfaces [4].

However, another group of studies came to the opposite conclusion – negative charge is better for the colonization of materials with cells *in vitro* and the material osseointegration *in vivo*. For example, negatively charged hydroxyapatite and β-tricalcium phosphate surfaces enhanced the adhesion and proliferation of human fetal osteoblasts, extracellular matrix formation by these cells, and the matrix mineralization in comparison with the positively charged and non-charged surfaces [5]. The negative surface charge of electrically-polarized hydroxyapatite ceramics was associated with increased osteobonding activity of this material after its implantation into tibial and femoral diaphyses of rabbits *in vivo* [6].

Finally, some studies found that there was no significant difference between positively and negatively charged materials in supporting the growth and differentiation of osteogenic cells [7,8].

Our earlier studies performed on thermally oxidized Ti, Nb and TiNb surfaces suggested that the positive charge of the material surface promoted the cell growth, while the negative charge supported the osteogenic cell differentiation [9,10]. An explanation was that the positively charged surfaces improved the adsorption of the negatively charged proteins mediating the cell adhesion, while the negatively charged surfaces adsorbed more Ca^{2+} ions, and thus promote the bone matrix mineralization [4,5]; for a review, see [1,10].

In this study, we investigated the adhesion, growth and osteogenic cell differentiation in human bone marrow mesenchymal stem cells cultured on poled electroactive ferroelectric $LiNbO_3$ single crystalline plates with positive or negative surface charge due to polarization perpendicular to the surface or with zero surface charge when polarization is parallel to the surface. The cell behaviour was evaluated in terms of the cell number, metabolic activity, markers of osteogenic cell differentiation (type I collagen, alkaline phosphatase and osteocalcin) and the bone matrix mineralization.

Our earlier study, performed on human osteoblast-like Saos-2 cells cultured on positively and negatively charged $LiNbO_3$ surfaces, showed that the cell behavior tended to be slightly better on positively-charged surfaces [11]. Since the Saos-2 cells are a cell line of osteosarcoma origin, which may be less sensitive to the material surface properties than primocultured and low-passaged cells, this study intend to verify the results obtained on Saos-2 cells on commercially available primary human mesenchymal bone marrow stem cells (passage 2).

Materials and Methods

Samples for cell experiments

The study was carried out on commercially available $LiNbO_3$ substrates (MTI Corporation). The materials were supplied in the following form: single crystalline plates, optical grade, dimensions 10×10×0.5 mm^3, two-sides polished, surface roughness <0.8 nm (determined by AFM), (0001) orientation poled perpendicularly to the surface (one surface with the positive charge and the opposite one with the negative charge) and (0100) orientation poled parallel to the surface with zero charge due to the polarization. The polarity of the surface was determined using the d_{33} meter (piezoelectric coefficient d_{33} = ± 23 pC N^{-1}). The measurements of zeta-potential, performed in our earlier study, showed that at near-physiological pH (i.e. pH ~6), the zeta potential was less negative on positively-charged than on negatively-charged $LiNbO_3$ surfaces [11].

Cell seeding

The samples were sterilized by 70% ethanol for 2 hours, inserted into 24-well cell culture plates (TPP, Switzerland; inner well diameter 15 mm) and seeded with human bone marrow mesenchymal stem cells (ScienCell Research Laboratories, Cat. No. 7500, passage 2). Each well contained 19 000 cells (approximately 10 000 cells/cm^2) and 1 ml of Mesenchymal Stem Cell Medium (MSCM, ScienCell Research Laboratories, Cat. No. 7501). After 6 days, when the cells reached confluence, one half of samples received osteogenic differentiation medium which was composed of α-MEM (Gibco, Cat. No. 11900-016), dexamethasone (10nM; Sigma-Aldrich, Cat. No. D1530-10UG), β-glycerolphosphate (20mM; Sigma-Aldrich, Cat. No. G9422) and ascorbic acid (50 μM; Sigma-Aldrich, Cat. No. 49752-10G). The second half of samples received α-MEM (Gibco, Cat. No. 11900-016). All the media contained foetal bovine serum (15%; Sigma-Aldrich, Cat. No. F7524-500ML), L-Glutamine (2mM; Gibco, Cat. No. A2916801) and gentamicin (40 μg/ml; LEK).

Cell number

The cell number was evaluated on day 1 as an important indicator of the initial cell adhesion. The cells were rinsed in phosphate-buffered saline (PBS), fixed with frozen 70% ethanol (-20°C) for 10 min and stained for 1 h with a combination of two fluorescence dyes, namely Hoechst #33258 (5 μg/mL; Sigma-Aldrich, Cat. No. B1155-25MG), which stains the cell nuclei, and Texas Red C_2-maleimide (1 ng/mL; Life Technologies, Cat. No. T6008), which stains the cell membrane and cytoplasm. The cells were then counted on microphotographs, and their morphology, i.e. the shape and spreading, was also evaluated.

Metabolic activity of cells

On the days 6 and 20, the cell number was estimated using the conversion of resazurin (Sigma-Aldrich, Cat. No. R7017) into fluorescent resorufin by viable and metabolically active cells. Briefly, the stock solution of the resazurin (4 mM) was added to the medium without phenol red to the final concentration of 40 μM. 1 mL of solution was added to the cells washed with PBS in order to remove formal medium. After 4-hour incubation at 37°C, the fluorescence was measured (Ex/Em = 530/590 nm) and corrected to background control (solvent mixture without cells) on a Synergy™ HT Multi-Mode Microplate reader (BioTek, USA). The cell number was recalculated per the substrate area (1 cm^2).

Type I collagen production

The total amount of collagen (i.e., an important component of the bone matrix) produced by the cells, i.e., intracellular collagen and collagen deposited on the tested materials, was determined using a Sircol kit (Biocolor Ltd., Carrickfergus, UK) on day 20 after cell seeding (6 days in MSCM + 14 days in differentiation medium or 6 days in MSCM + 14 days in α-MEM). The collagen was recovered by acid–pepsin digestion. The cells were rinsed with PBS, harvested with a cell scraper in 700 μL of pepsin solution (1 mg/mL dissolved in 0.5 M acetic acid), and lysed overnight at 4°C. The lysates were centrifuged and the supernatants were concentrated according to the Sircol kit manufacturer's protocol. Finally, the Sircol dye was bound to the isolated collagen, was dissolved, and the absorbance of the colored solution was measured. The absorbance was measured using a VersaMax ELISA Microplate Reader (Molecular Devices LLC) in Nunc-Immuno MicroWell 96-well cell culture plates (Sigma-Aldrich) with wavelength at 555 nm. The amount of total collagen was adjusted to the cell metabolic activity per sample.

Alkaline phosphatase (ALP) activity

The influence of the electrical polarization of the materials on the activity of alkaline phosphatase (ALP), i.e. an enzyme participating in bone matrix mineralization, in hMSCs was studied. After 20 days of cultivation (6 days in MSCM + 14 days in differentiation medium or 6 days in MSCM + 14 days in α-MEM), the cell layers were twice washed with PBS, and then the substrate solution (0.1 mg/mL p-nitrophenyl phosphate in substrate buffer [50 mM glycine, 1 mM $MgCl_2$, pH 10.5]) (Sigma-Aldrich) was added directly to the cells. The reaction was performed for 10 min at room temperature; the substrate solution was then removed and mixed with the same volume of the 1 M NaOH solution. The absorbance (at 405 nm) of the samples was measured together with the absorbance of the known concentrations of p-nitrophenol diluted in 0.02 M NaOH (9-90 μM) (Sigma-Aldrich). The results were normalized to the cell metabolic activity per sample.

Calcium deposition

The influence of the material surface polarization on calcium deposition by hMSCs was studied. After 20 days of cultivation (6 days in MSCM + 14 days in osteogenic differentiation medium or 6 days in MSCM + 14 days in α-MEM) the cell layers were rinsed with PBS, dried, and lysed in 0.5 M HCl for 24 h at 4°C. The calcium in the cell lysates and standards was directly determined by using the Calcium Colorimetric Assay (Sigma-Aldrich, Cat. No. MAK022-1KT) according to the manufacturer's protocol. The results were normalized to the cell metabolic activity per sample.

TABLE 1. Oligonucleotide primers for RT-PCR amplifications.

Gene	Primer sequence	Product size (bp)
ALP	Forward: 5′-GACCCTTGACCCCCACAAT-3′	68
	Reverse: 5′-GCTCGTACTGCATGTCCCCT-3′	
Collagen type I	Forward: 5′-CAGCCGCTTCACCTACAGC-3′	83
	Reverse: 5′-TTTTGTATTCAATCACTGTCTTGCC-3′	
OC	Forward: 5’-GAAGCCCAGCGGTGCA-3’	70
	Reverse: 5’-CACTACCTCGCTGCCCTCC-3’	
GAPDH	Forward: 5′-TGCACCACCAACTGCTTAGC-3′	87
	Reverse: 5′-GGCATGGACTGTGGTCATGAG-3′	

Real-time Q-PCR of markers of osteogenic cell differentiation

Real-time quantitative PCR (Q-PCR) was used to determine the effect of charge on the level of expression of genes for type I collagen, ALP and OC. Cells were grown on the tested materials in the growth or differentiation media for 20 days. Total RNA was extracted from MSCs using Total RNA purification Micro Kit (NORGENE Biotek Corp, Cat. No. 35300). The mRNA concentration was measured using NanoPhotometer™ S/N (IMPLEN). The cDNA was synthesized with the ProtoScript®M-MuLV First Strand cDNA Synthesis kit (New England BioLabs, Cat. No. E6300S) using 250 ng of total RNA and oligo-dT primers. The reaction was performed in T-Personal Therocycler (Biometra). Q-PCR primers were purchased from Generi Biotech Ltd. and are listed in TABLE 1. The primers were designed according to the literature (TABLE 1). Real-time quantitative PCR was performed using SYBR Green (Roche) in the total reaction volume to 20 µL and iCycler detection system (iQ™ 5 Multicolor Real-Time PCR Detection System, Bio-Rad) with cycling parameters of 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by a melt curve. Assays were conducted in quadruplicates. Data were analysed by the $2^{-\Delta\Delta Ct}$ method. The point at which the PCR product was first detected above a fixed threshold (termed cycle threshold, C_t), was determined for each sample. Changes in the expression of target genes were calculated using the equation:

$$\Delta\Delta C_t = (C_t^{target} - C_t^{GAPDH})_{sample} - (C_t^{target} - C_t^{GAPDH})_{calibrator}$$

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and data was normalized to the expression levels of cells grown on polystyrene in α-MEM medium (PS norm, calibrator).

Statistical evaluation

Cell number data were presented as mean ± S.E.M. from 36 measurements. The quantitative data (metabolic activity, ALP activity, type I collagen production, Ca deposition) were presented as mean ± S.D. (Standard Deviation) from 2-3 measurements. PCR data was presented as mean ± S.D. from 4 measurements. The statistical analyses were performed using SigmaStat (Jandel Corporation, USA). Multiple comparison procedures were made by the One-Way Analysis of Variance (ANOVA), Student-Newman-Keuls method. The value $p \leq 0.05$ was considered significant.

Results and Discussions

The cell number on day 1 after seeding did not differ significantly among the tested groups, although in average, it was slightly higher on positively-charged $LiNbO_3$ surfaces than on the negatively-charged surfaces (FIG. 1 A). The cells on all tested surfaces were of similar morphology, i.e. mostly polygonal and well-spread (FIG. 2). On days 6 and 20 of cultivation, the cell metabolic activity, i.e. an indicator of cell number, was similar on all tested surfaces (FIG. 1 B, C). At the same time, the two different types of cultivation media (i.e., standard growth medium and differentiation medium) did not cause any significant difference in the metabolic activity of cells on day 20 (FIG. 1 C).

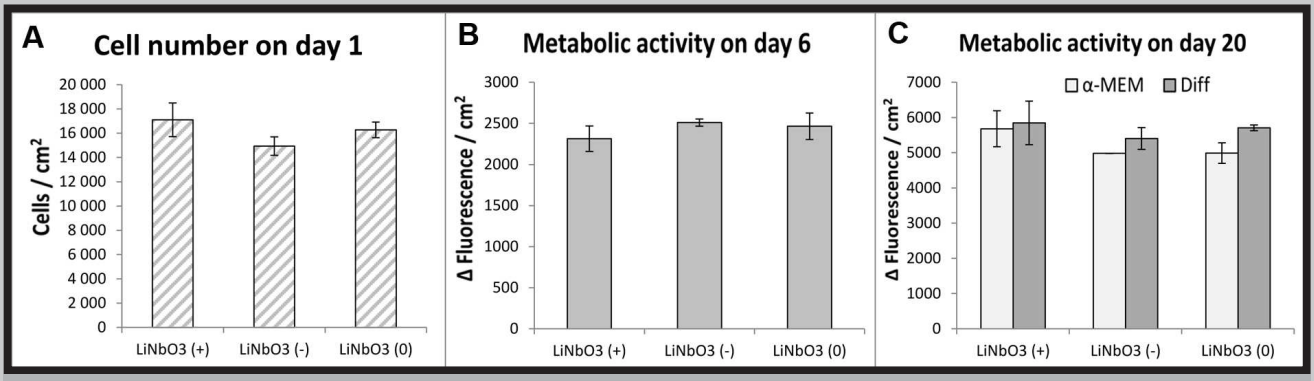

FIG. 1. Number (A) and metabolic activity (B, C) of human bone marrow mesenchymal stem cells on day 1, day 6, and day 20 after seeding on positively charged (+), negatively charged (-) and uncharged (0) $LiNbO_3$ surfaces. A: Mean ± S.E.M. from three samples (in total 36 values); B, C: Mean ± S.D. from three samples for each experimental group and time interval. C: For the last 14 days, the cells were cultured in standard medium (α-MEM) or osteogenic medium (Diff).

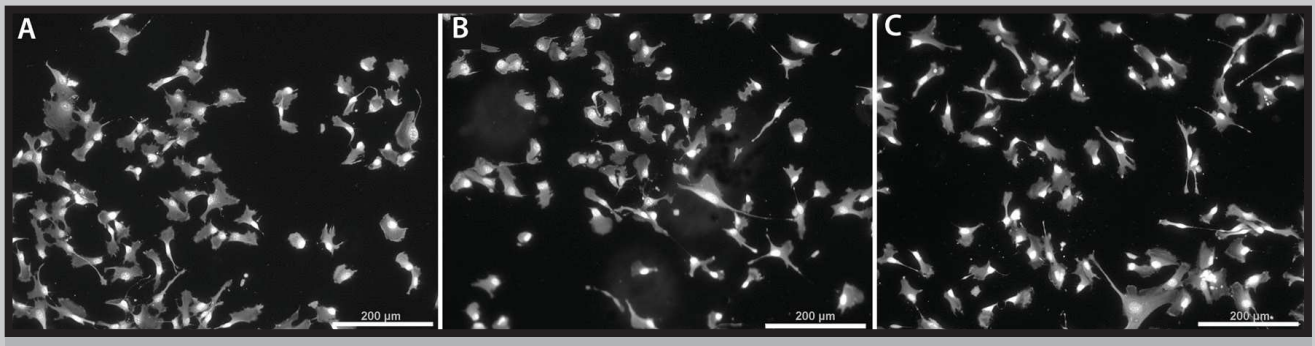

FIG. 2. Morphology of human bone marrow mesenchymal stem cells on day 1 after seeding on positively charged (A), negatively charged (B) and uncharged (C) $LiNbO_3$ samples. Cells stained with a combination of Hoechst #33258 and Texas Red C_2-maleimide. Olympus IX 51 microscope, objective 10x, DP 70 digital camera, scale bar = 200 µm.

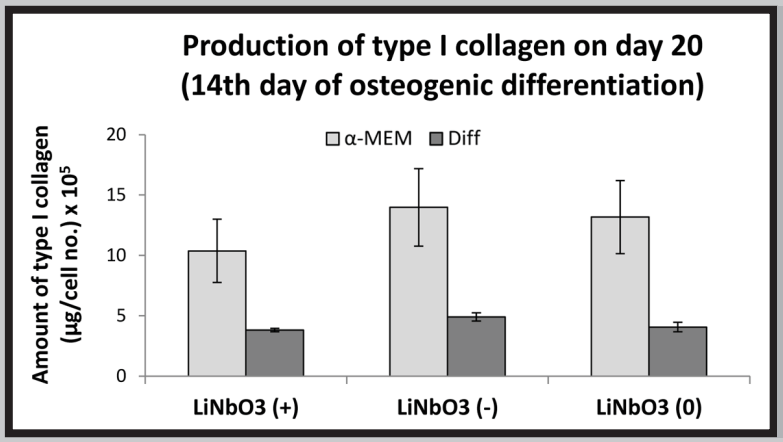

FIG. 3. Production of type I collagen per cell in human bone marrow mesenchymal stem cells in 20 day-old cultures on positively charged (+), negatively charged (-) and uncharged (0) $LiNbO_3$ surfaces. For the last 14 days, the cells were cultured in standard medium (α-MEM) or osteogenic medium (Diff). Mean ± S.D. from four measurements obtained from two samples for each experimental group.

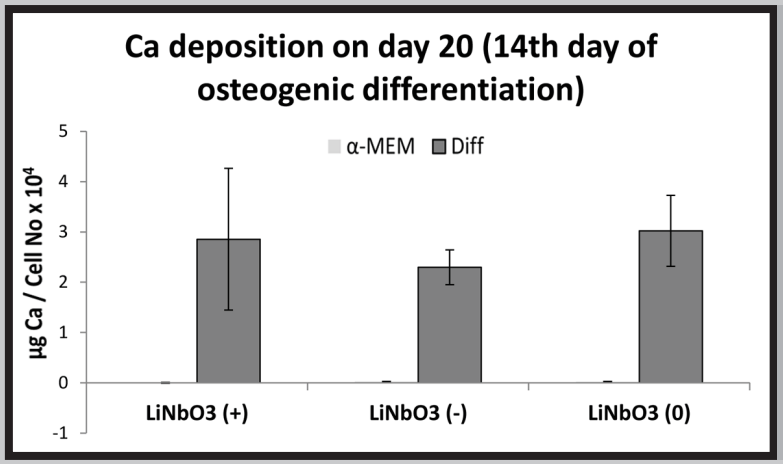

FIG. 4. Calcium deposition per cell in human bone marrow mesenchymal stem cells in 20 day-old cultures in standard medium (α-MEM) or osteogenic medium (Diff) on positively charged (+), negatively charged (-) and uncharged (0) $LiNbO_3$ surfaces. Mean ± S.D. from four measurements obtained from two samples for each experimental group.

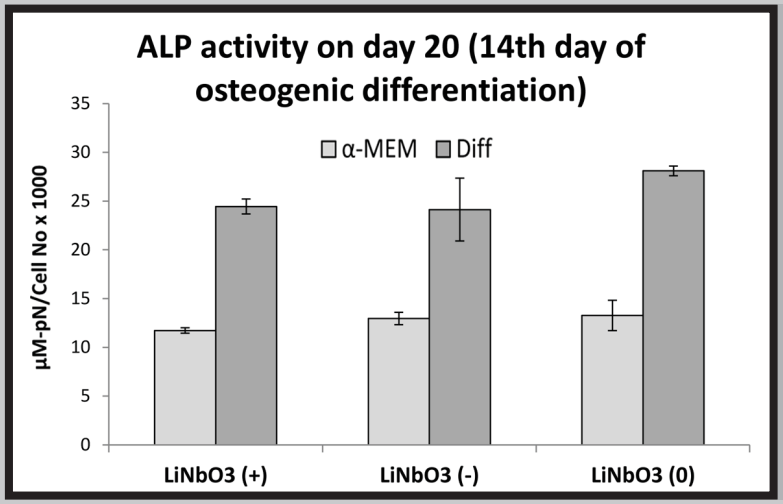

FIG. 5. Activity of alkaline phosphatase (ALP) per cell in human mesenchymal stem cells in 20 day-old cultures on positively charged (+), negatively charged (-) and uncharged (0) $LiNbO_3$ surfaces. For the last 14 days, the cells were cultured in standard medium (α-MEM) or osteogenic medium (Diff). Mean ± S.D. from four measurements obtained from two samples for each experimental group.

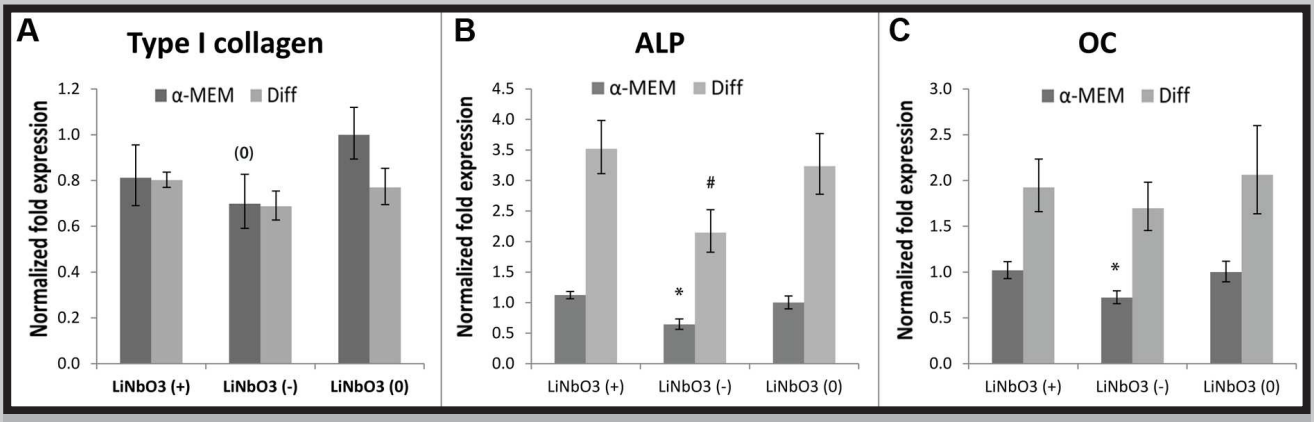

FIG. 6. Gene expression of type I collagen, alkaline phosphatase (ALP) and osteocalcin (OC) in 20 day-old cultures on positively charged (+), negatively charged (-) and uncharged (0) $LiNbO_3$ surfaces. For the last 14 days, the cells were cultured in standard medium (α-MEM) or osteogenic medium (Diff). Mean ± S.D. from 4 measurements for each experimental group. Statistical significance: * in comparison with the corresponding samples in α-MEM, # in comparison with the corresponding samples in osteogenic medium, and (0) in comparison with uncharged $LiNbO_3$ sample in α-MEM.

However, type I collagen was produced in higher amount by cells in the standard growth medium (α-MEM) than in osteogenic medium (FIG. 3). This result was surprising, because a higher production of type I collagen, considered as an early marker of osteogenic cell differentiation, can be expected in the osteogenic medium rather than in standard growth medium. An explanation might be that our method of collagen extraction was less successful in the case of cells in osteogenic medium, in which the extracellular matrix was strongly mineralized after 20 days of cultivation (14 days in osteogenic medium), as indicated by the markedly increased content of Ca^{2+} ions in comparison with the cultures in standard growth medium. In the latter cultures, the presence of Ca^{2+} ions was practically non-measurable (FIG. 4). In addition, the expression of type I collagen at the mRNA level was similar in cells cultures in both standard and osteogenic medium (see below). No significant differences in collagen production and matrix mineralization were found on the surfaces with different polarization (FIGs. 3 and 4).

The activity of alkaline phosphatase (ALP), i.e. an enzyme participating in bone matrix mineralization, considered as a medium-term marker of osteogenic cell differentiation, was significantly higher in cells grown for 14 days in the differentiation medium than in cells in the standard growth α-MEM medium. However, no differences in the ALP activity were observed in cells among the tested samples with different polarization (FIG. 5).

The evaluation of osteogenic markers at mRNA expression level revealed some significant differences among the cells cultured on the $LiNbO_3$ with various surface polarization. The cells on negatively-charged surfaces expressed in the standard growth medium expressed a significantly lower amount of type I collagen mRNA than the cells on non-charged $LiNbO_3$ (FIG. 6 A). The expression of mRNA for ALP was lower on negatively charged surfaces than on positively-charged and non-charged surfaces, which was apparent in both standard α-MEM and osteogenic media (FIG. 6 B). A similar trend was also found in the case of expression of osteocalcin, a late marker of osteogenic cell differentiation. The lowest expression of osteocalcin was found on negatively-charged surfaces in standard α-MEM (FIG. 6 C). Similar results were obtained in a study performed on gold nanoparticles with various surface functionalization. Positively charged Au nanoparticles (functionalized with amine groups) showed a higher cellular uptake, while negatively charged Au nanoparticles (functionalized with carboxyl groups) markedly reduced the ALP activity and calcium deposition in human bone marrow-derived mesenchymal stem cells [12].

The composition of the culture medium (i.e., standard growth α-MEM medium compared to osteogenic medium) did not significantly influence the expression of type I collagen (FIG. 6 A). However, the expression of ALP and osteocalcin was significantly increased in the medium with osteogenic factors (FIG. 6 B, C). The osteogenic medium might even mask the differences in the osteocalcin mRNA expression on materials with various polarization (FIG. 6 C).

Conclusions

The surface charge of $LiNbO_3$ due to ferroelectric polarization had no significant impact on the number, spreading, metabolic activity, production of type I collagen and activity of alkaline phosphatase in human bone marrow mesenchymal stem cells. However, the expression of osteogenic markers alkaline phosphatase and osteocalcin was higher in cells on positively-charged than on negatively-charged surfaces. The expression of type I collagen did not differ significantly between the positively and negatively-charged surfaces; however, on the negatively-charged surfaces, it was significantly lower than on the uncharged surfaces. In general, these results are in accordance with our earlier results obtained in human osteoblast-like Saos-2 cells on poled $LiNbO_3$ surfaces [11].

Acknowledgments

This study was supported by the Czech Science Foundation (grant No. 15-01558).

References

[1] Bacakova L., Filova E., Parizek M., Ruml T., Svorcik V.: Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. Biotechnol Adv 29 (2011) 739-767.
[2] Bacakova L., Filova E., Liskova J., Kopova I., Vandrovcova M., Havlikova J.: Nanostructured materials as substrates for the adhesion, growth, and osteogenic differentiation of bone cells. In: Nanobiomaterials in Hard Tissue Engineering. Applications of Nanobiomaterials. Volume 4, Ed. A. M. Grumezescu, Elsevier Inc., William Andrew Publishing, Oxford, Cambridge, 2016; pp. 103-153, ISBN 978-0-323-42862-0.
[3] Schneider G.B., English A., Abraham M., Zaharias R., Stanford C., Keller J.: The effect of hydrogel charge density on cell attachment. Biomaterials 25 (2004) 3023-3028.
[4] Kuwabara A., Hori N., Sawada T., Hoshi N., Watazu A., Kimoto K.: Enhanced biological responses of a hydroxyapatite/TiO_2 hybrid structure when surface electric charge is controlled using radiofrequency sputtering. Dent Mater J 31 (2012) 368-376.
[5] Tarafder S., Bodhak S., Bandyopadhyay A., Bose S.: Effect of electrical polarization and composition of biphasic calcium phosphates on early stage osteoblast interactions. J Biomed Mater Res B Appl Biomater 97 (2011) 306-314.
[6] Nakamura S., Kobayashi T, Yamashita K. Numerical osteobonding evaluation of electrically polarized hydroxyapatite ceramics. J Biomed Mater Res A 68 (2004) 90-94.
[7] Gao W, Feng B., Lu X., Wang J., Qu S., Weng J.: Characterization and cell behavior of titanium surfaces with PLL/DNA modification via a layer-by-layer technique. J Biomed Mater Res A 100 (2012) 2176-2185.
[8] Wolf-Brandstetter C., Hempel U., Clyens S., Gandhi A.A., Korostynska O., Oswald S., Tofail S.A., Theilgaard N., Wiesmann H.P., Scharnweber D.: The impact of heat treatment on interactions of contact-poled biphasic calcium phosphates with proteins and cells. Acta Biomater 8 (2012) 3468-3477.
[9] Jirka I., Vandrovcova M., Frank O., Tolde Z., Plšek J., Luxbacher T., Bacakova L., Stary V.: On the role of Nb-related sites of an oxidized β-TiNb alloy surface in its interaction with osteoblast-like MG-63 cells. Mater Sci Eng C Mater Biol Appl. 33(3) (2013) 1636-1645.
[10] Vandrovcova M., Jirka I., Novotna K., Lisa V., Frank O., Kolska Z., Stary V., Bacakova L.: Interaction of human osteoblast-like Saos-2 and MG-63 cells with thermally oxidized surfaces of a titanium--niobium alloy. PLoS One 9 (2014) e100475.
[11] Vanek P., Kolska Z., Luxbacher T., García J.A.L., Lehocky M., Vandrovcova M., Bacakova L., Petzelt J.: Electrical activity of ferroelectric biomaterials and its effects on the adhesion, growth and enzymatic activity of human osteoblast-like cells. J Phys D: Appl Phys 49 (2016) 175403.
[12] Li J.J., Kawazoe N., Chen G.: Gold nanoparticles with different charge and moiety induce differential cell response on mesenchymal stem cell osteogenesis. Biomaterials 54 (2015) 226-236.