INTEGRIN αv SIGNALING INFLUENCES PHENOTYPE AND MATURATION OF PRIMARY HUMAN OSTEOBLASTS ON ALUMINA SURFACE

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Abstract

Due to the growing interest in stem cells application in tissue engineering the better understanding of primary human osteoblasts behavior in vitro, on biomaterial surface, is required. Among other molecules integrins may be taken into account as being involved in these phenomena. Integrins are a family of cell adhesion receptors, which may regulate many cellular functions e.g., adhesion, motility, phenotype and cell maturation. The aim of this study was to determine the effect of the biomaterial surfaces and αv integrin signaling pathway on the behavior, phenotype and maturation of human osteoblasts in vitro. Human bone derived cells (HBDCs) obtained from adult femoral bone fragments were cultured on both alumina disks and tissue culture polystyrene (TCPS) dishes. After 7, 14, and 21 days of culture, localization and mRNA expression level of αv integrin subunits and BGLAP (osteocalcin) on polystyrene were analyzed in addition, we treated the cell cultures with monoclonal antibodies against human αv integrin to block its ligand-binding activity, on both alumina and TCPS substrates. We found that the αv integrin was present in focal contacts and cell cytoplasm at subsequent stages of cell maturation and the level of αv integrin mRNA was the highest in mature osteoblasts. Blocking αv integrin transduction pathway caused changes in cell activity and morphology, decreased cells proliferation on TCPS and reduced expression of alkaline phosphatase (ALP) on both materials. The results suggest that αv integrin is involved as an important receptor facilitating osteogenic differentiation.

Keywords: *integrin, human bone derived cells, osteogenic differentiation, cell adhesion, biomaterial*

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Introduction

Tissue engineering (TE) is a technique involving a combination of cells, biomaterials and biochemical or physicochemical factors to develop a construct used to improve or replace biological tissue. One of the most rapidly developing fields of medicine vividly interested in TE discoveries is reconstructive surgery, especially orthopaedics. In many instances orthopaedic surgery requires replacement of destroyed tissue; hence there is a growing need to develop a method to produce bone tissue *in vitro*. However, while more and more biomaterials with great properties for bone TE are designed, differentiation and maturation of osteoblasts still remain one of the greatest obstacles on a way to develop fully functional bone product.

Cell adhesion is a fundamental phenomenon, which supports the cell to sustain its structure, profoundly influences cell growth, differentiation, and migration as well as tissue morphogenesis, integrity and repair. Bone cell adhesion to extracellular matrix (ECM) directly influences cell spreading and growth, development of osteoblasts phenotype and bone tissue formation. Adhesion of bone cells onto the surface of the material plays a crucial role in host-implant interactions in biomaterial and tissue engineering. Osteoblasts interact with the surface through integrin membrane receptors binding first to proteins adsorbed on the biomaterial surface, and next, after the secretion of ECM elements, also into the ECM proteins [1].

Integrins are a family of heterodimeric surface receptors, exerting their function in both mechanical and signaling pathways. After ligand binding, integrins cluster and associate with cytoskeletal elements in a form of focal adhesion plaques. The structure of these proteins are supramolecular assemblies that provide anchorage forces and activate signaling cascades involved in cell cycle regulation and cell differentiation [2]. Osteoblasts express multiple integrins that bind to several matrix ligands. In particular, integrins *α2β*1 and *α5β*1 play a key role in osteoblasts function and activity [3,4]. Integrin *α2β*1 binds to collagen type I and has been reported to regulate osteogenic differentiation [5]. On the other hand, integrin *α5β*1 receptor has been demonstrated to be necessary for bone-like nodule formation *in vitro* when osteoprogenitor cells are grown on tissue culture polystyrene (TCPS) and other synthetic biomaterials [6]. Profound understanding of the interaction between cells and material surface is essential for the clinical success in regenerative medicine. *In vitro* studies indicate that cell adhesion to the surface is the crucial event involved in the phenotypic maturation of human osteoblasts. All types of bone cells expressed α1 and α5 subunits; however, only a subpopulation of osteoblastic cells expressed α2, *α*v, and *αvβ*3 subunits [7]. According to the recent knowledge, osteoblasts expressed numerous integrin subunits, namely: *α*1, *α*2, *α*3, *α*4, *α*5, *α*6, *α*v, *β*1, *β*3 and *β*5 [8,9].

Due to increasing knowledge on the role of integrins in differentiation and maturation of human osteoblasts and their potential clinical applicability, the interactions between biomaterials and integrins have become of great interest in TE. It is known that *αvβ3* integrin is a central molecule for osteoclastic bone resorption [10]. On the other hand, the expression of the integrin *β*1 subunit, which interacts with the *α*2 and *α*5, was increased in osteoblast-like MG63 cells cultured on titanium (Ti) compared to tissue culture on TCPS [11]. In addition, the properties of surface materials, such as topography, chemistry or surface energy play an essential role in osteogenic cells activity, adhesion and growth, mainly by affecting the localization and structure of the integrins [12-14].

In this report, we focused on the role of the *α*v integrin subunit in osteoblasts proliferation and function during differentiation of primary human bone derived cells (HBDCs). So far, the role of *α*v integrin subunit has been demonstrated in osteoclastogenesis, but not in osteoblasts [15]. Previously, immunolocalization studies revealed the presence of β3 and *α*v in focal contact areas [16]. Similar experiments were performed on human osteoblasts cultured on alumina and TCPS surfaces.

Materials and Methods

Primary cell culture of human osteoblasts

HBDCs were isolated with informed consent from adult femoral bone fragments removed at orthopaedic surgery. Experiments were repeated with cells obtained from three independent patients (aged 50-68 years). The procedure of HBDCs isolation was based on the protocols described with modifications by Kudelska-Mazur et al. [17]. Small pieces of bone were treated with collagenase (Sigma-Aldrich, St. Louis, USA) for 24 h and put into the culture medium. Cells were cultured until confluence in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Life Technologies B. V. Breda, Netherlands) enriched with 10% of heat-inactivated fetal calf serum (FBS; Gibco), 50 µg/ml Antibiotic-Antimycotic (Gibco) supplemented with 2% L-glutamine (Gibco) and 0.1 M L-ascorbic acid-2-phosphate (Sigma-Aldrich) - called culture medium - and incubated at 37°C in the atmosphere of 95% humidity and 5% CO_2. The cells of the first passage were used in all experiments. Cells were seeded onto 4-well dishes at a population density of $4x10^4$ cells per well on TCPS or alumina disks surface and cultured in medium supplemented with 10 nM dexamethasone (Sigma-Aldrich) and 100 nM 1α,25-dihydroxycholecalciferol (Sigma-Aldrich) (called differentiating medium).

Blocking of αv integrin subunit with an antibody

A monoclonal antibody against human αv integrin (clone P2W7, Sigma-Aldrich) was used for integrin blocking experiments. The antibody was added to the culture medium at concentrations of 0 (control culture without antibody), 1, 2.5 and 5 µg/ml, 24 h after seeding the human osteoblasts. Media were changed every five days. Antibody was always added on the same day when media were changed and was present in the cultures for the whole experiment period. In four time-points of the culture after addition of the antibody, i.e. 24 h, 7, 14, and 21 days, its effects on cell morphology, viability, and alkaline phosphatase activity were determined (both in cultures on TCPS and alumina). Morphology observations were performed using an inverted microscope equipped with phase contrast (for TCPS cultures) and fluorescent microscope (for alumina cultures fixed with 1% glutaraldehyde) connected to Nikon Digital Sight DS-U1 camera (Nikon Eclipse TE2000-u; Nikon, Japan). The experiments were repeated three times using cells obtained from different patients.

Biomaterial

The material used in experiments was pure (99.9%) alumina prepared by the Institute of Glass, Ceramics, Refractory and Construction Materials, Warsaw, Poland. Samples of the biomaterials had a form of disks, 15 mm in diameter and 2 mm of height and before the experiments the samples were sterilized with irradiation at a dose of 25 kGy. Alumina discs were placed at the bottom of each 4 wells in the culture plate. Next, the cells were seeded on the surface of biomaterials and on the bottom of the control wells on PS at a density of $4x10^4$ cells per well (1.9 cm^2).

Determination of cell count

The population of viable cells was assessed in the XTT (Sigma-Aldrich) assay, while the total cell number was determined by counting in the hematological camera. The number of viable cells was assessed after 24h as well as after 7, 14, and 21 days of culture. XTT assay is based on the ability of mitochondrial dehydrogenase enzymes of living cells to convert the XTT substrate (2.3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenyloamino)carboxyl]-2H-tetrazolium hydroxide) into a water-soluble formazan product. The concentration of color product of the reaction is proportional to the activity of mitochondrial respiration and thus to the number of viable cells. It was measured using an ELISA microplate reader (FLUOstar OPTIMA, BMG LABTECH GmbH, Germany) at a wavelength of 450 nm. The number of cells was calculated using the calibration curve referring XTT assay results to cell count in Burker camera using optical microscope (Nikon Eclipse TE2000-u) as a direct method of cell number determination. The charts present the results of one representative experiment performed in three independent repeats.

Osteoblasts differentiation marker analysis

After determining cell viability, the cultures were rinsed three times with PBS (Gibco) and lysed with 0.1% Triton X-100 (Sigma-Aldrich) overnight (ON) at 4ºC. Cellular alkaline phosphatase (ALP; Sigma-Aldrich) activity was measured using a colorimetric method based on the hydrolysis of p-nitrophenol phosphatase to p-nitrophenol. Absorbance of the reaction product was read at 405 nm using the FLUOstar OPTIMA reader (BMG LABTECH GmbH). ALP activity was calculated from the calibration curve according to the manufacturer's protocol (Alkaline Phosphatase Diagnostic Kit, Sigma-Aldrich). The enzyme activity was expressed in Sigma units /cell number. Experiments were repeated three times and the results of one representative study are presented.

Immunofluorescence localization of *αv* integrin subunit

HBDCs were seeded at cell density of $2x10^4$ cells per coverslide in cell culture medium. On the next day culture medium was exchanged for differentiating medium. At days 7, 14, and 21, HBDCs cells were washed in PBS and fixed with 3% formaldehyde in PBS (Gibco). Then, the cells were permeabilized in 0.1% Triton X-100 in PBS, washed three times in PBS and next incubated in 0.25% glycine in PBS for 30 minutes at room temperature. Non-specific binding was blocked with 3% bovine serum albumin (BSA) in PBS for 30 min, before incubation with the primary antibody. The cells were incubated overnight, at 4ºC with the mouse monoclonal anti-αv specific integrin (Sigma-Aldrich) antibody, diluted 1:100 in 3% BSA/PBS. Then, the cells were washed in PBS and incubated with biotin-conjugated secondary antibodies (Santa Cruz Biotechnology) (dilution 1:100 in 3% BSA/PBS), followed with Extravidin-TRITC (Sigma-Aldrich) (dilution 1:100 in 3% BSA/PBS). As a control, non-labeled cells and cells labeled with a secondary antibody (biotin-conjugated) only as well as with Extravidin-TRITC only were observed. After a final washing in PBS, specimens were mounted in Ultra Cruz Mounting Medium containing DAPI (Santa Cruz Biotechnology). The images of stained cells were captured using fluorescent microscope (Nikon Eclipse TE2000-u) equipped with Nikon Digital Sight DS-U1 camera.

Determination of mRNA level by semi-quantitative (sq) RT-PCR assay

Total RNA was isolated from cells harvested at different stages of differentiation using a High Pure RNA Isolation Kit (Roche Diagnostics U.S. Heagquarters, Indianapolis, USA). Specific transcripts were amplified by semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR) with 1 µg total RNA as the template and specific oligonucleotide primers, using a Titan One-Tube RT-PCR Kit (Roche Diagnostics) according to the manufacturer's instructions. Sequences of primers (synthesized by Singen Biotech, Poland) for the tested genes are shown in TABLE 1.

TABLE 1. The sequences of forward and reverse primers of osteocalcin (OC) αv integrin subunit and GAPDH.

Gene	Forward primer	Reverse primer	PCR product size (bp)
OC (*BGLAP*)	5'-ACTCCTCGCCCTATTGGC3-'	5'-AGAGCGACACCCTAGACCG3-'	300
αv integrin subunit (*ITGAV*)	5'-CTGATGCAGTGTGAGGAATTG3-'	5'-GCTAAGAGTTGAGTTCCAGCCT3-'	491
GAPDH	5'-TCAAGGAAGCTACGGGCA3-'	5'-TGGCAGAAATTACACACACACAC-3'	250

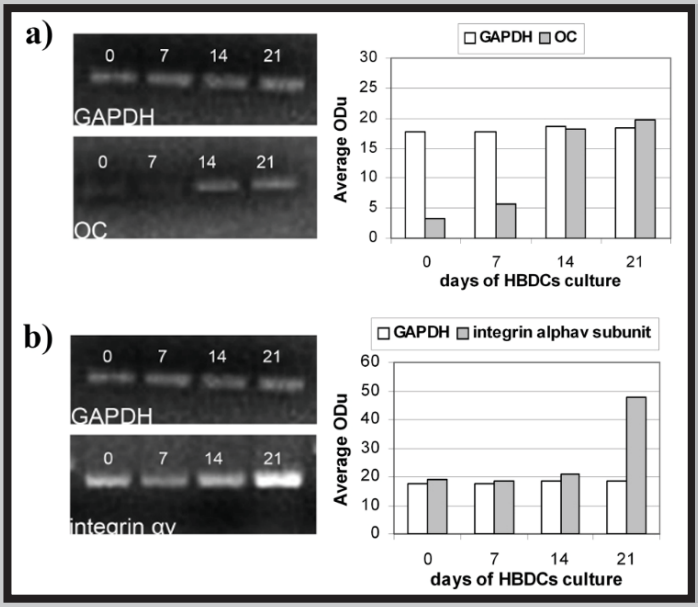

FIG. 1. Osteocalcin (OC) and integrin *α*v subunit expression. Changes in the level of osteocalcin (OC) and integrin *α*v subunit expression were examined during differentiation of HBDCs cultured on PS. RT-PCR products of OC and integrin *α*v subunit amplified from total mRNA of human osteoblasts cultured without differentiation medium (point 0 on the graph) and during differentiation of HBDCs *in vitro* (days 7, 14, 21) with the differentiation medium. The analysis of representative gels, and average optical density of bands (ODu) for OC (a) and integrin αv (b) are presented in the charts. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a reference gene.

The obtained cDNA fragments were separated on 2% Agarose LE gels (Roche Diagnostics). The gels were stained with ethidium bromide and the optical density of bands was analyzed with Gel Doc 2000 using Quantity One software (Bio-Rad, Hercules, CA, USA). To evaluate the expression levels of transcripts in a semi-quantitative manner, the optical densities (ODu) of amplified cDNA fragments bands were compared with that of the constitutively expressed GAPDH. The results of one representative out of three independent sqRT-PCR experiments performed are presented.

Results

Determination of osteoblastic phenotype of HBDCs

To determine the ability of HBDCs to differentiate in primary culture, we examined changes in expression of specific osteoblastic gene marker – osteocalcin by sqRT- PCR (FIG. 1a). OC mRNA was present at a very low level at point 0 and day 7 in human derived cells. During differentiation of HBDCs, the level of OC mRNA increased from day 14 (18.2 ODu) and reached the highest concentration at day 21 (19.6 ODu). The mRNA expression of GAPDH, serving as a reference gene, presented the same level in each time-point.

Expression of αv integrin subunit

As *α*v integrin subunit might have a considerable influence on differentiation, we determined its mRNA concentration in HBDCs by sqPCR (FIG. 1b). Concentration of mRNA of *α*v integrin was similar in the HBDCs during the first 2 weeks of the culture in differentiating medium reaching 19.1 ODu at day 0, 18.72 ODu at day 7 and 21.08 ODu at day 14. The highest increase (47.8 ODu) of *α*v integrin mRNA was observed at day 21, when the HBDCs were finally matured. The mRNA expression of GAPDH serving as control presented the same level in each time-point (FIG. 1a, b).

The integrin αv immunolocalization in HBDCs

We used a specific antibody against the *αv* integrin subunit for its localization on the surface of HBDCs cultured 7 (FIG. 2a), 14 (FIG. 2b) and 21 (FIG. 2c) days. Immunostaining followed by fluorescence microscopy analysis indicated that HBDCs expressed *αv* integrin subunits at the focal adhesion sites (toe-like structures; FIG. 2, arrows).

HBDCs morphology analysis after IgG block

To determine the role of *αv* integrin in adhesion related signals for HBDCs differentiation we blocked its extracellular domain with specific antibody. Cells morphology was observed after 24 h and on day 7, 14 and 21. Representative microphotographs obtained after 24 h and 21 days after treatment with anti-*α*v IgG are shown in FIG. 3. 24 h after addition of anti-*α*v IgG, in concentrations 1, 2.5, 5 μg/ml (FIG. 3b, c and d), there were no visible changes in morphology of treated with *α*v integrin specific IgG cells compared to control cultures (FIG. 3a). Human osteoblasts described as elongated spindle-shaped forms showed regular morphology. After 21 days of culture, the density of cells both maintained in absence and presence of anti-*α*v integrin IgG increased. We found that the morphology of HBDCs cultured with 1 μg/ml of anti-*α*v IgG (FIG. 3f) was similar to the control (FIG. 3e), but higher concentrations of IgG influenced HBDCs behavior. Cultures containing 2.5 μg/ml (FIG. 3g) and 5 μg/ml (FIG. 3h) of anti-αv antibody had reduced density of adherent cells and presented changes in their appearance. As shown in FIG. 3g and 3h, less well-spread and less spindle-shaped cells were observed.

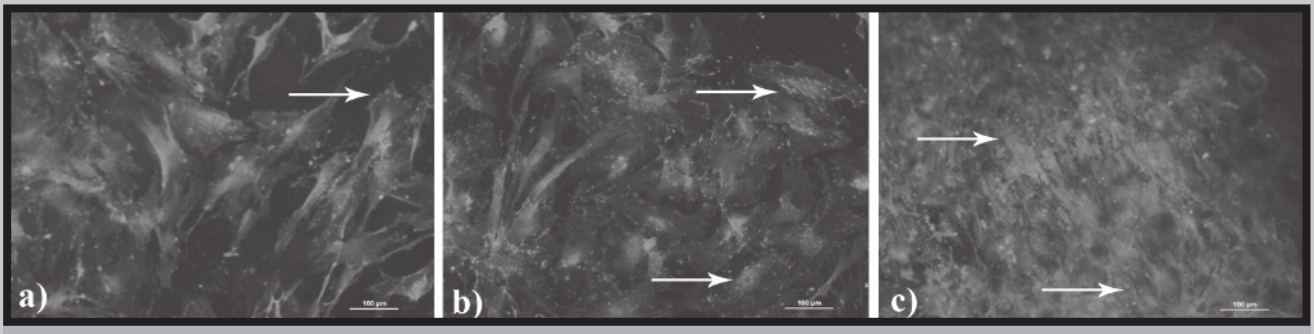

FIG. 2. Integrin subunits localization. The localization of *α*v integrin subunit in HBDCs was examined by immunostaining. Human osteoblasts cultured on coverslips were fixed at 7 (a), 14 (b) and 21 (c) days, stained with antibodies against *α*v subunit followed by secondary biotin conjugated antibodies, and next with Extravidin--TRITC. Fluorescence was detected using fluorescent microscope (Nikon Eclipse TE2000-u) equipped with Nikon Digital Sight DS-U1 camera. A monoclonal antibody against human *α*v integrin recognizes the extracellular domain of the *α*v subunit and representative images are presented. Arrows in the images indicate the integrin *α*v subunits at focal adhesion sites localization. Scale bar, 100 μm.

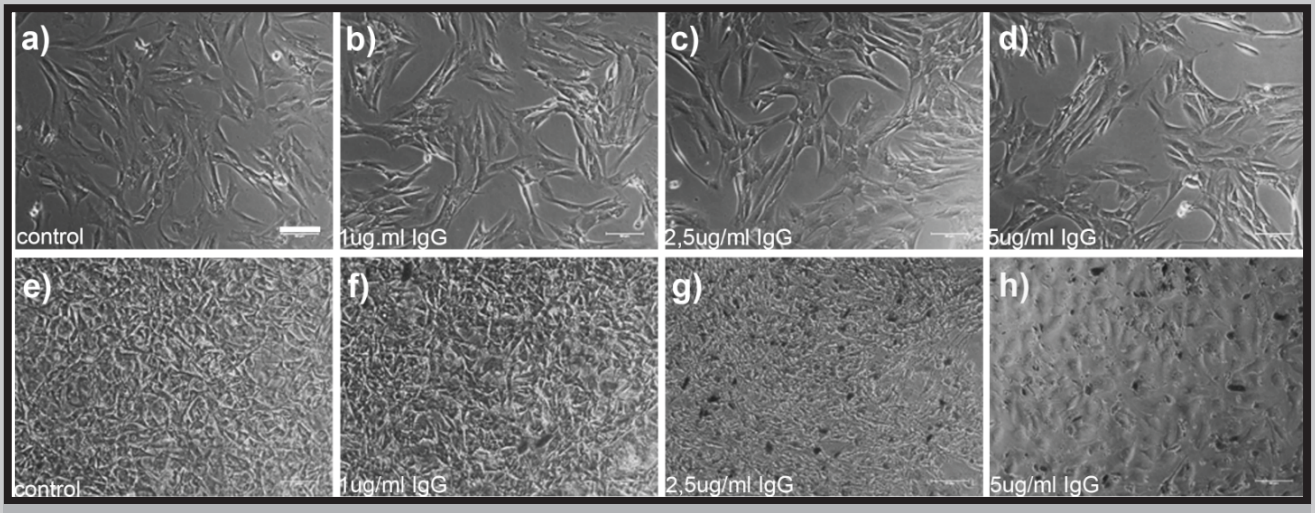

FIG. 3. Influence of integrin *α*v subunit inhibition on cell morphology. To compare the changes in human osteoblasts morphology during *in vitro* differentiation on TCPS, in the presence of antibodies (IgG) against the integrin *α*v subunit, cell cultures were analyzed using phase contrast microscope. Cultures of HBDCs were observed at every stage of their differentiation (24 h, 7, 14 and 21 days). Images from 24 h (b, c, d,) or 21 days of the culture (f, g, h) after treatment with three different concentrations of antibodies against αv integrin subunit are shown. Control samples cultured without antibodies are presented: a, e. Phase contrast micrographs. Scale bar, 100 μm.

Viability assay and effect of anti-αv antibody on HBCDs proliferation

Viability of HBDCs cultured in the absence or presence of different concentrations of *α*v integrin subunit specific antibody, in two different culture conditions – on TCPS or alumina surface was determined by XTT assay. The XTT assays results are presented in FIG. 4 (a – for TCPS, b – for alumina) as values of the absorbance read in an ELISA reader. The number of vital HBDCs in TCPS or alumina was determined from a calibration curve based on the absorbency of the formazan produced from XTT in the mitochondria of the living cells. Proliferation of HBDCs was inhibited in the presence of 1, 2.5 or 5 μg/ml of anti-*α*v IgG in cells cultured on TCPS, in comparison to control cultures (FIG. 4a). In contrast, the viability of cells cultured on alumina at different concentrations of anti-*α*v antibody and without specific IgG were similar (FIG. 4b). On day 14 of the culture, osteoblasts cell number on material surface suddenly dropped and bounced back at day 21 probably due to the large number of cells.

ALP activity effect of blocking αv integrin subunit

To identify the influence of *α*v integrin subunit IgG blockage on HBDCs differentiation we assessed ALP activity of cells maintained in different concentration of *α*v integrin specific IgG and on the studied surfaces. ALP assay was normalized to the number of viable cells determined with XTT assay. Mean values of alkaline phosphatese activity per cell are shown in FIG. 5. The experiments revealed considerable differences between surfaces, including TCPS (FIG. 5a) and alumina (FIG. 5b) showing that *α*v integrin blockade resulted in a decrease of the average ALP values for both cultures with more pronounced effect in cells cultured on alumina (FIG. 5b). At 1, 2.5 or 5 μg/ml of anti-*α*v IgG, after 21 days in differentiation medium HBDCs exhibited nearly 1 U/10^4 cells of ALP activity whereas in control cultures the respective value was 1.5 U/10^4 cells, as shown in FIG. 5b. In the HBDCs cultured on TCPS, the level of ALP activity in the presence of various concentrations of the anti-*α*v IgG was less pronounced in comparison to control culture; however, end-point results (after 21 days of culture) showed similar relation of *α*v integrin specific IgG concentration to ALP activity (FIG. 5a). On both surfaces *α*v integrin blockade specifically reduced ALP activity of differentiating HBDCs cultures.

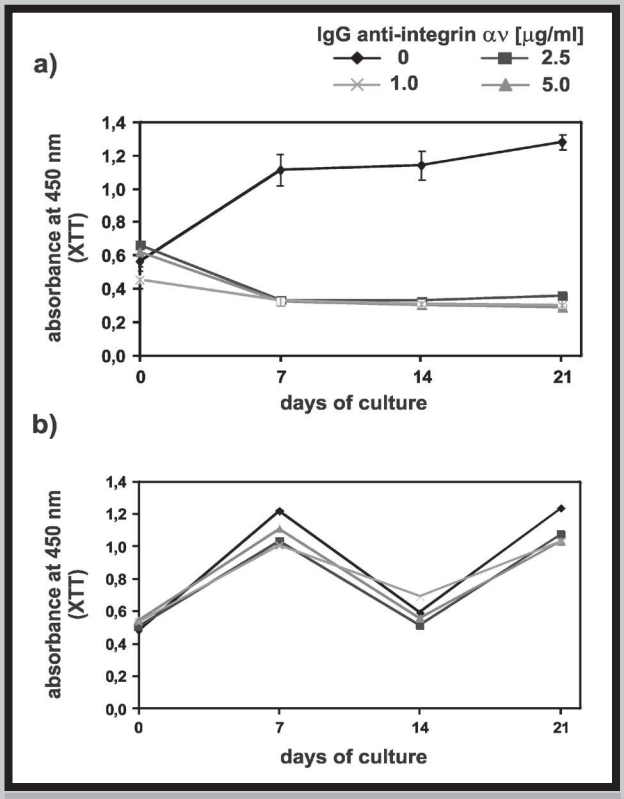

FIG. 4. Influence of integrin *α*v subunit inhibition on cell viability. The effect of anti-*α*v antibodies on the proliferation of HBDCs was examined by XTT assay. The results of XTT viability assay were determined in primary cultures of HBDCs on TCPS (a) or alumina (b) untreated of anti-*α*v antibody and treated with various concentrations of antibodies against *α*v integrin subunit (1, 2.5 or 5 μg/ml). Untreated cultures served as a control (0 μg/ml). The diagrams show the representative values from three independent experiments.

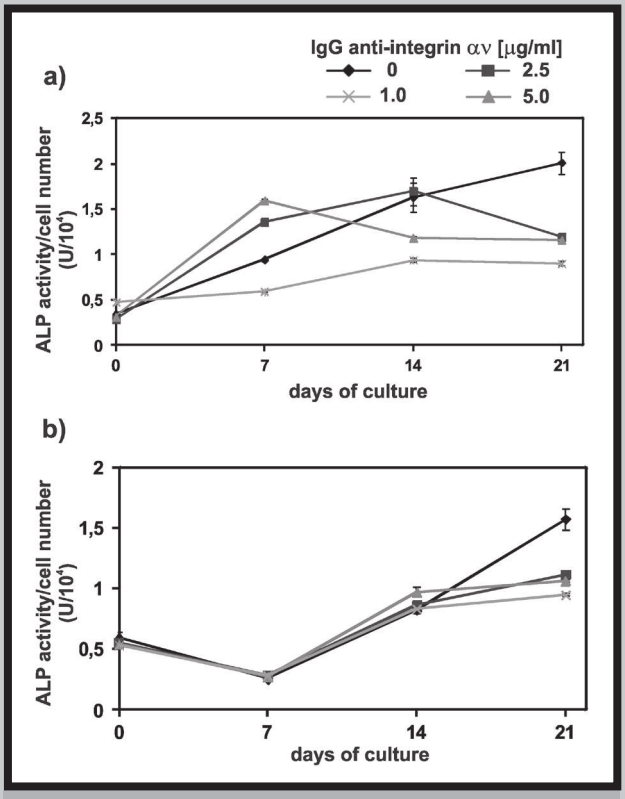

FIG. 5. Influence of integrin *α*v subunit inhibition on ALP activity. The effect of treatment of differentiating HBDCs with anti-integrin antibodies on the ALP activity was examined using colorimetric method. Primary human osteoblasts were cultured *in vitro* on TCPS (a) or alumina (b) without IgG (control) and in the presence of 1, 2.5 or 5 μg/ml of antibodies against the extracellular domain of *α*v integrin subunit. The antibody was added 24 h after cells seeding, and one week later, when the differentiation medium has been changed. The number of cells was calculated from the calibration curve for the XTT assay. ALP activity was counted in Sigma units and normalized to number of cells divided (number of cells x 10^4). The measurements were taken in four points of culture: 24 h, 7, 14 and 21 days after the addition of antibodies against *α*v integrin subunit, cell viability (XTT assay) and alkaline phosphatase activity tests have been determined. The diagrams show the mean values from three independent experiments.

Discussion

This study is the first step to understand the role of *α*v integrin in the process of osteogenessis in osteoblast-like HBDCs, cultured on tissue culture polystyrene dishes and on clinically used orthopaedic implant material – alumina. We used two different surfaces: alumina and TCPS to compare the behavior of cells on two different substrates in a shoulder signal of integrin *α*v. Because of its excellent biocompatibility and good mechanical properties alumina ceramic material is used clinically in artificial joints and as a filler for various bone defects [18]. We hypothesized that blocking of the integrin *α*v subunit will exert an impact on the processes of proliferation and cells differentiation *in vitro*. Our results indicate that osteoblastic differentiation depends on *α*v integrin signaling and mediates some, but not all, effects of cell proliferation or maturation of osteogenic phenotype. We focused on the interaction between integrin signaling and morphology, growth, adhesion, and differentiation process of HBDCs.

Cell adhesion is a fundamental cellular process directly influencing cell migration as well as tissue morphogenesis, integrity, and repair [19]. Integrin family is the largest group of cell adhesion molecules. It consists of major cell surface receptors for ECM proteins. Furthermore, integrins conduct signals from ECM to the cell and from the cell to ECM, influencing cellular behavior [20]. Human osteoblasts express variety of integrins including *α*v*β*3, *α*v*β*5, *α*2*β*1. Several studies have shown that integrins localize at the focal adhesion sites in many types of cell lines [21], but there were also reports indicating that some integrins were distributed on the cell membrane in dispersed manner [22]. The *α*v integrin subunits co-localized with *β*1 integrin subunits at the focal adhesion plaques in human osteoblasts (HOBs) [23]. The results obtained in this study, using sqRT-PCR technique confirmed the expression of *α*v integrin subunit in human osteoblast-like HBDCs. The mRNA expression of *α*v integrin subunit raised during differentiation and reached the highest level at day 21. We considered 21st day as the final step of the HBDC differentiation as it was the moment when osteocalcin concentration was the highest and later on it did not increase. The fact that integrin *α*v subunit increases during maturation suggests that it might play an important role in this process.

To stimulate the differentiation and maturation process of HBDCs *in vitro* we incubated HBDCs in the presence of standard differentiating medium (0.1M L-ascorbic acid-2-phosphate containing culture medium enriched with 10 nM dexamethasone and 1α,25-dihydroxycholecalciferol). Ascorbic acid-2-phosphate is essential for maturation of osteoblast-produced ECM protein, collagen type I [24]. 1α,25-dihydroxycholecalciferol stimulates gene expression of bone-related transcription factors, i.e., Runx2, Osterix and osteoblasts differentiation markers such as: ALP or osteocalcin. Alkaline phosphatase is an early marker of osteoblasts differentiation, which increases prior to calcium phosphate deposition and decreases after the onset of mineralization. On the other hand, osteocalcin is a late marker, increasing with calcification of the extracellular matrix [25]. Primary HBDCs are the population of cells, which demonstrate distinct level of cell maturation. Therefore during their differentiation we checked mRNA level of OC. Our results show that the highest level of osteocalcin mRNA is expressed in HBDCs after 14 and 21 days of culture. This indicates that HBDCs acquired a mature osteogenic phenotype.

To determine the specific roles of integrin in the adhesion, proliferation and differentiation of osteoblasts, many investigators have relied on the use of antibodies to block different integrin subunits. Thus, we decided to examine effects of blocking integrin αv subunits with specific monoclonal antibodies on cellular behavior of primary human osteoblast-like cells cultured on TCPS or alumina surface. Our results indicate that αv integrin affects osteoblasts differentiation through changes in induction of osteoblasts marker (ALP) and changes in cell proliferation and cell morphology. Treatment of HBDCs with monoclonal anti-αv antibody caused dose-dependent decrease in cell number and ALP activity in both TCPS and alumina surfaces. In our study, antibodies were added 24 h after seeding the cells when adherent HBDCs were observed in culture. Blocking antibodies were used at three different concentrations (1, 2.5, 5 μg/ml) in culture medium during 21 days of HBDCs culture *in vitro*. With the time of culture morphology of antibody treated cells was steadily changing when compared to control cells. Characteristic elongated spindle-shaped form and regular morphology of HBDCs after 24 h of incubation with the antibody were observed for control cells as well as for cells treated with all used concentrations of αv integrin subunit specific antibody. Whereas, after 21 days of culture, the samples exposed to 2.5 μg/ml and 5 μg/ml of anti-αv antibody demonstrated reduced density of adherent cells and changes in their appearance. On the other hand, the samples cultured in the presence of 1 μg/ml of αv integrin subunit specific antibody presented morphology and density similar to the control. Probably this concentration is insufficient to block all αv integrin subunits expressed on cell surface. As integrin expression and osteoblasts biology are closely related, the function of these molecules in early phases of the adhesion could represent an indicator of effectiveness of biomimetic surfaces in promoting adhesion, proliferation and function of osteoblasts [26]. Cells response to various concentrations of anti-αv antibody, suggests that αv integrin specific monoclonal antibodies used in the experiment probably block ligand binding sites of integrin subunit. Results of Wang et al. indicate that the antibody approach enables the blockade of β1 signaling by integrins that are exposed on the cell surface rather than at the interface between cell and its substrates [27].

Osteoblasts interact with surface of TCPS and other biomaterials [28,29] through integrins in a similar manner to the natural interactions between bone matrix environment. What is more, osteoblasts as well as osteoblast-like cells produce components of ECM *in vitro* depositing them on the surface of culture material. These components are responsible for cell adhesion to culture material in long-lasting cultures [30]. $\alpha 2\beta 1$ integrin binds to collagen type I, which is the major bone matrix protein [31]. Signaling of integrin $\alpha 2\beta 1$ has been reported to regulate osteoblastic differentiation [5] and blocking of integrin β1 with a specific antibody resulted in decreased adhesion strength [1]. Many researchers claim that integrin β1 is the major subunit in osteoblasts [27,32,33]. However, increasing integrin β1 and β3 mRNA levels, that are responsible for signal transduction necessary for osteogenic differentiation, was decreased or at least delayed [34]. Another integrin $\alpha 5\beta 1$ binding to fibronectin has been shown to be necessary for bone-like nodule formation *in vitro* when osteoblastic cells are maintained in tissue culture on PS [6]. As differences in HBDCs morphology and density can be observed after 21 days of treatment with αv integrin subunit specific antibody only, it seems that αv integrin subunit takes part in the interaction with ECM elements deposited on surfaces of TCPS or alumina rather than directly with the surface. This may also partly explain its increasing concentration and define the role of increasing amount of extracellular proteins produced by cells and adhered to the surface.

We used two different surfaces to determine whether the type of surface plays a role in the observed effects. Both materials are very well tolerated by cells. Tissue culture polystyrene is a standard material for *in vitro* culture and alumina is widely used in regenerative medicine [35,36]. However, cell behavior in both materials slightly differed. HBDCs cultured on TCPS were more susceptible to αv integrin subunit blockade and hence 3-fold reduction of proliferation was observed as early as after 7 days of treatment with specific antibody in all three studied concentrations. In contrast, blockade of αv integrin subunit with specific antibody did not influence the proliferation of cells cultured on alumina. The possible explanation of this phenomenon is different susceptibility of these materials to adherence of ECM proteins produced by cells.

The chemical composition and surface topography influences on the interaction between integrins, substrate, and cellular behavior. Cells on smooth surfaces tend to have a better-organized ECM and different organization of cytoskeleton. Osteoblastic cells have been shown to adhere to a wide variety of matrix proteins when coated on polystyrene dishes. Dedhar et al. demonstrated that up-regulation of fibronectin receptor in osteosarcoma cells correlated with phenotype and adhesion [37]. Although these studies have provided a great deal of evidence for mechanism involved in cellular attachment to tissue culture polystyrene, they do not necessarily correlate with attachment on implant materials. Other studies have shown altered profiles of integrin expression when primary osteoblasts are cultured on orthopeadic metals, as a function of both composition and topography [38].

We conclude that osteoblasts have differing abilities to use integrin-mediated mechanism for attachment on polystyrene and alumina, possibly by depositing different ECM proteins and changed the composition of integrin expression. It is possible that differences in composition of polystyrene and alumina result in the interaction of integrin with ECM proteins, affecting in different signaling pathways after blocking of integrin αv subunits. When the function of αv integrins was inhibited, the cells reduced capacity to differentiate which manifested with lower increase of ALP activity in the HBDCs on both materials. Thus, we conclude that integrin αv subunit seems to influence differentiation or maturation of osteoblastic phenotype in primary HBDCs *in vitro*. In our work we also observed that ALP activity in HBDCs treated with specific anti-αv antibody was lower on alumina than TCPS. Our results demonstrate that the specific effects of the αv integrin subunit blockade are time-dependent and slightly differ according to the type of biomaterial used.

Conclusions

In summary, we reported that blocking of integrin αv subunit with antibody influences proliferation and ALP expression in both alumina and TCPS cultures. Our data suggest involvement of the αv subunit in signal transduction in cellular behavior and maturation of HBDCs. Therefore, the investigation of molecular mechanism of integrin signaling governing human osteoblasts differentiation is very important for understanding the pathogenesis of human bone disease and our knowledge on cell activity after bone repairing interventions. Nevertheless, more intense studies of the interactions with other adhesion molecules are still needed to elucidate the role of integrin αv in behavior of human osteoblasts.

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