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POROUS COATED TITANIUM IMPLANTS DO NOT INHIBIT MESENCHIMAL STEM CELLS PROLIFERATION AND OSTEOGENIC DIFFERENTIATION

Boris Antonov[1], Ivan Bochev[2], Milena Mourdjeva[3], Plamen Kinov[1], Lubomir Tzvetanov[1], Ivan Sheitanov[4], Stanimir Kyurkchiev[5]
[1]University Hospital "Queen Giovanna - ISUL", Department of Orthopedics and Traumatology, Sofia, Bulgaria
[2]SAGBAL „D-r Shterev", Sofia, Bulgaria
[3]Bulgarian Academy of Sciences, Institute of Biology and Immunology of Reproduction, Department of Molecular Immunology, Sofia, Bulgaria
[4]Department of Rheumatology, University Hospital "St. Ivan Rilski", Sofia, Bulgaria
[5]Tissue bank "Bulgen", Sofia, Bulgaria
Correspondence to: Plamen Kinov
E-mail: plamenkinov@yahoo.com

ABSTRACT

The aim of this study was to investigate the proliferation and osteogenic differentiation of mesenchymal stem cells in contact with porous coated titanium implant. Human mesenchymal stem cells (BM-MSCs) were obtained from bone marrow aspirate from the femoral canal during elective hip replacement. The MSCs were isolated and cultured. The resulting cells were seeded on porous coated titanium plates obtained from prosthesis blanks that had all the characteristics of the final product. The same cells were grown on classic plastic polystyrene plates as a control specimens. The proliferation and osteogenic differentiation of the isolated and cultured mesenchymal stem cells were examined. Our results show that under the same conditions there were no significant differences between the study and control groups. Titanium implants with porous coated surface provide favorable conditions for the proliferation and differentiation of bone marrow stem cells and did not inhibit their development. In conclusion, it may be possible to advance this methodology to stimulate healing of bone defects and particularly massive osteolysis in the revision arthroplasty setting. MSCs may provide an alternative to the use of autologous and allogenic bone-grafts in orthopedic surgery.

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Keywords: mesenchymal stem cells, titanium plates, cell vitality and adhesion, cell proliferation and proliferative activity, osteogenic differentiation

Introduction

Hip replacement is one of the most successful surgeries with a well tracked and documented results over the last 50 years. Evolution in the development of this process does not stop to progress continuously. The endeavor is to make an implant that once inserted will not need revision. Numerous investigators are focused on various interrelated areas. One of them is the creation of an implant contact surface, that has a positive biological effect on bone building elements, resulting in a fast, seamless proliferation and differentiation of cells from the bone line in the contact zone. Providing stem cells with good functional capacity could help to resolve this problem.

The aim is rapid ingrowth of bone into the implant, creation of a larger contact area and a more endurable connection. All biomechanical, biochemical and biological approaches that potentiate bone integration are a step forward in the search end result - life-long prosthetic replacement.

The aim of this study was to investigate the proliferation and osteogenic differentiation of mesenchymal stem cells isolated from bone marrow (BM-MSCs) and placed in contact with the porous coated titanium implant. In order to fulfill our aim we compared the behavior of cells cultured on titanium plates with that of cells cultured on classic plastic plates as controls.

Materials and Methods

Aliquots of bone marrow aspirates (5-10 ml) were obtained from 5 patients undergoing elective hip replacement surgery (Clinic of Orthopedics and Traumatology, Queen Giovanna - ISUL University Hospital, Sofia, Bulgaria) after an informed consent was signed. Commercial pure titanium sheets were provided by Implant G Ltd. (Etropole, Bulgaria), specialized in the production of endoprostheses for large joints. The contact surface was uniformly porous with size of the pores ranging from 15.641 to 19.593 μm. (**Table 1**). Prior to use in a cell culture, square-shaped titanium specimens with dimensions 10 x 10 mm were steam sterilized at 121°C for 45 min.

TABLE 1

Characteristics of porous coated titanium plates

$\overline{R_z}$, μm	$R_{z\,min}$, μm	$R_{z\,max}$, μm	$R = R_{z\,max} - R_{z\,min}$, μm	σ, μm	$R = 6\sigma$, μm
17,761	15,641	19,593	3,952	1,42	8,52

Isolation and cultivation of human bone marrow mesenchymal stem cells (BM-MSCs)

BM-MSCs were isolated from bone marrow samples and further characterized following the routine procedure develeoped in our laboratory (2). Briefly, BM nuclear cells were obtained

after erythrocyte removal by ACK-lysing buffer (0.15M NH_4Cl; 0.1mM EDTA; 0.01M $NaHCO_3$; pH=7.2–7.4), then washed in Dulbecco's modified Eagle medium (LG-DMEM, PAA, Austria) and cultured in 10 cm^2 6-well plates (Orange Scientific, Belgium) at a concentration of 1.0×10^5 cells/cm^2 in LG-DMEM containing 10% fetal calf serum (FCS, PAA, Austria). After 7 days, nonadherent cells were removed and further on the medium was changed every 4th day of culture period. When culture reached 80-90% confluence, adherent cells were trypsinized and expanded in 25 cm^2 flasks (Orange Scientific, Belgium). Cells at second, third and fourth passages were used for experimental analysis.

Confocal microscopy

Human BM-MSCs (passage 3) were grown onto the titanium specimens for one week at 37°C, 5% CO_2. The titanium specimens were washed in PBS and fixed in paraformaldehyde (4% in PBS) for 15 min at RT. After washing twice with PBS, the cell nuclei were stained with Hoechst 33258 (1:1000, Sigma-Aldrich) for 5 min and after washing were mounted in Flouromount-G (Southern Biotech, USA). Fluorescent specimens were analyzed by a confocal scanning laser microscope (Leica TCS SPE, Leica, Germany).

Cell viability, adhesivity and proliferation activity

MTS assay was used to measure cell viability, adhesivity and proliferation. BM-MSCs (passage 4) were seeded onto the titanium specimens and tissue culture polystyrene (control) at an initial density of 5 x 10^3 cells/well in a 24-well plate (Orange Scientific, Belgium) for 5, 10 and 15 days, respectively. The culture medium (LG-DMEM + 10% FCS) was changed every 2 days during culture. After each incubation period an MTS assay was performed and analyzed according to the manufacturer's instructions (Promega, CellTiter 96® AQ One Solution Cell Proliferation Assay). 40 µl of MTS (tetrazolium compound) was added to each well containing 200 µl of standard culture medium and incubated at 37°C for 2 hours in a humidified, 5% CO_2 atmosphere. The MTS tetrazolium compound is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product is directly proportional to the number of living cells in culture. The absorbance of the solution from the experimental wells was measured at a wavelength of 490 nm with a spectrophotometric micro-ELISA reader (Dynatech AG, USA). Each experiment was repeated 3 times and the mean value was presented as the final result.

Osteogenic differentiation

To promote osteogenic differentiation, BM-MSCs (passage 3) were seeded onto the titanium specimens and tissue culture polystyrene (control) at a density of 1 x 10^4 cells/cm^2 into 24-well plates (Orange Scientific, Belgium) in triplicate and were cultured in LG-DMEM supplemented with 10% FCS, 100 nM dexamethasone (Sigma-Aldrich), 0.2 mM ascorbic acid-2-phosphate (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich) (6). The osteogenic induction medium was replaced every 3rd day for 3 weeks. For the negative control, cells were grown in LG-DMEM + 10% FCS. At the end of the induction period, osteogenic differentiation was determined by measuring the staining for alkaline phosphatase (ALP) activity, following the protocol of Leskela et al. (9). After washing of the cells with PBS, alkaline phosphatase buffer (0.05 M Na_2CO_3; 0.5 mM $MgCl_2$; pH=9.5) containing 0.1% Triton X-100 (Merck, Germany) was added to each well and the assayed plates were frozen (-70°C; 10 min.), whereupon immediately thawed. Afterwards 4-p-nitro-phenylphosphate (pNPP; 3.5 mM in ALP buffer; Sigma-Aldrich) was used as a substrate to assess the ALP activity and the absorbance of

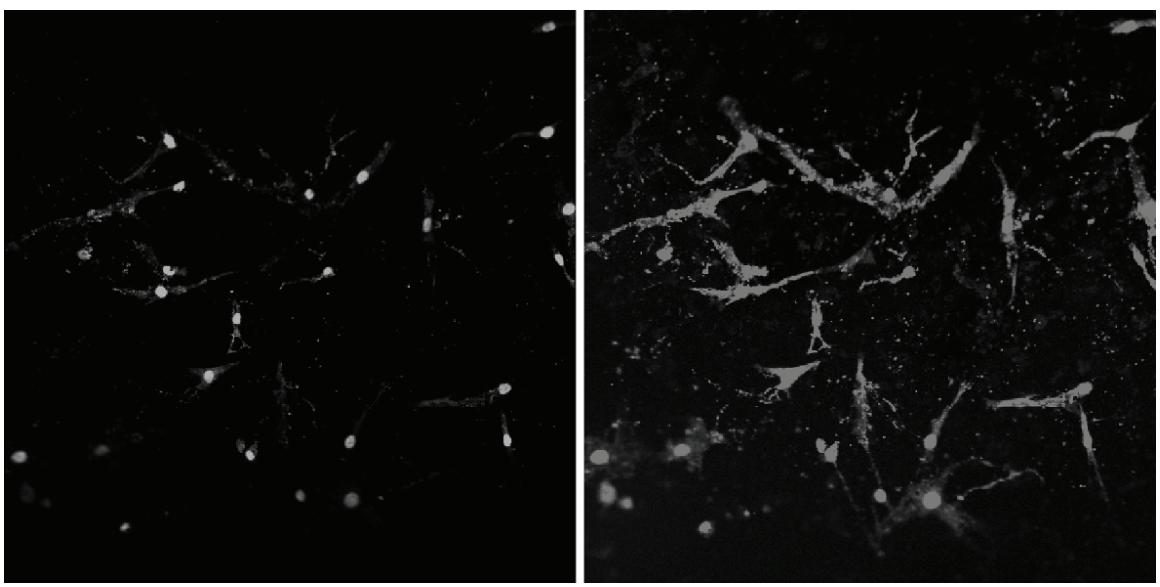

Fig. 1. Human BM-MSCs (passage 3) cultured on titanium specimens

Confocal microscope was used to scan the titanium surface at day 2 and day 7 after seeding. Cells were visualized by staining nuclei with Hoechst 33258. BM-MSCs spread over the titanium specimen. The morphology of BM-MSCs was typical with fibroblast-like shape

the final colored solution was measured at 405 nm on a micro-ELISA reader (Dynatech AG, USA).

Statistical analysis

Quantitative data are expressed as means ± standard deviations (SD). STATGRAPHICS Centurion XV 15.1.02 was used for analysis of data. Statistical significance of difference between diverse data sets was assessed by one-way analysis of variance (ANOVA). A level of $p \leq 0.05$ was considered significant.

Results and Discussion

Complete immunophenotypic characterization and identification of the bone marrow–derived MSCs was performed in our previous investigations (2). Some of the main characteristics of the *in vitro* cultured BM-MSCs are summarized in **Table 2** and it shows that cultured cells express specific mesenchymal markers (CD29; CD44; CD73; CD90; CD105) but do not express hematopoietic markers (CD34, CD45). Isolated cells also exert clonogenic capacities and have the potential for osteogenic and adipogenic differentiation (2). For the purpose of the current study which was to assess the ability of BM-MSCs to be cultured onto titanium specimens, human BM-MSCs (passage 3) were grown onto the titanium specimens for 7 days. Afterwards, the cell nuclei were stained with Hoechst 33258 and analyzed by a confocal microscope. As shown on **Fig. 1**, BM-MSCs spread over the titanium specimen at an even distribution without any gaps in the surface of the specimen. The morphology of BM-MSCs was typical with fibroblast-like shape.

TABLE 2

Characteristics of the *in vitro* cultured BM-MSCs

Parameter	BM-MSC
CFU-F (% cells seeded)	14 ± 2
CD34	-
CD45	-
CD29	+
CD44	+
CD73	+
CD90	+
CD105	+
osteogenic differentiation	+
adipogenic differentiation	+

Cell viability, adhesivity and proliferation activity

The results from the MTS assay indicated that BM-MSCs were able to effectively adhere to the surface of titanium specimens and to maintain at the same time their proliferative potential. One day after seeding onto titanium substrate most of the BM-MSCs were attached to it and viable as shown in **Fig. 2**. Regarding the cell growth dynamics on titanium surface, the data revealed that BM-MSCs entered the phase of exponential growth at day 5 after seeding. Their proliferative activity retained its stability until the last point of examination (day 15; **Fig. 2**).

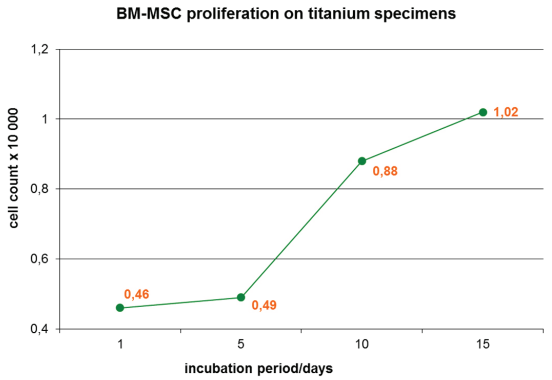

Fig. 2. Proliferation of BM-MSC cultured on titanium specimen analyzed by MTS test

BM-MSCs entered the phase of exponential growth at day 5 after seeding and retained proliferation activity until day 15

Osteogenic differentiation

To investigate the potential for osteogenic differentiation of BM-MSCs cultured onto titanium substrate, we characterized the ALP activity as an osteo-specific parameter. For BM-MSCs cultured on titanium specimens, the quantitative pNPP assay revealed significantly higher levels (6.5-fold; $p < 0.05$) of ALP activity in comparison to those for the undifferentiated control cells (**Fig. 3**), indicating a successful osteogenic differentiation. Moreover, the ALP activity of BM-MSCs differentiated on titanium substrate was comparable to that of the BM-MSCs cultured onto polystyrene surface and used as a positive control (**Fig. 3**). The latter are cells that have well-known and previously determined capacity for successful *in vitro* osteogenic differentiation (1).

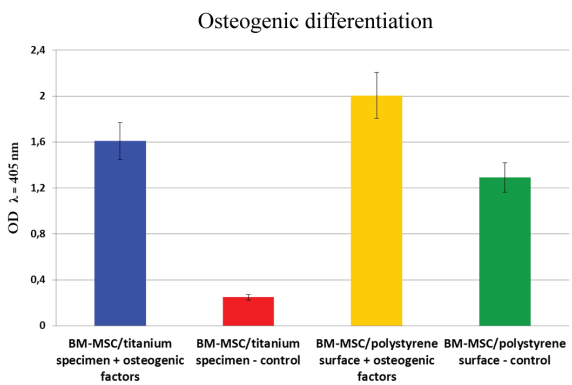

Fig. 3. Osteogenic differentiation of BM-MSCs cultured on titanium substrate

ALP activity of BM-MSCs differentiated on titanium substrate was comparable to that of the BM-MSCs cultured on polystyrene surface

Bone marrow has been shown to contain a population of several progenitor cells (3). One class of these multipotent progenitor cells is referred to rare mesenchymal stem cells. It is well established fact that they are capable of forming osteoblastic, chondrogenic and other connective tissue lineages

(5, 8, 13,16). In order to evaluate the influence of orthopedic implants on bone ingrowth, we examined the effect of critical-sized porous coated titanium plates (**Table 1**) on the growth and differentiation of cultured autologous mesenchymal stem cells. Morphometric analyses demonstrated that the pores of the implants had been loaded with mesenchymal stem cells. No substantial difference in vitality and adhesive capability between study group and controls was observed. Moreover, we were able to successfully induce MSCs osteogenic differentiation in the presence of titanium implant. The porous coated prosthetic titanium material did not influence inversely stem cell proliferation and differentiation.

The surgical treatment of extensive bone defects remains a great challenge in orthopedic and reconstructive surgery. The standard approach includes autologous bone grafting usually from the iliac crest or allogenic bone transplants. However, both methods have certain drawbacks including limited availability, donor site morbidity, risk of infection etc. (14). In previous studies, it has been demonstrated that bone marrow derived MSCs are promising alternative for treatment of extensive bone defects (12, 15). However, human studies are very limited. Our investigations along this line demonstrated that MSCs were capable of proliferation and osteogenic differentiation on porous coated titanium implants. These findings may expand the potential clinical armamentarium for treatment of extensive bone defects invariably present in loose arthroplasties.

One of the problems with allograft utilization is the lack of osteoprogenitor cells. In order to achieve bone formation and healing, four parameters have to be respected: utilization of growth factors, scaffolds, mesenchymal stem cells, and stable mechanics (7). Drawback of allografts is the lack of osteoinductive properties and MSCs. On the other hand, MSCs secrete a number of bioactive factors which modulate immune system and provide a milieu that stimulates bone regeneration and osteoblast proliferation (4, 10, 11).

We established a protocol for differentiation of bone marrow stem cells. Their clinical use is of great interest since they are easily obtained from bone marrow during the surgical procedure. However, medical and legal concerns handicap the clinical use of MSCs. Possible solution of this problem is autologous harvesting and immediate implantation. On the other hand, *in vitro* MSCs proliferation and differentiation may greatly improve bone regeneration and potentially lead to better clinical outcome. Further research is required in order to introduce the technique in clinical practice.

Our study has certain limitations: a) the behavior of MSCs *in vitro* may not reflect normal behavior of bone marrow stem cells; b) the *in vitro* results might not be translated *in vivo*; c) *in vivo* implants are under physiological load which might change stem cell differentiation and proliferation; d) in this initial experiment, the anatomical site for repair of porous coated was not investigated.

Conclusions

Autologous cultured bone-marrow-derived mesenchymal stem cells that had been loaded onto porous titanium plates were capable of proliferation and osteogenic differentiation. It may be possible to advance this methodology to stimulate healing of bone defects and particularly massive osteolysis in the revision arthroplasty setting. Obtaining autologous stem cells is feasible and well established process. It may provide an alternative to use of autologous and allogenic bone-grafts in orthopedic surgery. In this regard, the clinical effects of MSCs may have profound clinical applications that will change the current paradigm in treating bone deficiencies and orthopedic diseases.

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