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PROCEEDINGS OF THE INTERNATIONAL CONFERENCE NANOMATERIALS: APPLICATIONS AND PROPERTIES
Vol. **1** No 2, 02NNBM24(3pp) (2012)

Application of Nanocomposite Coatings with Different Structural Physical and Chemical Characteristics in Tissue Engineering

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(Received 19 June 2012; published online 09 August)

The research covers the results of experimental studies of the effect character of nanocomposite coatings with different physical and chemical parameters (type, roughness, hydrophilic-hydrophobic characteristics) on structural and functional properties (adhesive potential, phenotype, gene expression) of mesenchymal stem cells (MSCs). On the tested nanocoatings (Al_2O_3, ZrO_2, Ta_2O_5) the capability of oxide coating Al_2O_3 to enrich the *in vitro* cultured bone marrow (BM) with the cells of MSCs phenotype markers as well as to increase the expression rate of *ido* gene in them, which may extend the spectrum of their therapeutic application in clinics, has been found.

Keywords: Nanocomposite coatings, Mesenchymal stem cells, *ido* gene.

PACS numbers: 81.15. – z, 87.85.Lf

1. INTRODUCTION

Rushing investigations in the field of cell and tissue engineering has demanded the intensifying of the works on introducing the novel technologies, methods, approaches, including the use of different on their origin nanomaterials [1, 2].

Even now there has been proved the possibility of controlling the functional state of cells by modifying the surface of nanocomposite coatings whereon they are cultured [3]. There was demonstrated the effect of physical and chemical parameters of the matrices (titanium and its alloys, ruthenium, niobium, different polymers) on functional characteristics of cultured osteoblasts, fibroblasts, macrophages [4]. However in spite of numerous reports on studying the behaviour of different cells when culturing on various nanocarriers in respect of the stem compartment cells the existing data are apparently insufficient. It is commonly known that during stem cell culturing *in vitro* they manifest a high plasticity potential. To implement it the certain culturing conditions and standard media providing differentiation towards the lineage needed have been designed. The modification of surface coatings whereon they are cultured may obviously also be additional and quite significant factor of controlling the functional status of stem elements.

In this connection the research aim was investigation of the effect peculiarities of nanocomposite coatings with different physical and chemical parameters on structural and functional characteristics (adhesive potential, phenotype, gene expression) of cultured on them cells with MSCs features.

2. MATERIALS AND METHODS

In the present study the samples were glass substrates (Petri dishes), uncoated and oxide-coated (Al_2O_3, ZrO_2 (MS) magnetron-sputtered and Ta_2O_5 (EB) e-beam-evaporated) with different roughness parameters – 20, 200 and 400 nm. The Al_2O_3 (MS) coatings deposition was performed in a high-vacuum pumping system with base pressure of about 10^{-3} Pa. The main process parameters were: magnetron discharge power 1 – 8 kW, oxygen source power 1 kW, deposition rate 8 μm/hour [5]. The Ta_2O_5 films evaporation process was carried out at initial vacuum of 7×10^{-4} Pa, operational mode vacuum of 3×10^{-3} Pa, anode current of 50 mA and calculated evaporation power of 350 W [6]. The deposition rate under these conditions was 28 nm/min.

The surface roughness was measured by means of a Hommel T-2000 profilometer and the surface topography was estimated by AFM (Quesant Instrument Corporation, USA). The coatings' structure was investigated by means of XPS and XRD. The X-ray photoelectron spectroscopy measurements were carried out using an ESCALAB MkII (VG Scientific) electron spectrometer at a base pressure in the analysis chamber of 5×10^{-8} Pa (1×10^{-6} Pa during the measurement), using an Alα X-ray source (excitation energy $h\nu = 1486.6$ eV).

Other parameters, such as surface free energy (SFE), its polar and dispersion components and fractional polarity were determined by means of the Wu and Owens-Wendt-Rabel-Kaelble methods. The contact angles were measured on a Kruss K12 Tensiometer at 20 °C [6].

BM cells were obtained from femoral bones of CBA/H mice and cutured in Iscove's medium containing 10% FBS, 50 units/ ml penicillin, 50 units/ml streptomycin in 3 cm glass Petri dishes without or with studied nanocoatings with seeding density of $0.5\text{-}1 \times 10^6$ cells/cm² at 37°C, 5%CO_2. Adhesive potential of BM cells was assessed in 48 hrs of culturing according to the routine method [7]. Cell cultures were visually controlled using light phase contrast microscope (Axiovert 25, Zeiss, Germany).

BM cells were passaged with the density of $0.5\text{-}1.0 \times 10^4$ cells/cm² when achieving subconfluent monolayer. At the end of each passage the cells were taken-off with trypsin-EDTA solution (Sigma) according to the standard methods [8], inactivated with the medium containing 10% FBS, washed-out and their structural and functional characteristics were estimated.

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The MSCs phenotypical characteristics were determined by a FACS Calibur cytofluorimeter using fluorochromal monoclonal antibodies to CD73, CD105, CD106, CD90, CD44 structures.

The data obtained with cytofluorimetrical analysis were statistically processed with WinMDI 2.8.

The expression rate of *ido* gene in cultured MSCs (passage 1 and 2) on various substrates was assessed by PCR with the stage of reverse transcription (PCR-RT) during an hour after thawing [9] on the presence of their amplicons. Total RNA was isolated by means of Diatom RNA Prep 100 (Isogene Lab Ltd, Russia) from 1x10^5 cells of each sample. The resulted mixture of nucleic acids was treated with DNase I according to the instructions of the producer ("Syntol" Ltd, Russia). RT reaction was set using random-oligonucleotides and revertase (M-Mlv) (R&D institute, "Reverta L", Russia). The primers to the studied genes were designed using the data base "GenBank" (NCBI BLAST, USA): *ido*-NM_008324.1 and were synthesized at "Medbioservis Ltd" (Kiev, Ukraine). The amplification products were detected with capillary electrophoresis in chip analyzer ("Agilent" 2100, USA).

The amount of transcripts of the studied targets was compared on the base of relative quantitative estimation of amplification products. In brief, there were prepared consecutive ten-fold dilutions of initial cDNA preparation from each the sample studied. To every dilution there was added a reactive mixture and the amplification was performed. The less target consequences were in initial preparation, the earlier (i.e. at smaller dilutions) amplifications stopped. The dilution logarithm (lg) cDNA served as the index of expression rate of the investigated genes. The results were levelled in respect to the expression rate of *beta actin* gene (housekeeping gene) (NM_007393.3). Reactive mixture with no cDNA was a negative control.

The findings were statistically processed with Student's method using MS Excel software.

3. RESULTS AND THEIR DISCUSSION

Receptor repertoire of cells represents a complicated system due to which the cells interact each other, structures of intercellular space and finally implement numerous functional activities. One of the main places in a wide spectrum of membrane receptors is taken by the cell adhesion molecules. There is noted that the surface relief acts as the factor modulating an adhesive cell potential [4].

The influence of coatings' deposition and treatment conditions on BM adhesive potential was studied. The adhesive potential of BM cells was statistically and significantly different depending on substrate materials. The best results were obtained on the glass and glass/Al_2O_3 (MS) surface. The statistical difference between cell adhesive and survival parameters depending on surface roughness in the range 20-400 nm was observed. The cell adhesion decreases with the rise of surface roughness parameters in the range 20-400nm.

The structure of Al_2O_3 (MS) and ZrO_2 (MS), magnetron sputtered and Ta_2O_5 e-beam evaporated thin films was investigated by means of XPS and XRD methods. X-ray diffraction profiles of Al_2O_3 (MS), ZrO_2 (MS), and Ta_2O_5 (EB) as deposited coatings demonstrate the amorphous nature, no peaks were observed. The structural analysis of Al_2O_3 (MS) and ZrO_2 (MS) oxide coatings by means of XPS method was performed. The photoelectron spectra of Al_2O_3 (MS) and ZrO_2 (MS) oxides demonstrate that oxide coatings have a strong stoichiometric composition with binding energy peaks E(Al2p) – 74.4 eV, E(O1s) – 531.3 eV for Al_2O_3 (MS) films. The binding energy peaks E(Zr3d) – 182.4 eV, E(O1s) – 530.2 eV for ZrO_2 (MS) coatings were observed.

The surface free energy SFE and its polar and dispersion parts, fractional polarity were estimated by Wu method for two liquids system and by Owens-Wendt-Rabel-Kaeble' methods for liquid system: α-bromonaphthalene- formamide-ethylene glycol-diidomethane- glycerol-water. The surface free energy changes but is not dependent from the surface roughness parameters in the range 20, 200 and 400 nm. The surface free energy was in the range 52-53 [mN/m] for Al_2O_3 (MS), 48-51 [mN/m] for ZrO_2 (MS), 41-44 [mN/m] for Ta_2O_5 (EB) coatings. The results allow the separating of the influence of roughness and surface free energy effects on the regularities of cell/nanomaterial interactions.

The results presented in Fig. 1 demonstrate the dependence of adhesive properties of BM cultured cells both on coating type and surface roughness (20, 200, 400 nm) whereon it was applied. The reduced adhesive potential of BM cells was shown with a rise in embedding roughness.

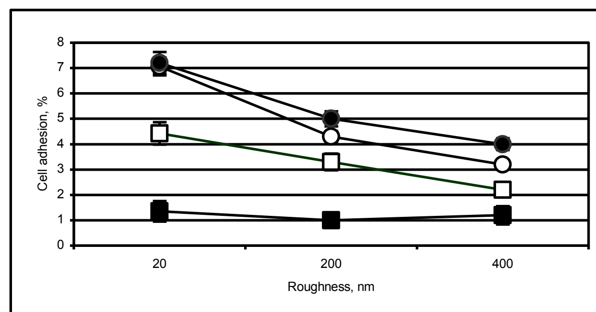

Fig. 1 – Adhesive potential (percents) on various substrate materials with roughness parameters within the range of 20-400 nm after 48 hrs' culturing: (◦) glass, (•) glass/Al_2O_3 (MS), (▫) glass/ZrO_2 (MS), (▪) glass/Ta_2O_5 (EB).

Percent of adhesive cells to glass with Al_2O_3 coating was the highest one, and the lowest one was that with Ta_2O_5. Taking into account that in addition to topography of substrate surface the cell adhesion is also affected by their hydrophobic-hydrophilic characteristics (Table 1) [10], one can conclude that the highest hydrophilic properties are inherent to Al_2O_3 coating. The Ta_2O_5 coating demonstrates the least value of surface energy and more hydrophobic origin. So, it is evident that the cell adhesion is affected both by roughness rate and hydrophobic-hydrophilic characteristics of the coatings.

By means of cytofluorimetric research method we have estimated the content of cells with MSCs phenotype markers : $CD73^+$, $CD106^+$, $CD105^+$, CD90+, $CD44^+$ in BM culture, passaged on glass (control) and on glass with Al_2O_3 coating and roughness of 20 nm (experiment) (Fig. 2), demonstrating the highest adhesion to BM cells.

Table 1 – Contact angles (water) and values of surface energy of coatings

№	Samples	Contact angles with water (hydrophility), grad	Complete surface energy (interval for different roughness values) [mN/m]
1	Glass control	34-36	60-62
2	Glass /Al_2O_3	47-52	52-53
3	Glass /ZrO_2	52-57	48-51
4	Glass /Ta_2O_5	61-65	41-44

It has been established that content of $CD73^+$, $CD105^+$ and $CD106^+$ cells cultured on Al_2O_3 after the 1st passage was 1.4 -1.5 times higher, after the second it was 1,2-1.6 times higher if compared with the corresponding to each passage control (glass). The increased content of $CD73^+$, $CD105^+$ and $CD106^+$ cells cultured on Al_2O_3 at the second passage was statistically and significantly higher if compared with the 1st one. This fact strongly testifies to the capability of Al_2O_3 coating to selectively enrich the cultures with BM cells, expressing the MSCs markers.

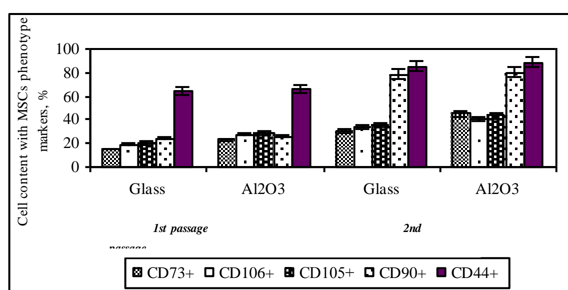

Fig. 2 – Content of cells with MSCs phenotype markers when culturing BM on glass with Al_2O_3 coating and without it

One of the key characteristics of MSCs is their immune modulating activity [11], implementation of which is related to *ido* gene expression in these cells. Namely to this gene belongs the key role in activation of immune system suppressor link. In this connection in the studied MSCs the expression of *ido* gene was assessed.

The data presented in Fig 3 and 4 testify to the increased content of transcripts of *ido* gene in MSCs after their culturing on Al_2O_3 nanocoating after the 1st passage in 3.6 times and in 7 times after the second one if compared with the those cultured on glass. It is important that after the second passage the expression rate of ido gene in the cultured on glass MSCs reduced twice. The fact of involvement of the embedding into activation in MSCs of *ido* gene expression responsible for the production of IDO enzyme is an interesting one not only from the point of view of fundamental studies but also the widening of spectrum of their clinical application.

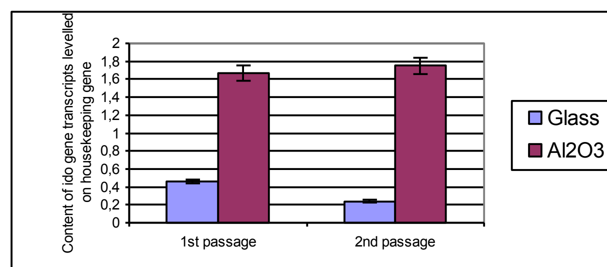

Fig. 3 – Semi-quantitative analysis of *ido* gene expression in MSCs cultured on various matrices

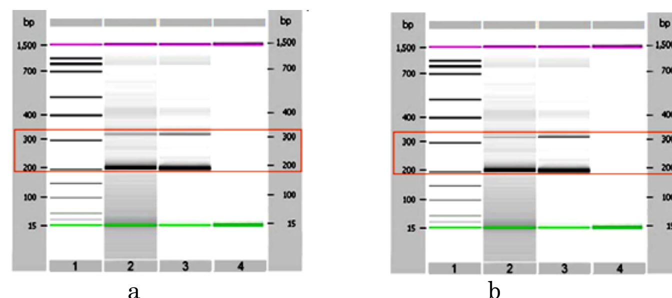

Fig. 4 – Detection of *ido* gene transcripts in MSCs (a - 1st passage, b - 2nd passage cultured on different matrices . *Ido* gene (upper marker), house-keeping gene (lower marker); groups: 1 – fragment length marker, 2 – glass, 3 - Al_2O_3 (20 nm), 4 – blank control

4. CONCLUSION

Our data demonstrate that the cell adhesive potential is different for various oxide coatings deposited by magnetron sputtering and e-beam evaporation. The best results were obtained in the case of magnetron-sputtered oxide coatings with minimal roughness within the range of 20 – 400 nm, the intermediate values of the surface free energy within the range of 50 – 60 mN/m and the bulk of SFE polar components and fractional polarity.

The possibility of directed modification of structural and MSCs functional characteristics by culturing substrates has been proved. In particular, it has been found the capability of Al_2O_3 oxide coating applied to a glass substrate with 20 nm roughness to selectively enrich the cultures with BM cells with MSCs phenotype markers, as well to increase the expression rate of *ido* gene in them.

The established fact of the rise of the content of *ido* gene transcripts in MSCs after culturing on Al_2O_3 coating focuses to their possible use when treating the pathologies of immune genesis.

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