Molecular markers of adhesion, maturation and immune activation of human osteoblast-like MG 63 cells on nanocrystalline diamond films

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1. Introduction

Nanostructured materials have recently become very important for constructing advanced bioinspired tissue replacements. Materials composed of various nanoparticles, such as nanocrystals, nanotubes or nanofibres, resemble the architecture of natural extracellular matrix (ECM), which contains nanofibres of various proteins, and in the case of bone, also inorganic nanocrystals. The nanoroughness of the material surface, i.e., the presence of irregularities smaller than 100 nm, is considered to mimic the folding of physiological ECM molecules and to improve their adhesion to the material surface from the cell culture media, body fluids or cell secretions. On nanostructured surfaces, it is believed that ECM molecules are adsorbed in an appropriate amount and spectrum, and particularly in an appropriate geometrical conformation that can provide access to specific amino acid sequences in these molecules (e.g., RGD, KRSR) for cell adhesion receptors, i.e., integrin and non-integrin adhesion receptors on the cell membrane [4–6].

Various materials have been used for constructing nanostructured surfaces. Examples include synthetic polymers, such as a copolymer of poly lactide and poly glycolide (PLGA) etched with NaOH [7], carbon nanofibres [2], nanotubes or fullerenes [8], ceramics, metals, such as titanium and its alloys or oxides [1,3] and various carbon-based or inorganic materials [4–6]. However, all these materials may have some drawbacks, e.g., possible retention of NaOH in the polymers and its subsequent cytotoxic action [7], potential cytotoxicity of carbon nanotubes and fullerenes [8], as well as the release of cytotoxic and immunogenic ions from metallic materials [for a review, see [10]].

Diamond & Related Materials
However, adhesion and cytoskeletal proteins participating in the process of cell attachment and spreading have not yet been systematically studied in bone-derived cells growing on NCD layers. On other hard biocompatible layers for potential biomaterial coating, represented by nanocomposite hydrocarbon plasma polymers enriched with Ti, the vascular endothelial cells displayed higher concentrations of talin, an integrin-associated protein, as well as von Willebrand factor, a marker of endothelial cell differentiation [9]. A higher concentration of talin and vinculin, another integrin-associated protein, was also found in human osteoblast-like MG 63 cells cultured on a terpolymer of polytetrafluoroethylene, polyvinylidifluoride and polypropylene mixed with single-wall carbon nanohorns [8]. Therefore, in the present study, an enzyme-linked immunosorbent assay (ELISA) was applied for semiquantitative studies on the concentration of integrins αv and β3 (i.e., receptors for vitronectin, fibronectin and collagen), talin and vinculin (integrin-associated proteins present in focal adhesion plaques), and β-actin (a component of cytoplasmic cytoskeleton) in human osteoblast-like MG 63 cells cultured on NCD films deposited on silicon substrates. In addition, cell maturation was evaluated by the concentration of osteocalcin and osteopontin, i.e., calcium-binding non-collagenous ECM glycoproteins important for the bone tissue mineralization process. Potential immune activation of cells on NCD films was estimated by the concentration of intercellular adhesion molecule-1 (ICAM-1), an immunoglobulin cell adhesion molecule binding cells of the immune system (for a review, see [18]). Since not only nanoroughness but also micron- and submicron-scale roughness is important for the cell–material interaction [19–21], the NCD layers were also constructed with a hierarchically organized submicron- and nano-scale structure, i.e. with nanostructures superposed on submicron-sized irregularities prefabricated on the silicon substrates by grinding them. It is believed that materials organized on two or more length scales bear a closer resemblance to biological matrices than those with single scale features, and thus these materials should be more advantageous in biomedical applications [20].

2. Material and methods

2.1. Preparation and characterization of NCD layers

The preparation and physicochemical characterization of NCD films were described in detail in our earlier studies [8,15–17,22]. Briefly, the films were grown on (100) oriented silicon substrates (40 mm in diameter) by a microwave plasma enhanced chemical vapor deposition (PECVD) method in an ellipsoidal cavity reactor (AIXTRON – P6, Germany). Prior to the NCD growth, the silicon substrates were either mechanically lapped to roughness up to 300 nm or polished to atomic roughness (RMS – 1 nm). Therefore, the deposited NCD films were either nanostructured (nano-NCD) or hierarchically submicron- and nanostructured (submicron-nano-NCD). The Si substrates were hermetically “sealed” in a nanocrystalline diamond layer (i.e. overcoated on both sides) to minimize any potential unwanted biochemical reaction of the Si substrate with the cells and their environment. Finally, the NCD films were chemically cleaned and treated in oxygen plasma to achieve a hydrophilic surface character. The control samples were polystyrene tissue culture dishes (GAMA GROUP a. s., Ceske Budejovice, CR) and microscopic glass coverslips (Menzel Glaser, Germany); both of them had a flat surface (RMS – 1 nm).

The Raman spectra of the deposited NCD films were measured using a 514.5 nm excitation wavelength laser, which enabled us to determine the diamond character of the deposited films (i.e., the sp3 hybridization). The typical Raman spectrum of an NCD film displayed one dominant peak centered at wavenumber 1333 cm⁻¹ (optical phonon in diamond) [23]. In addition, X-ray photoelectron spectroscopy (XPS) determined that the ratio of carbon in sp3 hybridization, characteristic for diamond, to sp2 C was more than 95% [24]. As confirmed by atomic force microscopy (AFM), two types of NCD morphologies were prepared: a) nanostructured surfaces with RMS of 8.2 nm, and b) surfaces with a hierarchically organized submicron- and nanostructure (i.e., surfaces of RMS roughness up to 301 nm) were further patterned with nano-scale features of RMS roughness as low as 7.2 nm). The AFM images were published earlier [16].

The surface wettability of the films was determined from the contact angle measured by a static method in a material–water droplet system using a reflection goniometer (SEE System, Masaryk University, Brno, Czech Republic). The measured wetting angle was approximately 30° for both types of NCD surfaces, i.e., nanostructured and hierarchically submicron-nanostructured, and this value was stable without significant changes with time [17].

2.2. Cells and culture conditions

The samples (diameter 40 mm) were sterilized with 70% ethanol for 1 h, placed into polystyrene Petri dishes (GAMA GROUP a. s., Ceske Budejovice, Czech Republic; diameter 50 mm) and seeded with human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK). Each dish contained 100 000 cells (i.e., about 5095 cells/cm²) and 6 ml of Dulbecco’s modified Eagle’s Minimum Essential Medium (DMEM; Sigma, Cat. No. D5648) supplemented with 10% fetal bovine serum (FBS; Gibco, Cat. No. 10270-106) and gentamicin (40 μg/ml, LEK, Slovenia). The cells were cultured for 5 days at 37 °C in a humidified air atmosphere containing 5% of CO₂.

2.3. Evaluation of the number, volume and protein content of cells

On day 5 after seeding, the cells were rinsed with phosphate-buffered saline (PBS; Sigma, Cat. No. P4417), detached by mild trypsinization (trypsin-EDTA, Sigma, Cat. No. T4174; 5 min, 37 °C), resuspended in PBS and counted in a Bürker haemaocytometer. In the suspension, the cells were spherical and their diameters (d) were measured in a ViCell XR analyzer (Beckman Coulter, U.S.A.). The volume of cells (V) was then calculated as $V = 4/3\pi d^2$. The protein content was determined by a modified Lowry method in cell homogenates prepared by destruction of 10⁶ cells per 1 ml of distilled and deionized water in a sonicator (Bandelin Sonoplus HD 3080, BANDELIN electronic GmbH & Co.) for 10 s [18].

2.4. Enzyme-linked immunosorbent assay (ELISA)

The concentration of αv- and β3-integrins, talin, vinculin, β-actin, osteocalcin, osteopontin and ICAM-1 (CD 54) was measured in homogenates of MG 63 cells after 5-day cultivation. Trypsinized cells were resuspended in PBS, centrifuged, resuspended in distilled and deionized water (10⁶ cells/ml) and kept in a freezer at −70 °C overnight. The cells were then homogenized by ultrasonication for 10 s in a Bandelin Sonoplus HD 3080 sonicator (BANDELIN electronic GmbH & Co.), and the total protein content was measured using a modified method originally developed by Lowry [18]. Aliquots of the cell homogenates corresponding to 1–50 μg of protein in 50 μl of water were adsorbed on 96-well microtiter plates (Maxisorp, Nunc) at 4 °C overnight. After washing twice with PBS (100 μl/well), the non-specific binding sites were blocked by 0.02% gelatin in PBS (100 μl/well, 60 min) and then treated by 1% Tween (Sigma, Cat. No. P1379, 100 μl/well, 20 min). Primary antibodies, diluted in PBS and represented by polyclonal rabbit anti-human integrin αv (dilution 1:400 to 1:800, Chemicon, Cat. No. AB 1930), monoclonal mouse anti-human β3-integrin (dilution 1:200, Chemicon, Cat. No. MAB 1981, clone LM534), monoclonal mouse anti-chicken talin (dilution 1:200, Sigma, Cat. No. T3287, clone 8D4), monoclonal mouse anti-human vinculin (dilution 1:400, Sigma, Cat. No. V9131, clone hVIN-1), monoclonal mouse anti-β-actin (dilution 1:200, Sigma, Cat. No. A5441, clone AC-15), rabbit anti-human osteocalcin (1:49) purified antiserum IgG (dilution 1:500, Bachem Group, Peninsula Laboratories)
Inc., CA, U.S.A.; Cat. No. T-4743.0400), polyclonal rabbit anti-human osteopontin (dilution 1:500, Alexis, Cat. No. ALX-210-309) or monoclonal mouse anti-human ICAM-1 (anti-CD54) antibody (dilution 1:200, Exbio s.r.o., CR, Cat. No. 11-228, clone MEM 111), were applied for 60 min at room temperature (50 µl/well). As secondary antibodies, goat anti-mouse F(ab)’2 IgG fragment (Sigma, Cat. No. A3682, dilution in PBS 1:1000) was used after mouse monoclonal primary antibodies, and goat anti-rabbit IgG (Sigma, Cat. No. A9169, dilution 1:5000), was used after rabbit polyclonal antibodies or antisera. Both secondary antibodies were conjugated with peroxidase and applied for 45 min (50 µl/well). This step was followed by double washing in PBS and orthophenylene diamine reaction (Sigma, Cat. No. P1526, concentration 2.76 mM) using 0.05% H2O2 in 0.1 M phosphate buffer (pH 6.0, dark place, 100 µl/well). The reaction was stopped after 10–30 min by 2 M H2SO4 (50 µl/well) and the absorbance was measured at 490 and 690 nm by a Versa Max Microplate Reader ( Molecular Devices Corporation, Sunnyvale, California, U.S.A.).

The quantitative results were expressed as means±Standard Error of Means (S.E.M.). The statistical analyses were performed using SigmaStat (Jandel Corporation). Multiple comparison procedures were performed by the One Way Analysis of Variance (ANOVA), Student–Newman–Keuls method, using SigmaStat software (Jandel Corp, U.S.A.). p values equal to or less than 0.05 were considered significant.

3. Results and discussion

3.1. The cell number, volume and protein content in cells on NCD films

On day 5 after seeding, the cell number and total content of proteins evaluated in cells cultured on both NCD films were lower than in cells on the control polystyrene (PS) culture dish. The cell population density on the control PS reached 416 405±10 227 cells/cm², whereas on the nano-NCD and submicron-nano-NCD it was only 343 914±16 818 cells/cm² and 325 929±19 614 cells/cm², respectively (Ps<0.05 for both values). This could be due to a higher tendency of cells on both NCD films to detach themselves, though the cell viability, measured by a LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Molecular Probes, Invitrogen) or by a ViCell Analyser was very high and similar to that on polystyrene [16,17]. The tendency to cell detachment could be explained by the relatively high hydrophilia of O-terminated surfaces (contact angle about 30°). Highly hydrophilic surfaces are known to bind the adsorbed ECM molecules with relatively weak forces [5,6], which could lead to the detachment of these molecules especially at later culture intervals, when they bind a large number of cells. In accordance with these findings, extremely hydrophilic oxygen-terminated nanostructured diamond surfaces (contact angle<2°) almost completely resisted the adhesion of human mesenchymal stem cells derived from the bone marrow, whereas less hydrophilic nanodiamond surfaces (contact angle 86°) gave good support for the attachment, spreading and growth of these cells [25]. Also in our recent study [26], hydrogen-terminated NCD films (contact angle of 85–90°), deposited on glass substrates, increased the number of adhered human osteoblast-like MG 63 cells in comparison with unmodified glass substrates and standard polystyrene cell culture dishes. Moreover, on day 7 after seeding, when the cell layer was confluent, the cells adhered firmly to the NCD substrates without considerable cell detachment and loss [28]. On the other hand, highly hydrophobic material surfaces (contact angle more than 90°) often adsorb cell adhesion-mediating ECM molecules in a rigid and denatured form, which leads to a lower accessibility of cell adhesion motifs in these molecules to cell adhesion receptors, and to a reduced cell adhesion [4,5]. It is generally known that the cell adhesion and growth are optimal on moderately hydrophilic surfaces [4,5]. These surfaces could be achieved by a lower concentration of oxygen on the material surface, e.g. by a shorter exposure of NCD films with hydrogen.

The total protein content in the cells on the tested surfaces decreased proportionally to the cell numbers. The highest content of protein was found in cells cultured on the control PS (24 µg of total protein per 100 µl of cell homogenate containing 10^6 cells/ml), whereas the values measured in cells on nano-NCD and submicron-nano-NCD were lower (19 µg and 18 µg of total protein per 100 µl of cell homogenate, respectively). This might be due to the secretion of some protein molecules into the cell culture medium or their incorporation into a newly formed ECM [21], which was mostly degraded during cell trypsinization and thus not included in the protein content measurement.

At the same time, the cell volume remained similar in cells on PS (4650±365 µm³) and nano-NCD (4428±419 µm³), or even showed a slight tendency to increase in cells on submicron-nano-NCD surfaces (5231±349 µm³), though this difference was not significant. Possible explanations, which remain to be further investigated, include increased hydration of the cells caused either by stimulation of the Na⁺/K⁺/Cl⁻ cotransport system or by the influence of oxygen present on the NCD surface, as well as cell ageing (for a review, see [18]).

3.2. Specific markers of cell adhesion, cytoskeletal organization, differentiation and immune activation in cells on NCD films

The concentration of integrins with α, chain, measured by ELISA per mg of protein, was similar in cells on all tested surfaces. The absorbance, expressed as a percentage of the value obtained from the cells on the control PS dishes (100.0±0.02%), was 97.8±2.5% on the nano-NCD and 98.8±5.8% on the micro-nano NCD (Fig. 1A). Similarly, the absorbance found for β-integrins in cells on the PS dishes, nano-NCD and micro-nano NCD amounted to 100.0±3.7%, 94.9±2.8% and 104.2±17.7%, respectively (Fig. 1B).

The group of α-integrins comprises receptors for vitronectin (e.g., αvβ1, αvβ3, and the β-integrins mainly receptors for

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collagen (α2β1, α2β1), fibronectin (α5β1), and laminin (α7β1), i.e., ECM molecules which could be spontaneously adsorbed to the material surface from the serum supplement of the culture medium or deposited by the cells themselves [5]. Thus, the MG 63 cells on all tested surfaces might be equally active in binding these ECM molecules. However, the primary antibody used in this study recognized all αv or β1 chain-containing integrin molecules, i.e., including those not engaged in binding their ligands and not clustered in focal adhesion plaques. This issue needs further investigation using antibodies against phosphorylated integrins or integrins in an active conformation. Another approach would be a sequential biochemical method of cross-linking integrin–ligand complexes, followed by extracting bulk cellular components in a detergent and detecting bound integrins by ELISA [27].

On the other hand, the concentration of talin, i.e., an important integrin-associated protein present in focal adhesion plaques, increased significantly in cells on the submicron-nano-NCD (absorbance 136.0±6.2%) in comparison with the values for cells grown on polystyrene dishes.

Fig. 1. Concentration of αv-integrins (A), β1-integrins (B), talin (C), vinculin (D), β-actin (E), osteocalcin (F), osteopontin (G) and ICAM-1 (H) in osteoblast-like MG 63 cells on day 5 after seeding on polystyrene cell culture dishes (PS), nanostructured NCD films (nano-NCD) or NCD films with hierarchically-organized submicron- and nanostructure (submicron-nano-NCD). Measured by ELISA per mg of protein; absorbance values of cells from the NCD layers were expressed as % of the values obtained from the control cells grown on polystyrene dishes. Mean±S.E.M. from 2 experiments (each performed in tri- or quadruplicates), ANOVA, Student–Newman–Keuls method. *: P≤0.05 compared to the values on polystyrene dishes.

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control PS dishes (absorbance 100.0±1.7%). On nano-NCD surfaces, the concentration of talin also showed a tendency to increase (120.0±6.0% of the value on PS) but this difference remained non-significant (Fig. 1C). The concentration of vinculin, another integrin-associated protein, was significantly higher in cells on both NCD films. In cells on nano-NCD, the absorbance reached 119.8±3.4% of the control value (100.0±1.7%) in cells on PS dishes, and on the submicron-nano NCD it was even 126.0±6.1% (Fig. 1D). Both talin and vinculin take an important part in transmission of the signal from integrin receptors to the actin cytoskeleton, various enzymes, transcription factors and genes, and thus are involved in the control of cell adhesion, growth, differentiation, viability and other functions by the physical and chemical properties of the cell adhesion substrate [28], for a review see [10].

The high activity of MG 63 cells on both NCD layers in forming focal adhesion plaques was further confirmed by immunofluorescence staining, which showed that in these cells, the talin-containing focal adhesion plaques were bigger, more numerous and more brightly stained than those in cells grown on the control microscopic glass coverslips (Fig. 2A–C). Similar results were obtained in human fetal osteoblastic hFOB cells cultured on poly(l-lactic acid) (PLLA) surfaces patterned with nano-sized pits. These cells displayed a higher concentration of integrins with αv chain, an integrin-associated protein paxillin, increased activity of focal adhesion kinase, and they also adhered over a larger surface area than the cells on flat non-patterned PLLA [28]. An enlarged cell adhesion area was obtained when OCT-1 osteoblast-like cells were seeded on PLLA with submicron- and micron-sized pits [29] or in MG 63 cells cultured on hierarchically submicron- and nanostructured diamond substrates in our earlier study [16]. Enhanced attachment of another osteoblast-like cell line, SaOS-2, was shown on Ti surfaces patterned with micron- and submicron-scale structures, created by anodic oxidation, in comparison with flat Ti surfaces [30]. Enhanced osteoblast adhesion was also observed on compacts made of Se metal particles of micron and submicron diameter range with an additional surface nanostructure created by chemical etching [3].

The concentration of the cytoskeletal protein β-actin was similar in MG 63 cells growing on all tested surfaces. The absorbance values were 93.3±3.4% on nano-NCD, 90.5±2.2% on submicron-nano-NCD and 100.0±5.4% on PS dishes (Fig. 1E). Immunofluorescence showed that in MG 63 cells on all tested surfaces, β-actin molecules were distributed diffusely rather than assembled into the distinct cables observed e.g. in endothelial or muscle-type cells. Relatively thin and short β-actin fibers were apparent usually at the cell periphery (Fig. 2D–F). A rich and well developed actin cytoskeleton has often been regarded as desirable behavior of cells on artificial materials [5,16,18]. On the other hand, thick actin stress fibers are typical for the classical “two-dimensional” cell culture system, i.e. conditions less physiological for cells, and can also be associated with a lower expression of differentiation markers in cells. In addition, the development of muscle-like cells is typical for softer and more elastic substrates, whereas harder and more rigid substrates direct the cell differentiation towards the osteogenic phenotype [31]. A well developed β-actin cytoskeleton associated with a lower concentration of osteocalcin was observed in MG 63 cells cultured on relatively soft matrices made of a terpolymer of polytetrafluoroethylene, polyvinylidifluoride and polypropylene mixed with 4 wt.% of multi-walled carbon nanotubes [8].

Osteogenic cell maturation on various materials has often been induced by creating nano-, submicron- and micron-scale irregularities on these surfaces, and has usually been manifested by higher production of osteocalcin and bone matrix mineralization [19–21]. However, in the cells on our NCD layers, the concentration of osteocalcin and osteopontin was similar to the values obtained in the cells on flat PS surfaces. In the case of osteocalcin, the absorbances of cell samples taken from nano-NCD and submicron-nano-NCD were 101.7±9.2% and 107.4±3.6% of the control value obtained from cells on

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PS (100.0±2.1%), respectively, and the distribution of this molecule was also similar in cells on all tested surfaces (Fig. 2G–I). For osteopontin, the absorbance on nano-NCD and submicron-nano-NCD amounted to 106.2±19.0% and 101.5±4.8% of the control value on PS (100.0±4.8%), respectively (Fig. 1F and G). The absence of significant changes in the osteocalcin and osteopontin concentration might be due to a relatively immature state of the MG 63 cells, which are of osteosarcoma origin and thus not capable of expressing a highly differentiated phenotype [19,20].

A positive finding was that the concentration of ICAM-1, i.e. an immunoglobulin adhesion molecule binding β2-integrin receptors on monocytes, macrophages, leukocytes and lymphocytes (for a review, see [18]), was not increased in MG 63 cells on both NCD layers in comparison with standard PS culture dishes. The absorbance of cell samples taken from nano-NCD and submicron-nano-NCD were only 73±1.3% and 85.9±14.1% of the control value on PS dishes (100.0±2.1%), respectively (Fig. 1H). This finding suggests relatively low attractiveness of the cell–NCD film constructs for inflammatory cells. However, a further more complex investigation of cell immune activation, including the production of interleukins and tumor necrosis factors [32], would be desirable.

4. Conclusion and further perspectives

The nanocrystalline diamond films organized on the nanoscale (RMS 8.2 nm) or hierarchically on the submicron- and nanoscale (RMS 301.0 and 7.6 nm, respectively), increased the concentration of integrin-associated focal adhesion proteins talin and vinculin in human osteoblast-like MG 63 cells in cultures on these layers in comparison with conventional flat cell culture polystyrene (RMS ~1 nm). Also talin-containing focal adhesion plaques were larger and more numerous on both NCD surfaces than on the control microscopic glass coverslips. The concentration of αv- and β1-integrins, β-actin, osteocalcin and osteopontin, as well as the spatial distribution of β-actin, osteocalcin and osteopontin, was similar in cells on both types of NCD films as well as on the control flat surfaces. The cells on NCD films did not increase their concentration of the immunoglobulin molecule ICAM-1, a marker of cell immune activation. Thus, both NCD films could be used for surface modification of bone implants (e.g., bone-anchoring parts of joint prostheses or bone replacements) in order to improve their integration with the surrounding bone tissue, as well as for constructing biosensors, for which improved cell-substrate adhesion is also needed.

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