MICRO- AND NANOPATTERNED SURFACES FOR GUIDED ADHESION, GROWTH AND PHENOTYPIC MATURATION OF CELLS

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Abstract

Micropatterned surfaces were created by UV light-irradiation of polytetrafluoroethylene through a metallic mask, by successive plasma polymerization of acrylic acid and 1,7-octadiene, or by creation of prominences and grooves by deposition of fullerenes C60 through a metallic mask. All these surface types were capable of inducing regionally-selective adhesion, proliferation and phenotypic maturation of vascular endothelial cells, vascular smooth muscle cells or human bone-derived MG 63 cells. Nanopatterned surfaces created by tethering GRGDSG oligopeptides through polyethylene oxide chains on a polymeric surface promoted spreading, formation of focal adhesion plaques and DNA synthesis in vascular smooth muscle cells. Surfaces nanopatterned with nanocrystalline diamond gave good support for the adhesion, growth and metabolic activity of osteoblast-like MG 63 cells.

Key words: *surface patterning, microstructure, nanostructure, biofunctionalization, endothelial cells, vascular smooth muscle cells, bone cells*

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Introduction

Patterned surfaces are material surfaces containing domains with different chemical and physical properties, such as chemical composition, surface energy, wettability, electrical behavior, or surface roughness and topography. These surfaces were originally developed for applications in electronics, e.g. for constructing semiconductors [1,2]. A marked expansion of patterning technology into the biological disciplines started in the early 1990s, when surfaces containing highly hydrophilic domains non-adhesive for cells, and less hydrophilic domains promoting adhesion and growth of bovine vascular endothelial cells, were created by photolithography [3]. Recently, patterned surfaces have provided a suitable tool for all applications in which we require regionally-selective adhesion of cells, controlled spreading, directed migration and growth, specific spatial organization of cells, induction of their differentiation and functioning, preferential adhesion and growth of certain cell types, interaction and cooperation of various cell types, and also temporal control of these events. These applications involve tissue engineering and various other biotechnologies, such as cell microarrays for use in advanced genomics, proteomics, drug discovery or construction of biosensors [2].

For biological purposes, material patterning usually occurs on a micro- or nanoscale. On micropatterned surfaces, cell proliferation, differentiation or apoptosis is controlled by the size and shape of the cell spreading area, which is determined by the size and shape of the cell-adhesive microdomains [4,5]. On nanopatterned surfaces, cell adhesion, spreading, survival, proliferation activity and differentiation are controlled by manipulating the number, size, shape, chemical composition and spatial distribution of cell-material contacts [6,7].

In this study, we have investigated the adhesion, growth and maturation of vascular and bone-derived cells on three types of micropatterned surfaces and two types of nanopatterned surfaces. Micropatterned surfaces were prepared using three different techniques, namely (1) irradiation of synthetic polymers with ultraviolet (UV) light through a metallic mask, (2) successive plasma polymerization of hydrophilic and hydrophobic compounds, and (3) variation in the material surface roughness and topography by deposition of carbon nanoparticles (fullerenes C_{60}) or hybrid metal-fullerene composites through metallic masks. Nanopatterned surfaces were created by (1) functionalization of the material surface by oligopeptides GRGDSG, i.e. synthetic ligands for integrin adhesion receptors, or by (2) deposition of nanocrystalline diamond.

Material and methods

Preparation of micropatterned surfaces

UV-light irradiation

Polytetrafluoroethylene (PTFE) foils (25μm in thickness; Goodfellow Ltd., Cambridge, UK) were irradiated with UV light generated by an Xe2*-excimer lamp (Heraeus-Noblelight, Hanau, Germany; center wavelength 172nm, spectral bandwidth 16nm, intensity about 20mW/cm^2) for 10, 20 or 30 min through a nickel contact mask with holes 100μm in width with center-to-center distances of 300 μm. The irradiation was performed in a reactive atmosphere of NH_3 (purity of 99.995%, Linde, Höllriegelskreuth, Germany). The spot pattern covered 8.7% of the total sample area [8].

Plasma polymerization

Acrylic acid (AA) and 1,7-octadiene (OD) were polymerised on the inner surface of 24-well multidishes (Costar, Falcon, Becton Dickinson, Lincoln Park, NJ) using a radio frequency (RF) signal generator (Coaxial Power Systems Ltd., UK). After polymerization of the first polymer, AA, in the form of a continuous layer on the dish bottom, the OD was polymerized through a mask consisting of a copper transmission electron microscope grid (Sjostrand Copper 3.05 mm, Athene Grids UK), with stripe-like gaps that were

75 and 150μm in width and 67.5 or 135μm apart. The AA strips occupied 47% of the resulting patterned surface [9].

Deposition of fullerenes C_{60} and hybrid metal-fullerene composites

Fullerenes C_{60} (purity 99.5%, SES Research, U.S.A.) were deposited as micropatterned films on to microscopic glass coverslips (Menzel Glaser, Germany; diameter 12mm) by the evaporation of C_{60} in the Univex-300 vacuum system (Leybold, Germany) in the following conditions: room temperature of the substrates, C_{60} deposition rate ⩽10 Ĺ/s, temperature of C_{60} evaporation in the Knudsen cells about 450°C, time of deposition up to 50 minutes. The thickness of the layers increased proportionally to the temperature in the Knudsen cell, and the time of deposition. Micropatterned layers were created by deposition of C_{60} through a metallic mask with rectangular openings with an average size of 128 per 98μm (about 12,500μm^2) and 50μm spacing. Due to the divergent fullerene beam, however, the C60 molecules could also invade the underside of the ribbing and form a light backing film [10].

Hybrid C60/Ti films of micropatterned morphology were created in a similar manner by co-deposition of C_{60} and Ti in a ratio of 1:1 (i.e., one C_{60} molecule per one Ti atom) [11].

Preparation of nanopatterned surfaces

GRGDS-functionalized polymer surfaces

Circular glass coverslips (12mm in diameter, Dispolab, Brno, CR) were silanized with dimethyldichlorsilane, and a uniform poly-L-lactide (PLLA, Mw=365 000) film was cast on the silanized surface from a polymer solution in dioxane by spin-coating (PWM32 Precision Spin Coater, Headway Research, USA). The surface of the PLLA films was then modified with 1:4 mixtures of poly(DL-lactide) (PDLLA) and poly(ethylene oxide-block-poly(DL-lactide) copolymer (PDLLA-b-PEO), in which 5% of the copolymer molecules carried a synthetic extracellular matrix-derived ligand for integrin adhesion receptors, the GRGDSG oligopeptide, attached to the methoxy end group of the PEO chain [7].

Nanocrystalline diamond films

Nanocrystalline diamond (NCD) films were grown on (100) oriented silicon substrates (12mm in diameter) by a microwave plasma-enhanced CVD method in an ellipsoidal cavity reactor (AIXTRON-P6, Germany). The silicon substrates were polished to atomic flatness (rms roughness about 1 nm). Prior to the deposition process, the substrates were mechanically seeded in an ultrasonic bath using 5-10 nm diamond nanoparticles (NanoAmando®) for 40 minutes. The nucleation procedure was then followed by the growth step, provided at a constant methane concentration (1% CH_4 in H_2) and at a total gas pressure of 30mbar. The substrate temperature was 860°C. The silicon substrates were overcoated with an NCD film on both sides, i.e. on the top and bottom side, respectively. Thus, hermetic sealing of the Si substrate minimized any unwanted bio-chemical reaction. Finally, the deposited NCD films were treated in oxygen plasma to enhance the hydrophilic character of the diamond surface [12].

Cell source and culture condition

The patterned surfaces were seeded with vascular endothelial cells in the form of commercially available cell lines, namely human umbilical vein endothelial cells (HUVEC) of the line EA.hy926 [8] or bovine pulmonary artery endothelial cells (line CPAE, ATCC CCL-209, Rockville, MA, U.S.A.). Other cell types used in this study were vascular smooth muscle cells (VSMC) derived from the thoracic rat aorta by an explantation method [13] and used in passage 4, or human osteoblast-like cells (line MG 63, European Collection of Cell Cultures, Salisbury, UK). The cell seeding densities ranged from about 6,500 to 32,000cells/cm^2. The EA.hy926 endothelial cells were cultured in a Dulbecco-modified Eagle Medium (Life Technologies, Vienna, Austria) with 10 % fetal bovine serum (FBS; Life Technologies), 100units/ml penicillin/streptomycin (Life Technologies), 2.5μg/ml amphotericin B (Sigma) and 1% HAT supplement (Sigma). The CPAE endothelial cells were grown in Minimum Essential Eagle Medium with 2mM L-glutamin, Earle's BSS with 1.5g/l sodium bicarbonate, 0.1mM non-essential amino acids, 1.0 mM sodium pyruvate (all chemicals from Sigma) and 20% of FBS (Sebak GmbH, Aidenbach, Germany). For VSMC and MG 63 cells, Dulbecco's Modified Eagle's Medium (Sigma, Cat No. D5648), supplemented with 10% of FBS and 40μg/ml of gentamicin (LEK, Ljubljana, Slovenia).

Results and discussion

Micropatterned surfaces created by UV light irradiation

The degree of selectivity of the cell adhesion to the irradiated microdomains was dependent on the time of exposure of these spots to the irradiation. On PTFE irradiated for 10 minutes only, the cells adhered almost homogeneously to the polymer surface, whereas on the polymer irradiated for 20 or 30 minutes, the cells adhered to the modified spots with a high degree of selectivity, i.e. 70 to 90% of all adhered cells with a mean value averaged over all samples of 79.84±12.35% . This is a very high value when we consider that only 8.7 % of the surface was covered with spots. This cell behavior was probably due to a pronounced formation of polar oxygen-containing groups and positively charged amine groups on the polymer surface, which are known to improve the adsorption of adhesion-mediating molecules (e.g., fibronectin and vitronectin) from the serum of the culture medium (for a review, see [14]). The selectivity of the cell colonization also depended on the time of cultivation. During approximately the first four days of the culture, the adhered cells at the spots proliferated, and this enhanced the differences between the cell population densities on the spots and on the unmodified polymer surface. However, with prolonged time of cultivation (approx. one week and more), the cell clusters were not confined only to the modified spots but extended to the neighborhood. Similar cell behavior was observed with increasing cell seeding densities. The preferential growth of cells on the modified microdomains also depended on the cell type, being more apparent on HUVEC than on VSMC cells. VSMC often migrated out of the irradiated spots and tried to bridge the unmodified regions between these domains (FIG.1A,B) [8,13].

Micropatterned surfaces created by successive plasma polymerization

On hydrophilic AA domains (advancing water contact angle of about 48ş), both endothelial CPAE cells and VSMC (FIG.1C) adhered and grew preferentially rather than on the hydrophobic OD domains (advancing contact angle of ~76ş). On day 1 and 7 after seeding, the percentage of both CPAE and VSMC cells on AA domains was about 85%. This difference decreased with time of cultivation, especially in VSMC, due to their migration and spanning the hydrophobic OD regions. Thus, on day 7 after seeding, the percentage of cells on the AA domains was about 74% for CPAE but only 63% for VSMC. Nevertheless, both cell types on AA domains were more mature, which was manifested by more apparent Weibel-Palade bodies containing von Willebrand

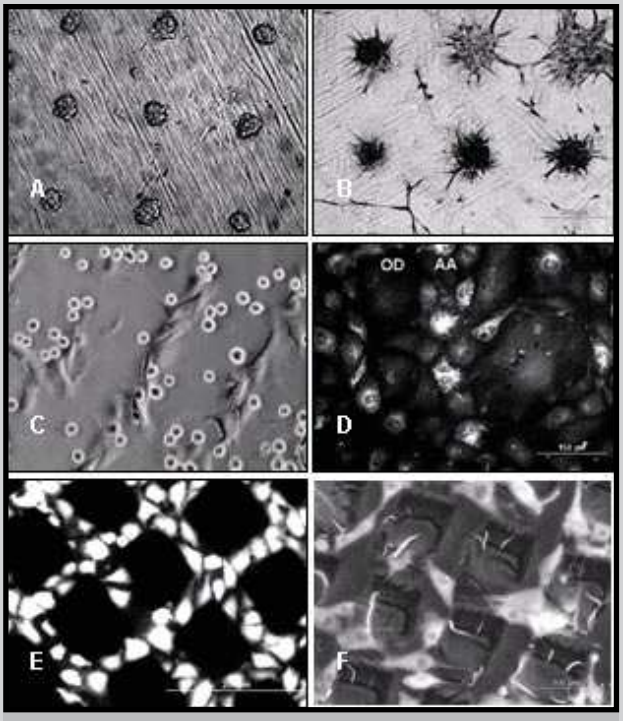

FIG.1. Vascular or bone-derived cells on micropatterned surfaces created by UV light-irradiation through a metallic mask for 20 min in an ammonia atmosphere (A,B), successive plasma polymerization of acrylic acid and octadiene (C,D) and deposition of fullerenes C_{60} through a metallic mask (E,F). A: Human umbilical vein endothelial cells, line EA.hy926, day 3 after seeding; B: Rat aortic smooth muscle cells, day 7 after seeding; C: Rat aortic smooth muscle cells, 6 hours after seeding; D: Immunofluorescence of von Willebrand factor in endothelial CPAE cells, day 3 after seeding; E, F: Human osteoblast-like MG 63 cells on surfaces patterned with fullerene C60 prominences of 1043±57nm in height, day 7 after seeding (E), or on binary C60/Ti films with prominences of 351±18nm in height, day 3 after seeding (F).

factor in endothelial CPAE cells (Fig. 1D) and better developed alpha-actin-containing filaments in VSMC. In addition, the enzyme-linked immunosorbent assay (ELISA) revealed that the concentration of alpha-actin per mg of protein was significantly higher in VSMC on AA strips [9]. Similarly, also in studies by other authors, the maturity, function and resistance of endothelial cells against shear stress were enhanced on micropatterned surfaces [15,16].

Micropatterned surfaces created by deposition of fullerenes and metal-fullerene composites

Depending on the temperature and the time of deposition, fullerenes formed bulge-like prominences of various heights. When seeded with human osteoblast-like MG 63 cells, the surfaces with lower prominences (up to 326±5nm) were almost homogeneously covered with cells, whereas on the surfaces with the highest prominences of 1090±8 nm, the cells adhered and grew preferentially in the grooves among the prominences (FIG.1 E, F), and this selectivity increased with time of cultivation. Although these grooves occupied only approximately 41 % of the surface, they contained from 80% to 98% of the cells, and the cell population density in the grooves was about 5 to 57 times higher than on the bulges [10]. This cell behaviour may be due to a synergetic action of hydrophobia and other physicochemical properties of the fullerene bulges less appropriate for cell adhesion, such

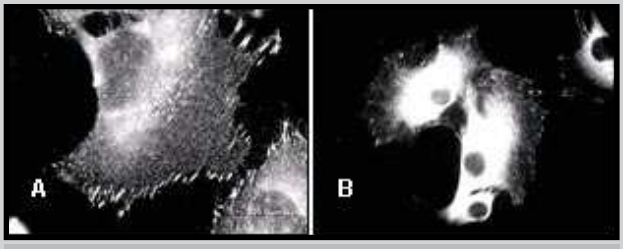

FIG.2. Rat aortic smooth muscle cells (A) and human osteoblast-like MG 63 cells (B) on surfaces nanopatterned by tethering GRGDS adhesion oligopeptides through PEO chains (A) or deposition of nanocrystalline diamond (B). Immunofluorescence of vinculin (A) and talin (B), day 3 after seeding.

as their steep rise and the tendency of spherical ball-like fullerene C60 molecules to diffuse out of the prominences towards the grooves [10]. Preferential growth of MG 63 cells in the grooves among the prominences was also observed on microstructured hybrid Ti/C60 films, as well as pure Ti films [11].

Nanopatterned surfaces created by functionalization with ligands for cell adhesion receptors

On PDLLA surfaces, the adhesion and growth of VSMC was similar as on standard cell culture polystyrene or microscopic glass coverslips. However, the copolymer PDLLA-b-PEO almost completely inhibited the adhesion, spreading and growth of cells. This behavior was due to the high hydrophilicity and mobility of the PEO chains, which disabled the adsorption of cell adhesion-mediating proteins from the serum of the culture medium (e.g., fibronectin, vitronectin). However, functionalization of PEO chains with the GRGDSG oligopeptide almost completely restored the cell adhesion. The cell spreading areas on these surfaces reached average values of 991μm^2 compared to 958μm^2 for PDLLA. In addition, the cells on GRGDSG-grafted copolymers were able to form vinculin-containing focal adhesion plaques (FIG.2A), to synthesize DNA and even proliferate in a serum-free medium, which indicates specific binding to the GRGDSG sequences through their adhesion receptors [7].

Nanopatterned surfaces created by deposition of nanocrystalline diamond (NCD)

On NCD films (rms roughness of 8.2nm), the osteoblast-like MG 63 cells adhered and grew better than on conventional flat tissue culture polystyrene surfaces. The cells on NCD surfaces were better spread, i.e. adhering by a larger cell-material contact area, and formed well-apparent focal adhesion plaques containing talin and vinculin, i.e. proteins associated with integrin adhesion receptors on cells (Fig. 2B). As revealed by ELISA, these cells also contained a higher concentration of vinculin, measured per mg of protein. In addition, the cells on NCD films usually reached higher population densities than those on standard cell culture polystyrene dishes, and were metabolically more active, as demonstrated by the XTT test [12,17-19]. The beneficial effect of surfaces with nanoscale roughness on cell adhesion and growth has been explained by the adsorption of cell adhesion mediating molecules in an appropriate geometrical conformation, enabling good accessibility of active sites in these molecules to the cell adhesion receptors [20].

Conclusion

All types of micropatterned surfaces created in this study promoted regionally-selective adhesion, growth and

phenotypic maturation of vascular and bone-derived cells. Nanopatterned surfaces provided good support for the adhesion, spreading, growth and metabolic activity of these cells. All these surfaces could be useful for tissue engineering, construction of cell arrays and biosensors.

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VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON BIOFUNCTIONALIZED CELLULOSE-BASED SCAFFOLDS

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Abstract

Viscose, dialdehyde cellulose and oxidized 6-carboxycellulose with 2.1 or 6.6wt.% of –COOH groups were prepared. The materials were subsequently functionalized with arginine or chitosan. Both unmodified and biofunctionalized materials were seeded with vascular smooth muscle cells. The morphology of the adhered cells indicated that oxidized 6-carboxycellulose with 2.1% content of –COOH groups was the most appropriate of all tested materials for potential use in tissue engineering. The shape of the cells on this material was elongated, which demonstrates adequate adhesion and viability of the cells, while the morphology of the cells on other tested materials was spherical. Moreover, the stability of 6-carboxycellulose with 2.1wt.% of –COOH groups in the cell culture environment was optimal, with a tendency to degrade slowly with time. The highest stability was found on the viscose samples, whereas there was very low stability on oxidized 6-carboxycellulose with 6.6 wt. % of –COOH groups, and also on dialdehyde cellulose. Functionalization with arginine or chitosan increased the number of adhered cells on the materials, but not markedly. We did not obtain a significant elevation of the cell population densities with time on the tested samples. These results suggest the possibility of using a cellulose-based material in such tissue engineering applications, where high proliferation activity of cells is not convenient, e.g. reconstruction of the smooth muscle cell layer in bioartificial vascular replacements.

Key words: *oxidized cellulose, tissue engineering, biofunctionalization, chitosan, arginine, vascular smooth muscle cells*

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Introduction

Cellulose, composed of glucose monomers, is a polysaccharide commonly occurring in nature. Oxycellulose is cellulose oxidized by oxidizing agents, such as NO_2 or $NaClO_2$, which induce conversion of the glucose residues to glucuronic acid residues, i.e. compounds containing –COOH groups [1]. The concentration of these groups modulates the pH, swelling in a water environment, degradation time, drug loading efficiency and other behavior of the material [2]. In addition, –COOH groups, which are polar and negatively charged, can be used for functionalizing the oxidized cellulose with various biomolecules.