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ADHESION AND GROWTH OF VASCULAR SMOOTH MUSCLE CELLS IN CULTURES ON CARBON- FIBRE-REINFORCED CARBON COMPOSITES COVERED WITH PYROLYTIC CARBON

L. Bacáková*, K. Balík, S. Zizka****

* Institute of Physiology, Acad. Sci. CR, Prague
** Institute of Rock Structure and Mechanics, Acad. Sci. CR, Prague

Abstract

Biocompatibility of two-dimensionally reinforced carbon-carbon composites infiltrated and coated with pyrolytic carbon was evaluated in vitro by seeding them with smooth muscle cells derived from the rat aorta. The cells adhered to the composites in numbers comparable with those found on standard plastic culture dishes and these numbers tended to be positively correlated with the open porosity of the material surface. In contrast, the following proliferation was rather negatively related to the open porosity. The maximum population density of cells growing on the composites was similar or lower than that on standard culture plastic. These results suggest relatively good biocompatibility of the pyrolytic-carbon infiltrated and coated carbon composites and their suitability for future biomedical applications.

Introduction

The carbon-fibre-reinforced carbon composites (CFRC) are indispensable materials for specialised technical and

industrial applications (e.g. the aerospace industry) owing to their uniquely combined properties, such as low thermal expansion, high thermal shock resistance, good strength retention at high temperatures as well as their electrical conductivity [7,16,19]. Their mechanical properties, namely density, porosity and modulus of elasticity, can be tailored to be similar to those of bones and make them attractive for use also in orthopaedic and dental surgery [4,6,7, 19]. For the construction of heads and cups of joint prostheses, inert materials not allowing the adhesion of cells are preferred [7,9]. However, in artificial substitutes of bones, the colonisation of the material with cells and its integration with the surrounding tissue is desirable. Our preliminary studies performed on cultured vascular smooth muscle cells (VSMCs) showed that the CFRC are suitable for cell attachment and growth [2,4]. However, as shown by scanning electron microscopy, the surface roughness of pristine unmodified composites was often too high for optimal adhesion of cells. Moreover, the CFRC were also prone to release carbon microparticles, especially during cyclic stress [2]. Both problems could be solved by covering the material surface with a stronger biocompatible layer. Therefore, in this study we evaluate the initial adhesion and subsequent growth of rat VSMCs in cultures on CFRC infiltrated and coated with pyrolytic carbon.

Measured parameters	Pyrolytic carbon-coated samples				Nunclon Multidishes
	No.1	No. 2	No. 3	No. 4	
Open porosity (%)	5.77	6.16	6.20	6.70	not measured
Initial adhesion (cells/cm^2, day 1)	8000±900**	12300±1800	8400±1100**	14100±1000*	12300±600
Cell volume (μm^3, day 4)	2029±30**	1831±30***	2084±40**	2513±30	2961±130
Doubling time (h, days 1-4)	23.2±1.3	33.7±4.7*	23.8±2.7	29.5±2.5*	23.2±1.3
Population density (cells/cm^2) day 4 day 10	74400±4800** 85800±7600***	53900±2300*** 74800±6100***	68100±5900**197 800±14400	76400±4100** 70900±3900***	106000±6900185 600±3900

TABLE 1. Adhesion and growth of vascular smooth muscle cells on pyrolytic carbon-coated CFRC with different porosites.

Materials and methods

Two-dimensionally reinforced composites were prepared from plain-woven cloth (Torayca carbon fibres T800) and phenolic resin. The prepregs were stacked in 5 layers, cured, cut into 40x8x1 mm pieces and carbonised at the heating rate of 50°C/hr up to 1000°C in nitrogen. After three-step impregnation with phenolic resin, the samples were graphitised up to 2200°C in argon. Pyrolytic carbon was deposited in a tumbling bed reactor [13] with the inner diameter of 48 mm and hot zone, 124 mm long. The bed rotated at 20 rpm. As carbon source, propane in the concentration of 11% in nitrogen was used [5]. The deposition was performed at ambient pressure and the reaction temperature was 850°C. The gas flow rate was 1.8 l/min, total deposition time was 36 hours and the experiments were interrupted every 6 hours to examine the deposition process. Open porosities of the samples were obtained from densities determined on the basis of water penetration according to ASTM C-20. Optical properties of the deposited pyrolytic carbon layer, its thickness and depth of infiltration were studied using a polarised-light optical microscope.

For cell culture, the 40-mm-long beams were cut with a diamond saw into 8x8x1 mm samples. They were washed in distilled and deionized water, autoclaved and placed on the bottom of plastic Nunclon Multidishes (diameter 1.5 cm, NUNC, Denmark). The VSMCs were obtained from the intima-media complex of the thoracic aorta of adult male Wistar rats by explantation method [3]. In passage 10, the cells were seeded on the composites at a density of 17000 cells/cm^2 in 1 ml of Dulbecco Minimum Essential Medium supplemented with 10% of fetal calf serum and gentamicin (40mg/ml). The adhesion and growth of cells were evaluated by counting cells in the Bürker haemocytometer after their detachment from the growth substrate by 0.2% trypsin in phosphate-buffered saline (PBS) on days 1, 4 and 10 after seeding. The cell suspensions obtained by trypsinization were also used for the measurement of cell diameters in light microscope with a calibrated ocular grid and calculation of cell volume. For evaluation of cell shape and degree of spreading, the VSMCs growing on the composites were visualised by staining with propidium iodide in PBS (5 mg/ml) after fixation in 70% ethanol and observed in fluorescence microscope. The degree of cell differentiation was estimated by immunofluorescence staining with monoclonal antibodies against VSMC-specific cytoskeletal protein alpha-actin [19] and the possible interaction of the cell-colonised material with the immune system by staining against vascular cell adhesion molecule-1 (VCAM-1) [11]. The antibodies were purchased from Sigma, St. Louis, U.S.A. (anti alpha-actin) and Exbio, Prague, CR (anti VCAM-1).

Four groups of pyrolytic carbon-covered CFRC (labelled No.1-No.4) were evaluated. For each group, time interval and type of experiment, the samples were used in duplicate. As control samples, cells grown on Nunclon Multidishes (NUNC, Denmark) were taken.

Results and discussion

Thickness of the deposited pyrolytic carbon layer was 1-2 mm, its density was 1.55-1.57 g/cm^3 and its open porosity 5.77-6.70 % (TABLE 1). In the pores and cracks, the optical microstructure of pyrolytic carbon was laminar, whereas on the surface, it was finely granular. The granular microstructure was visible also in fluorescence microscope after staining cells on composites, especially in group No.3 (FIG. 1). The presence of this structure can be explained by rotation of the samples in the tumbling bed reactor [13].

The number of initially adhered VSMCs on day 1 tended to increase proportionally to the open porosity (TABLE 1).

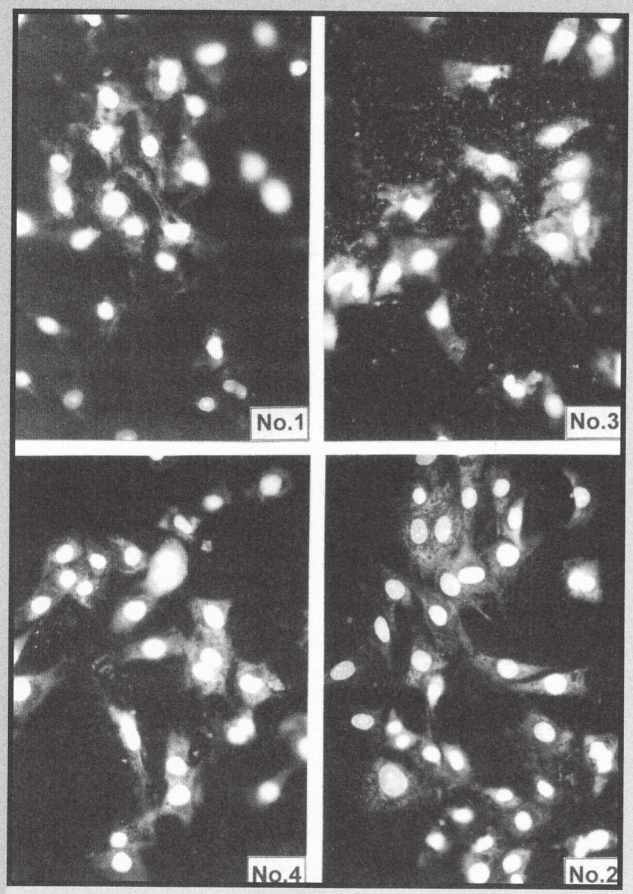

FIG. 1. Rat vascular smooth muscle cells in cultures on CFRC coated with pyrolytic carbon layer with different open porosities (P). No.1: P=5.77%, No.3: P=6.16%, No.4: P=6.20%, No.2: P=6.70%. Day 3 after seeding, fixed in 70% ethanol,stained with propidium iodide, obj.40.

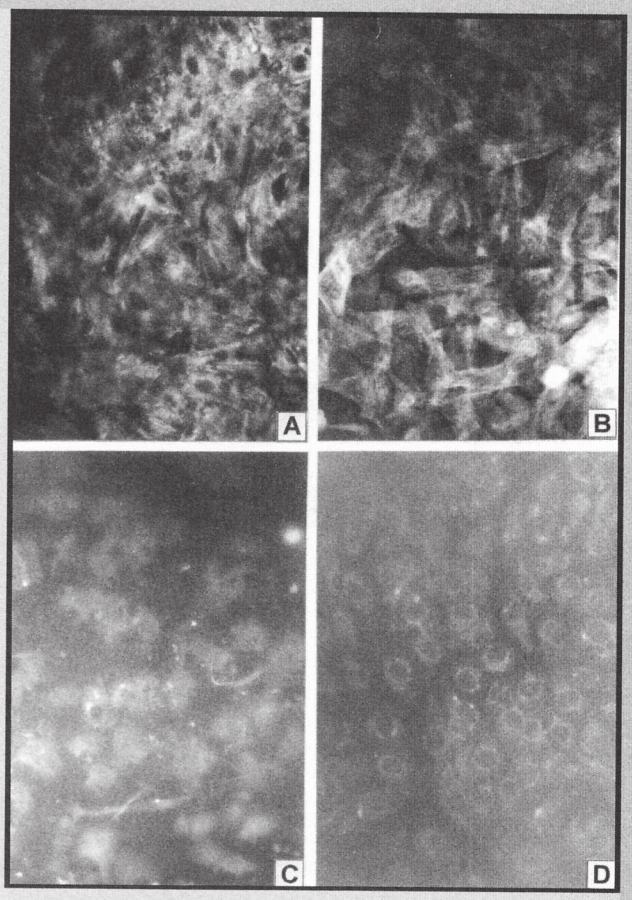

FIG. 2. Immunofluoresce staining of alpha-actin (A,B) and VCAM-1 (C,D) in rat vascular smooth muscle cells in cultures on CFRC coated with pyrolytic carbon (A=No.4, C=No.3) and in cultures on Corning coverslips (B,D). Day 10 after seeding, Axioplan fluorescence microscope, obj.40.

On samples with the lowest porosity (5.77%), it was significantly lower than that on Nunclon Multidishes. At intermediate porosity (6.16%), it increased to values comparable with the control samples and on surfaces with the highest porosity (6.70%) it was even significantly higher than on stand-ard culture dishes. At the highest porosity, the cells had also the highest volume comparable with that on standard culture surfaces, and adhered on the largest area (TABLE 1, FIG. 1). However, the following proliferation of cells was rather inversely related to the open porosity. On samples with the lowest porosity of 5.77%, the doubling time of cell populations was shortest and comparable to that of the control cells, whereas on the most porous or granular composites (groups No. 2 and 3, respectively), the cells proliferated most slowly (TABLE 1). On day 4 after seeding, the population density of cells on all carbon samples was lower than that on Nunclon Multidishes but on the sample No.4, the cells continued to proliferate and on day 10 after seeding, they reached a similar population density as the control cells (TABLE 1).

Our findings on adhesion and proliferation of VSMCs on pyrolytic-carbon-coated CFRC with different open porosities are similar to those obtained in chick vascular and corneal cells cultured on polymethylmetacrylate growth supports [14] or human osteoblast-like cells grown on titanium [15]. Increased roughness of these materials, measured by the size of surface irregularities and distance between them, enhanced the adhesion and migratory potential of cells but slowed down their proliferation. Similarly, the initial adhesion of osteoblasts on three-dimensional biodegradable polymeric scaffolds or porous ceramics in vitro and their migration inside these materials were maximal at the pore size of 300-500 mm (from the range of 150-710 mm) but the cell proliferation was not affected by the pore size [11]. The beneficial effects of certain surface topographies of artificial materials on cell adhesion and migration can be explained by adsorption of extracellular matrix molecules (e.g. vitronectin and fibronectin) in amounts and conformation optimal for binding the RGD sequence of these molecules to integrin receptors on cells [1,14]. After initial attachment and spreading, the cells usually degrade molecules previously adsorbed on the growth support and replace them by their own de novo synthesised matrix, which can annihilate the effects of physicochemical surface properties of the artificial growth support on further growth and differentiation of cells [2,3,14].

The VSMCs growing for 10 days on all tested pyrolytic carbon-coated samples displayed intense immunofluorescent staining for alpha-actin, which was arranged in clearly visible microfilament bundles, and only weak diffuse staining for VCAM-1. Both staining intensities were similar to those found in the control cells cultured on Nunclon Multidishes or Corning coverslips (FIG. 2) and suggested a satisfactory degree of differentiation [18] and possible low binding of monocytes, lymphocytes, eosinophils and basophils to cells colonising the material [10]. Relatively low immunogenicity was found also in the pyrolytic-carbon-coated silicone [8] and Dacron [10].

In general, adhesion, growth and differentiation of VSMCs on pyrolytic-carbon-coated CFRC are comparable to those found on standard tissue culture plastic. Our present study supports previous findings on beneficial effects of pyrolytic carbon on adhesion, growth and differentiation of various types of cells, such as endothelial and vascular smooth

muscle cells in vitro [2, 17] and cells of the cartilage in situ [12]. It can be concluded that the bi-directional CFRCs infiltrated and coated with pyrolytic carbon exhibit a relatively good biocompatibility in vitro and they are promising materials for future biomedical applications.

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IMPROVED BIOCOMPATIBILITY OF CARBON-FIBRE-REINFORCED CARBON COMPOSITES IN VITRO AFTER THEIR POLISHING AND COATING WITH A CARBON-TITANIUM LAYER

L. Bacáková*, V. Stary**, O. Kofrooová***

*Institute of Physiology, Acad. Sci. CR, Prague
**Faculty of Mechanical Engineering, Czech Technical University, CR, Prague
***Institute of Microbiology, Acad. Sci. CR, Prague

Abstract

The surface of unidirectionally reinforced carbon-carbon composites was modified either by polishing or coating with a carbon-titanium layer. In culture conditions, the composites were seeded with vascular smooth muscle cells derived from the rat aorta. On both types of modified samples, the number of initially adhered cells, degree of their spreading and their subsequent growth were significantly higher than on untreated samples, and in the case of carbon-titanium-covered composites, also higher than on standard plastic culture dishes Sterilin. These results obtained in vitro suggest possible good biointegration of the polished and carbon-titanium-covered carbon-carbon composites with the surrounding tissue in situ after their use in transplantation medicine for the construction of artificial implants.

Introduction

The carbon-fibre-reinforced carbon composites (CFRC) are promising materials for the construction of artificial implants, especially those of bones, joints and dental roots [1]. Their physical properties, namely density and porosity, are very close to those of bones, and their chemical composition is suitable for adhesion and growth of several kinds of cells in vitro as well as in situ [1,2]. However, the surface of unmodified newly fabricated composites usually seems to be too rough to ensure optimum degree of cell adhesion, which can be explained by heterogeneous composition of the CFRC, i.e. carbon fibres protruding from the carbon matrix. Moreover, the carbon matrix is brittle, which can lead to the release of carbon microparticles to the surrounding tissue, particularly on cyclic stretching [2]. These disadvantages of the CFRC could be minimised by polishing their surface and/or by covering it with a thin biocompatible layer. Thus, in this study we evaluate the initial adhesion and subsequent growth of rat aortic smooth muscle cells in cultures on CFRC either polished with SiO_2 or (in non-polished state) coated with a layer of carbon and titanium, a metal widely used in orthopaedic surgery and reconstructive dentistry [3,4,5,6,7].