ADHESION AND GROWTH OF CELLS IN CULTURE ON CARBON-CARBON COMPOSITES WITH DIFFERENT SURFACE PROPERTIES

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Summary

The biocompatibility of unidirectionally reinforced carbon-carbon composites (carbon fibre T300, phenolformaldehyde resin based matrix) with different surface roughness and chemical composition was tested in cell culture conditions. The surface of the composites was polished, covered with amorphous or pyrolytic carbon and seeded with rat aortic smooth muscle cells. Coating with amorphous carbon significantly lowered the number of initially adhered cells. In these samples, the surface roughness had no significant effect on the number of initially adhering cells nor their subsequent proliferation. In contrast, coating with pyrolytic carbon improved significantly both cell adhesion and growth, especially on the polished surfaces. In addition, the layer of pyrolytic carbon was more resistant to mechanical damage than the film of amorphous carbon. It is concluded that polished composites covered by pyrolytic carbon could be suitable for the future application in medicine and biotechnology.

Introduction

In recent years, various types of artificial materials are widely applied in medicine and biology. They are used for construction of tissue and organ transplants, fabrication of cell culture growth supports and for experimental studies of cell-extracellular matrix interactions. Among the artificial materials, the carbon materials are distinguished for their excellent biocompatibility. They belong to the most advantageous substrates for adhesion and growth of several types of cells *in vitro* [1, 12, 13-18]. Advantages of the carbon materials were proved also at *in vivo* conditions. This includes, for instance, the glass-like and pyrolytic carbon, electrographite and carbon-carbon composites (for review see [15]), carbon-enriched hydroxyapatite [8], calcium carbonate [9], tyrosin-derived polycarbonates [6] and polymers reinforced with carbon fibers [5, 7]. These materials were succesfully used for contructions of mechanical parts of pacemakers, artificial joint and bone prostheses, dental implants as well as external and internal bone fixations.

The aim of this study is to evaluate the biocompatibility of carbon fibre reinforced carbon (CFRC) composite materials in cell culture conditions. The CFRC materials are the most mature examples of fibre reinforced composites in which the chemical nature of the fibre and matrix is identical. Their excellent mechanical properties similar to those of bone make them an attractive material for implants in orthopaedic and dental surgery [18]. However, there are also some unsolved problems in their possible medical application. Their surface, especially of those reinforced unidirectionally, is too rugged to permit sufficient adhesion, spreading and subsequent replication of cells. In addition, these materials are prone to release carbon particles which is caused by the brittle nature of the carbon matrix. The carbon microparticles can be released during cyclic loading of the implant and may irritate biological components of the graft as well as the surrounding tissue. A possible solution of these two main problems may consist in polishing the implant's surface and covering it by a stronger compatible layer. Therefore, we evaluate the adhesion, subsequent proliferation and morphology of cells cultured on surfaces of unidirectionally reinforced carbon composites treated by polishing followed by the deposition of thin films of diferent physical and chemical properties, i.e. amorphous or pyrolytic carbon. As an appropriate cell type, the vascular smooth muscle were chosen. As known from our earlier studies [1, 2, 17], these cells respond very sensitively to environmental conditions including changes of physical and chemical surface properties of growth supports.

Material and methods

1. Preparation of CFRC composite samples

The polyacronitryle-derived carbon fibres T300 (Torayca, Soficar, France) were soaked with the UMAFORM LE phenolformaldehyde resin (Synpo Ltd., Semtin, Czech Republic) and wound onto a rotating drum to form a prepreg, i.e. a layer of parallel fibres embedded in the matrix precursor. After drying partially the still sticky prepreg was cut to 150 mm sections and stacked in a heated mold. At 120 °C and under a uniaxial pressure 0.5 MPa the thermosetting resin hardened and in this way a carbon fiber reinforced polymer composite was formed. This material was carbonised at 1000 °C in N_2 (when almost all non-carbon heteroatoms were removed from the polymer and the matrix was converted to glass-like carbon) and then graphitised at 2500 °C in Ar_2 at atmospheric pressure. The prepared beams were cut with a diamond saw. One half of these specimens was ground and polished, in the final step with colloidal SiO_2 (grain size 0.06 m). The roughness of the surface was measured by Talysurf (Rank Taylor Hobson Ltd., England).

For the first set of experiments, part of the polished as well as unpolished samples (culture area of 8 x 10 mm, thickness 6 mm) was covered with a thin layer of amorphous carbon (a-C:H; thickness about 20nm) prepared by the CVD method (high frequency discharge in n-hexane, power 125W, bias -300 V, time of exposition 8 minutes). Finally, we had 4 groups of substrates for cell growth: A – without treatment, B – polished only, C – a-C:H film on the untreated surface, D – a-C:H film on the polished surface.

For the second set of experiments, polished and unpolished CFRC composites (cultivation area 8x10 mm, thickness 3 mm) were covered by a 5 mm pyrolytic carbon film deposited during 30 min. from butane at 1400 °C and pressure 230 Pa [3, 4]. In this case, we compared 3 groups of substrates: E – without treatment, F– pyrolytic carbon on the untreated surface, G – pyrolytic carbon on the polished surface.

2. Cell culture on the composites

The samples of CFRC composites were sterilized in 96% ethanol for 24 hours, washed in distilled and deionized water and placed on the bottom of plastic Nunclon Multidishes (Denmark, diameter 1.5 cm). The smooth muscle cells were obtained from the intima-media complex of the thoracic aorta of four female Wistar rats (Ipcv: Wist, age 8 weeks, Institute of Physiology, Acad. Sci. ČR) by explantation method [2]. In passage 40, the cells were seeded on the composites at a density of 17000 cells/cm² (i.e. 30000 per one dish) in 1.5 ml of Dulbecco Minimum Essential Medium (SEVAC, Prague) supplemented with 10% of fetal calf serum (Sebak GmbH, Germany) and gentamicin (40 g/ml, Lek, Slovenia). The cultures were grown at 37°C and 95% humidity in air atmosphere containing 5% CO_2.

The number of initially adhering cells was calculated one day after seeding (in our preliminary experiments we found that more than 90 % of the cells used in this study adhered and spreaded during the first 24 hours without significant cell division). Then, the medium was changed in order to prevent the additional attachment of initially unadhered cells, and 8 days after seeding, the increase in cell number on the tested samples was evaluated. The cells were detached from the growth substrate by 0.2% trypsin (Sigma, St. Louis, U.S.A.) in phosphate-buffered saline (10 min, 37°C) and counted in the Brker haemocytometer. For each time interval and group of composites, 6-8 samples were evaluated (meanS.E.M.). The results were compared by Student's t-test for unpaired data. Values p<0.05 were considered significant.

Results and discussion

1. Samples covered with amorphous carbon (a-C:H)

The surface roughness was slightly lower in the covered than in uncovered samples and significantly lower in polished than unpolished samples (Tab. 1). However, the roughness of the composites did not affect significantly the number of initially adhering cells nor their subsequent growth. On the polished surfaces of both covered and uncovered samples, the numbers of cells on days 1 and 8 tended to be higher but these differences were not significant (Fig. 1).

The chemical composition of the surface had more pronounced effects on the cell adhesion and growth. On samples covered with amorphous carbon, the number of initially adhering cells on day 1 after seeding was significantly lower than on corresponding uncovered surfaces (Fig. 2). This can be due to a relatively lower content of carbon in the a-C:H layer in comparison with the CFRC composite material which can be considered as pure carbon. Similarly, on the polyethylene doped chemically with carbon black, the number of adhered vascular smooth muscle cells was proportional to the carbon content in this substrate [17]. Another explanation of the lower cell adhesion could be an unappropriate surface polarity, high content of free radicals or presence of unsaturated sp3 bonds in the a-C:H layer [1, 10-14]. On day 8 after seeding, the difference in the number of cells growing on both covered and uncovered samples disappeared. This may be related to the deposition of various extracellular matrix molecules on the tested surfaces. These molecules are produced by the cells themselves or adsorbed from the serum in the culture medium [10, 13]. They can gradually separate the cells from the original growth support.

The uncovered samples released microparticles of material which were fagocyted by cells. The release of particles was prevented by covering the surface with amorphous carbon but this film was not sufficiently resistant to mechanical damage during handling the specimens (sterilization, washing, placing into dishes, removal of the cells). The latter suggests that the film of amorphous carbon would not be a suitable material for medical use.

2. Samples covered with pyrolytic carbon

The surface roughness of the samples was higher than in composites used in experiments with amorphous carbon. Also the difference between polished and unpolished samples was more apparent (Tab. 2). The beneficial effect of surface polishing on cell adhesion and growth was more pronounced in comparison with amorphous carbon. (Fig. 2). These results indicate that some optimal degree of the surface roughness exists for good adhesion, proliferation and survival of cells. Similar range optimal for cell adhesion and growth was described for the surface polarity of ion implanted polymers [1]. The roughness of the cultivation substrate was found to be in correlation with its surface polarity (wettability), protein adsorption, number of adhering cells, cell adhesion area, expression of cell specific markers and migration capacity of cells [13].

In contrast to the amorphous carbon, covering the surface with pyrolytic carbon significantly improved the initial adhesion as well as subsequent growth of cells. On the days 1 and 8 after seeding, the number of cells was significantly higher than on control uncovered samples. Similarly to these results, better adhesion and growth of endothelial cells on Dacron– and Teflon-made blood vessel prostheses coated with pyrolytic carbon was described by Sbarbati et al. [16].

Like amorphous carbon, the film of pyrolytic carbon prevented the release of microparticles of composite material into surrounding environment. Moreover, it was resistant to mechanical damage during handling necessary for establishment of tissue culture. The stability of the pyrolytic carbon layer, together with its significantly beneficial effect on cell adhesion and growth, indicate good integration of this material with its biological surrounding and recommend it for the possible medical application.

Roughness parameter	A		B		C		D	
	cross	along	cross	along	cross	along	cross	along
R_a [µm]	1.97	0.69	0.81	0.37	1.88	0.78	0.51	0.26
S [µm]	17	69	38	106	19	72	24	48

TAB. 1. Parameters of the surface roughness of samples coated with amorphous carbon.

A. without treatment, B. polished only, C. a-C:H film on the untreated surface, D. a-C:H film on the polished surface.
"Cross" and "along" refer to directions perpendicular and parallel to the direction of the fibres; R_a is the aritmetic mean of the departures of the roughness profile from the mean line; S is the mean spacing of adjacent local peaks.

Roughness parameter	E		F		G	
	cross	along	cross	along	cross	along
R_a [μm]	5.50	1.34	4.60	1.09	0.81	1.71
S [μm]	30	21	26	22	16	21

TAB. 1. Parameters of the surface roughness of samples coated with pyrolytic carbon.

E. without treatment, F. pyrolytic carbon on the untreated surface, G. pyrolytic carbon on the polished surface. "Cross" and "along" refer to directions perpendicular and parallel to the direction of the fibres; R_a is the aritmetic mean of the departures of the roughness profile from the mean line; S is the mean spacing of adjacent local peaks.
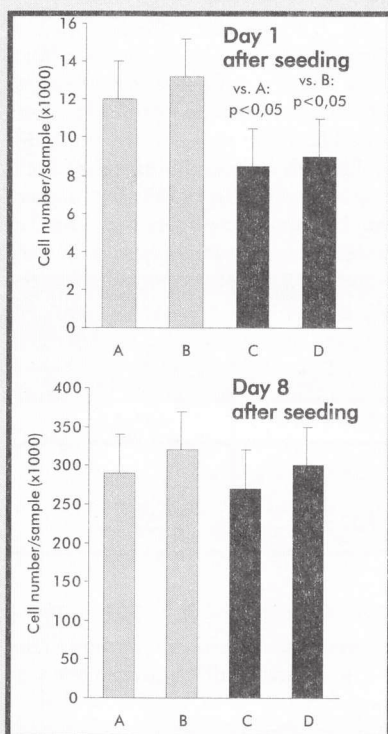

FIG.1. Number of smooth muscle cells in cultures on CFRC composites treated by polishing and coating with amorphous carbon:

A-without treatment, B-polished only, C- a-C:H film on the unpolished surface, D- a-C:H film on the polished surface.
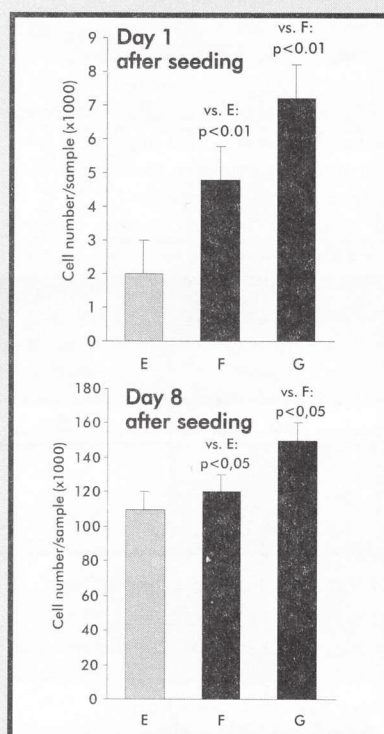

FIG.2. Number of aortic smooth muscle cells in cultures on CFRC composites treated by polishing and coating with pyrolitic carbon:

E- without treatment, F- pyrolitic carbon on the unpolishing surface, G- pyrolitic carbon on the polished surface

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