Adhesive and Metabolic Activity of Bone-Marrow Cells on Titanium and Titanium Modified by C and CN_x Films

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Abstract – The interest attracted to solid-state metal carriers, which act as the internal fixation device and the three-dimensional matrix of osteogenic cells, remains high as before. The determining factors include a high mechanical strength, biological compatibility and absorbability. Monocrystalline titanium with different degrees of surface roughness, titanium samples coated with a diamond-like carbon (DLC) or nitrogen-containing carbon (CN_x) film, and samples of porous titanium, which were prepared by compaction of titanium sponge particles, were used as the matrix in this study. Intraoperative bone-marrow cells, which were taken from patients at the age of 14 to 54, were used in the experiments. The cell suspension with the concentration of $3–5\cdot10^9$ cells/l was cultivated in the medium 199, which contained 50% horned cattle serum, at 37 C for 4 hours in humid air. The study revealed that (1) titanium samples with and without coats had not a toxic effect in short-term cultures, (2) uncoated titanium samples had the largest biological inertness, (3) the number of adhered cells considerably increased with the surface roughness, (4) the CN_x films offered some advantages when used as substrates for the cell adhesion and caused activation of metabolic processes in bone-marrow cells, and (5) the number of adhered cells on porous titanium samples was significantly larger in statistical terms.

1. Introduction

Studies of solid-phase carriers made of various materials (metals and their alloys, polymers and various ceramics), which act as the internal fixation device and the three-dimensional matrix of osteogenic cells, present great interest [1–4]. The main factors that determine applicability of materials as implants are their biological compatibility and mechanical strength. The biological compatibility is determined mainly by the chemical composition of the implant material. Properties of the material surface, such as its energy, relief and roughness, are important for deposition of cells [3]. Toxicity of the implant material should be considered too. Properties of the implant surface can be modified by a variety of coats, specifically diamond-like carbon (DLC) films. It was shown that DLC films, which are doped with other elements, can change the ratio of proteins absorbed on the surface and, hence, improve adhesion and growth of cells [5]. Surface properties can be improved if, for example, osteoinductive agents are added [5]. Porosity of materials is also important for implants since it provides diffusion of physiological fluids, which contain nutrients and metabolites, and the migration of osteogenic cells to the implant, leading to the integration of the implant and the bone tissue [6]. The present study deals with the influence of surface roughness of titanium samples and those, which were modified with carbon and nitrogen-containing carbon (CN_x) films, on the adhesive and metabolic activity of bone-marrow cells. Tentative results concerning adhesion of the cells on porous titanium samples were obtained.

2. Experimental

The substrates for adhesion of bone-marrow cells were monocrystalline titanium disks of diameter 5 mm and 2 mm thick, which were polished or treated with a diamond paste. Diamond-like carbon (DLC) and nitrogen-containing carbon (CN_x) films ~20 nm thick were deposited on the polished titanium samples by the method of pulsed arc sputtering of graphite. The CN_x films were sputtered at the nitrogen pressure of the working chamber equal to P=1 Pa.

The average roughness of the sample surface Ra and the range of heights R_z over 10 most protruding points on the surface were determined using a CMM-2000T instrument by the method of scanning tunneling microscopy (STM). For this purpose, randomly chosen regions (40×40) m in size were scanned on the surface.

The surface morphology was examined in a Philips 515 scanning electron microscope (SEM).

One more subject of study was porous titanium samples, which were prepared by compaction of particles of the TG-OP-1 titanium sponge. The samples were compacted in special molds under different conditions ($1.5\cdot10^3$ to $5\cdot10^3$ kgF). The compacts were annealed for 2 hours in a vacuum at $P=10^{-2}$ Pa and t=1100 °C. The annealed samples underwent chemical purification in a mixture of nitric and hydrofluoric acids. The volume fraction of pores was determi-

ned by the gravimetric method. Bone-marrow cells were deposited on samples having the volume fraction of pores equal to ~50 %.

Bone-marrow cells, which were taken from patients at the age of 14 to 54, were used in the experiments concerned with adhesion of cells on different samples and metabolic processes. The cells were sampled from lumbar vertebra bodies or the crest of the upper flaring portion of the ilium during surgical operations.

The cell suspension with the concentration of $3–5 \cdot 10^9$ cells/l was cultivated in the medium 199 containing 50 % of horned cattle serum (HCS) at 37 °C for 4 hours in humid air. Titanium samples were placed in hollows of disposable flat-bottomed plastic cups. The suspension of bone-marrow cells in the amount of 200 μl was poured into the cups [1]. The samples were rinsed once in the HCS medium 199 so as to remove loose cells and then were immersed in 0.25-% tripsin-EDTA (Sigma) for 5 minutes to re-slurry the cells. The cells were reanimated in the medium 199 with 50 % HCS and their number was counted in the Goryaev chamber. The morphology of adhered cells was analyzed in light and scanning microscopes. Before examination in the scanning microscope, adhered cells were dehydrated in methanol and a thin layer of titanium was applied on the cells.

The index of metabolic deviations in the cells was the total activity of respiratory dehydrogenase, which was estimated considering the capacity of the cell suspension to reduce nitroblue tetrazolium to the insoluble form of violet formazan [7]. The spectrophotometrically modified NCT test was used for this purpose. The reference was bone-marrow cells, which were incubated in the same conditions without any additions.

3. Results and Discussion

3.1. Analysis of titanium samples

The average surface roughness of the polished disks was R_a=30 nm and R_z=200 nm as determined by the STM method. The corresponding values for the disks treated with a diamond paste were R_a=105 nm and R_z=700 nm. The surface roughness of polished titanium samples changed little after sputtering of thin DLC and CN_x films.

The concentration of nitrogen in CN_x was 18 at.% (CN0.25) as measured by the X-ray spectroscopy method.

Figure 1 presents the porous titanium surface, which was observed in a scanning electron microscope. It is seen from this figure that the sample had pores (200–400) μm in size, which were necessary for accumulation, migration and adhesion of bone-marrow cells [4].

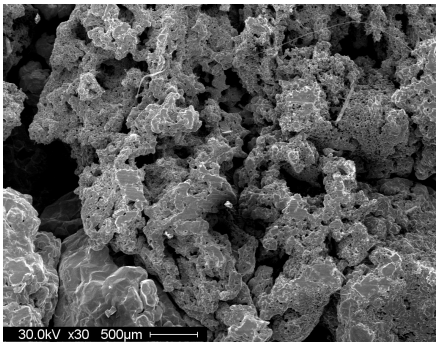

Fig. 1. SEM image of the porous titanium surface

3.2. Adhesion of bone-marrow cells

The number of cells, which adhered to titanium and DLC-coated titanium samples in an interval of 4 hours, differed little. The number of cells adhering to titanium samples with the CN_x film was significantly larger in statistical terms (p<0.05) than their number on samples of polished titanium (Fig. 2).

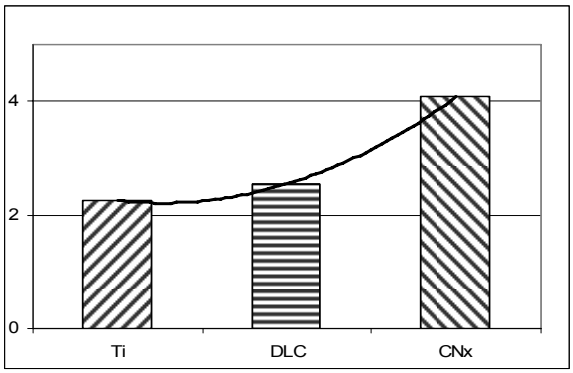

Fig. 2. Percentage of adhered cells relative to the total amount of cells added

The morphological analysis of adhered cells did not reveal any symptoms of degenerative-dystrophic changes in these cells. This fact suggested that the samples were not toxic to karyocytes in short-term cultures. All the adhered cells, which were described in morphological terms, had the blood-borne origin. The absolute majority of these cells (97 %) referred to mature blood cells. Correspondingly, 3 % of the cells was related to peripheral progenitors having different degrees of maturity. Lymphocytes and neutrophils dominated among mature cells. Remarkably, the percentage of adhered lymphocytes was higher on the titanium sample with the CN_x film than on samples of polished titanium and DLC-coated titanium (Fig. 3).

Most probably, adhesion of cells during the short-term incubation was determined not so much by the effect of the surface material on the cells as by its affinity for macromolecules of the medium [3, 5]. This supposition was confirmed in experiments concerned with the study of the titanium surface after its

incubation in the cell-free medium. The increase in the titanium surface roughness (Fig. 4) proved unambiguously that macromolecules were deposited on the titanium surface. It is seen in Fig. 4 that the titanium surface roughness increased with time and then reached some constant value. This observation is an indication that macromolecules covered the titanium surface probably during the first 10 minutes of incubation.

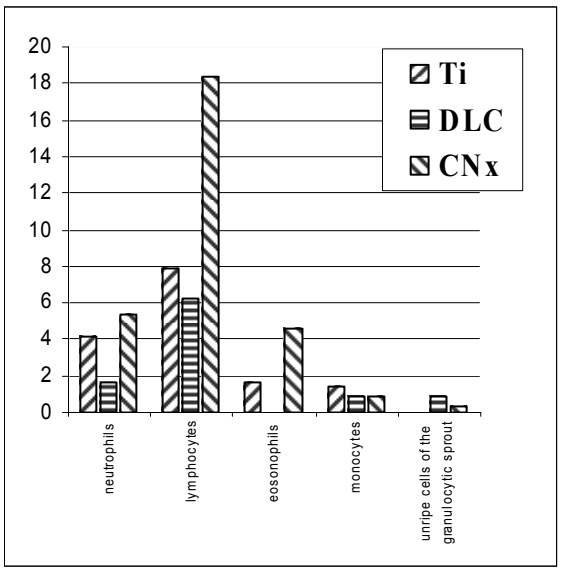

Fig. 3. Percentage of different adhered cells on titanium, DLC and CN_x relative to the total amount of cells added

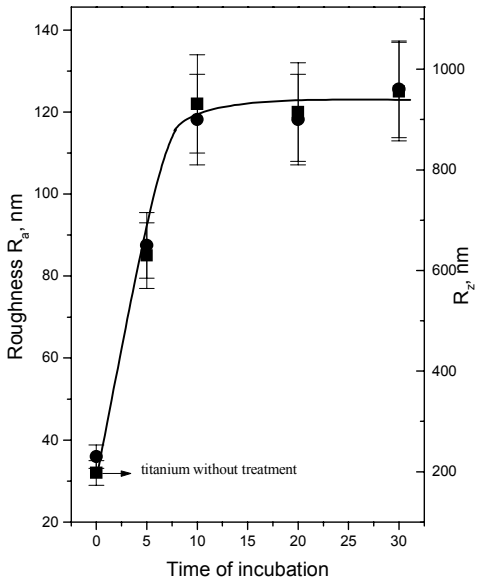

Fig. 4. Dependence of the roughness of polished titanium samples on the incubation period in the medium without bone-marrow cells

It is known [1] that the adhesive activity of cells depends on surface properties. One can reasonably think that adhesion is more active on a "protein-lipid substrate" than on "bare" metal. Therefore the affinity of the surface for protein-lipid components of the medium may be significant for adhesion of cells in short-term cultures in vitro. It is not improbable that macromolecules adhered more actively to the CN_x film thanks to the presence of active CN groups, leading to the increase in the number of adhered cells on titanium with the CN_x film.

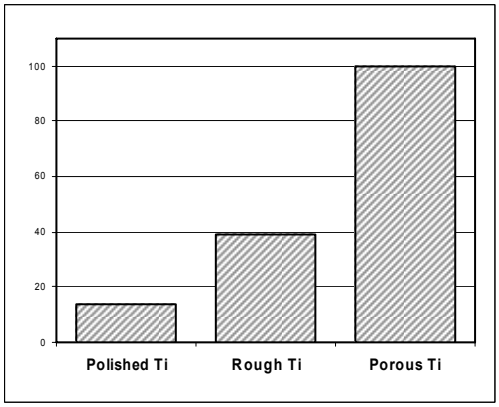

Fig. 5. Relative adhesion of cells on titanium samples with different degrees of surface roughness

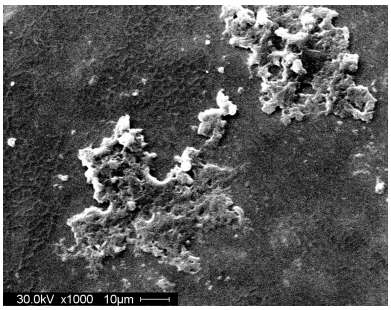

Fig. 6. SEM image of morrow cells on titanium

When titanium with different degrees of the surface roughness and porous titanium were used as the substrate, discrepancies were larger (Fig. 5). Colonies of cells on the rough titanium surface were clearly seen in a scanning microscope (Fig. 6). This observation was one more proof of the literature data concerning the effect of roughness on adhesion properties [1, 2].

3.3. Metabolic processes

The total activity of NADPH-oxidase was enhanced to some extent during incubation of bone-marrow cultures with DLC- and CN_x-coated titanium (Fig. 7). The variation dynamics of this index corresponded to changes of the adhesive activity of karyocytes on the corresponding samples.

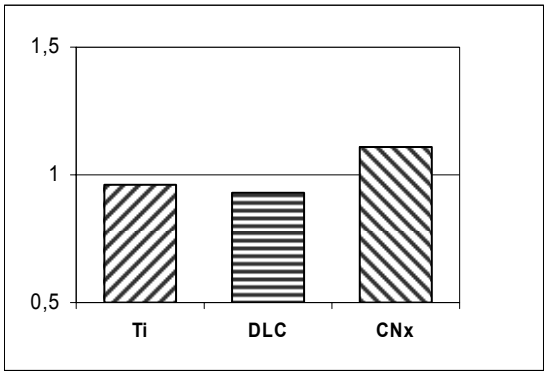

Fig. 7. Summary activity of dehydrogenises of karyocytes during the incubation with titanium and titanium with DLC or CN_x films

4. Summary

1. Coated and uncoated titanium samples were not toxic in short-term cultures.
2. Uncoated titanium samples were characterized by the largest biological inertness and caused the least functional and metabolic changes in bone-marrow cells. The number of adhered cells considerably increased with growing surface roughness of titanium samples.
3. Samples, which had a coat of nitrogen-containing carbon films, had some advantages as substrates for adhesion of cells. The stage of adhesion is necessary for saturation of implants with bone-marrow precursors of osteogenesis.
4. The number of cells adhering to porous titanium was significantly larger.

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