The Use of Catalytic Carbon Deposits as 3D Carriers for Human Bone Marrow Stromal Cells

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We studied the possibility of using 3D structures based on carbon catalytic deposits as carriers for human bone marrow stromal cells. It was found that carbon catalytic deposits obtained by gas deposition method using $FeCl_3 \times 6H_2O$ as the catalyst are a biocompatible material for human bone marrow stromal cells promoting adhesion, proliferation, and distribution of cells within the 3D carrier, and therefore can be used for tissue engineering.

Key Words: *bone marrow stromal cells; catalytic carbon deposits; tissue engineering; 3D structure*

Carbon materials of different structure and shape are widely used in biology, experimental medicine, and clinical practice. Since 1970s carbon fibers were used as the implants for restoring cartilage tissue (knee joint *etc*.) and for replacement of bone defects during intraosseous or surface implantation [4]. Potentials of carbon fibers as cell-free implants for restoration of ligaments and tendons were proven and *in vivo* biocompatibility of the used carbon materials was demonstrated [7]. Thus, long-term (15 years) study of the use of carbon implants for restoring the knee joint revealed no infiltration of newly formed cartilage with lymphocytes or other inflammatory cells [6]. Despite the fact that carbon material is not completely inert, the authors found no signs of ionic exchange between the implant and tissue after intraosseous implantation [10]. The bone tissue was formed *de novo* from both the inside and outside of the carbon implant. At present, carbon materials are practically not used as cell-free implants; however, they can be considered as potential carriers for tissue engineering *in vitro*.

Tissue engineering is aimed at replacement of damaged tissue with a composite consisting of cells and a carrier capable of restoring or maintaining tissue structure and function. Of crucial importance is the choice of cell type and carrier responsible for the tissue formation and development. Mesenchymal stromal cells are promising for tissue engineering due to high proliferative activity and ability to differentiate into different mesenchymal cells in response to corresponding inductors [12].

In most cases, carriers for tissue engineering should be biocompatible, provide a scaffold for 3D cell growth, have extensive area for cell population and growth relative to the total volume of the carrier, and exhibit certain mechanical characteristics. In recent years, carbon materials in the form of nanotubes and nanofibers are more and more often used in tissue engineering [8,13,15]. Carbon nanotubes are usually manufactured using the following three methods: electroarc [9] or laser synthesis [14] or gas deposition method [1,2,5]. The latter is most widely used in industry and implies the use of various metals as catalysts placed in the flow of reagent gas (hydrocarbons, CO, CO_2, *etc*.) at high temperatures (>700°C). Variation of the reaction conditions (temperature, gas pressure, time, catalyst, *etc*.) allows the formation of catalytic carbon deposits (CCD) of different diameter

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and length, from monolayer nanotubules to multilayer tubules with a diameter of several microns.

Here we studied the possibility of using 3D structures based on CCD as carriers for human bone marrow stromal cells.

MATERIALS AND METHODS

Bone marrow cells were isolated from fragments of the iliac crest spongy bone tissue obtained during planned surgery with strict adherence to deontology rules. The study was approved by Ethic Committee of the Institute of Cryobiology and Cryomedicine Problems, National Academy of Sciences of Ukraine. The suspension was obtained by repeated cell washout from the bone fragment with medium 199 and centrifuged at 150*g* for 10 min; the pellet was resuspended in α-MEM containing 15% FCS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mmol/ml L-glutamine. The cells were cultured at 37°C, 5% CO_2, and 95% humidity. The medium was changed after 72 h and then every 3-4 days. After attaining 70% confluence, adherent cells were treated with trypsin and subcultured 1:3. Cells of passages 3 and 4 were used for further studies.

CCD were prepared using the method of catalytic decomposition of methane [1-3]. Iron salts $FeCl_3 \times 6H_2O$ were used as the catalyst of initiation of the growth of carbon structures and sulfur powder served as a promoter of the reactions. The catalytic mixture was placed on a graphitic plates positioned inside a vertical cylindrical carbon-carbon heater. The process was carried out in atmosphere of natural gas containing 96-98% methane (CH_4) at 1100°C (0.4-0.8 m^3/h gas flow and 12-24 h process duration). Under these conditions, growth of hollow carbon fibers was observed with a pyknometerical density of 1.66-1.68 g/cm^3. CCD structure was studied by SEM under a JSM-6700F electron microscope (Jeol).

For further population with cells and preparation of compact 3D structures, CCD samples were gently compressed and sterilized at 160°C for 90 min. For population of the carrier, concentrated cell suspension (10^5 cells in 20 μl) was dropwise applied to the 3D carrier, incubated at 37°C for 3 h, and transferred to wells of a 24-well plate containing 1 ml nutrient medium for further culturing.

Metabolic and proliferative activities of cells in cultured in CCD carriers were determined using REDOX Alamar Blue (AB) indicator. To this end, 10% AB was added at different terms of culturing (days 2, 7, and 10) and incubated for 2 h at 37°C. The medium containing AB was taken and the degree of AB reduction was determined using a Tecan GENios plate reader at excitation and emission wavelengths of 550 and 590 nm, respectively. The data were presented as the difference between the experimental and control sample (without cells) and expressed in arbitrary units of fluorescence (AUF).

For histological study, the carbon carriers with cells after 10-day culturing were fixed in 70% ethanol, dehydrated in ascending alcohol concentrations, and embedded in paraffin. Serial paraffin sections (15 μ) were stained with azur-eosin after Romanovskii–Giemsa and examined under a light microscope.

The data were processed statistically using Student's *t* test, the differences were significant at $p<0.05$.

RESULTS

Before population, the obtained spatial CDD structures looked like gray-black fibrous fragments (~4 mm in diameter and 2 mm thickness) consisting of thin carbon firers loosely packed in a elastic structure with whitish spots. Electron microscopy provided more information of CCD microstructure (Fig. 1).

CCD represented a 3D matrix consisting of interlaced twisted fibers (tubes) with a diameter up to 10 μ. The hollow structure of the fibers was confirmed by their considerable low pyknometric density compared to theoretical value. All fibers had smooth insignificant, but visible variations of diameter, which was probably related to peculiarities of the technological process. The butt ends of some fibers were closed.

Twenty-four hours after seeding of stromal cells on 3D CCD matrices and subsequent *in vitro* culturing of these biocomposites, adherent and flattened fibroblasts-like elements were observed on the surface of carbon fibers. Metabolic activity of cells cultured in 3D structure was assessed using AB indicator (Fig. 2). To exclude the contribution of cells migrated from the matrix and adherent to plastic to the total level of AB reduction, the samples every time were transferred to new wells of the plate.

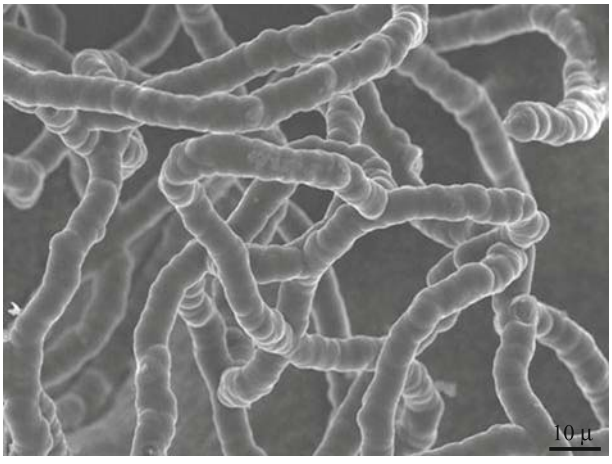

Fig. 1. SEM of catalytic carbon deposits.

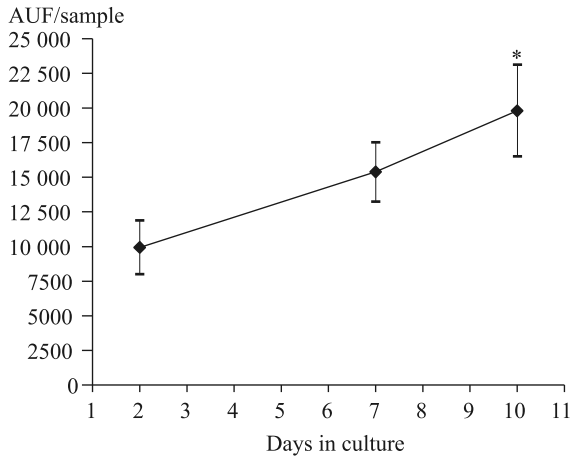

Fig. 2. Reduction of AB by stromal cells during culturing in 3D CCD carrier. *p<0.05 compared to day 2 in culture.

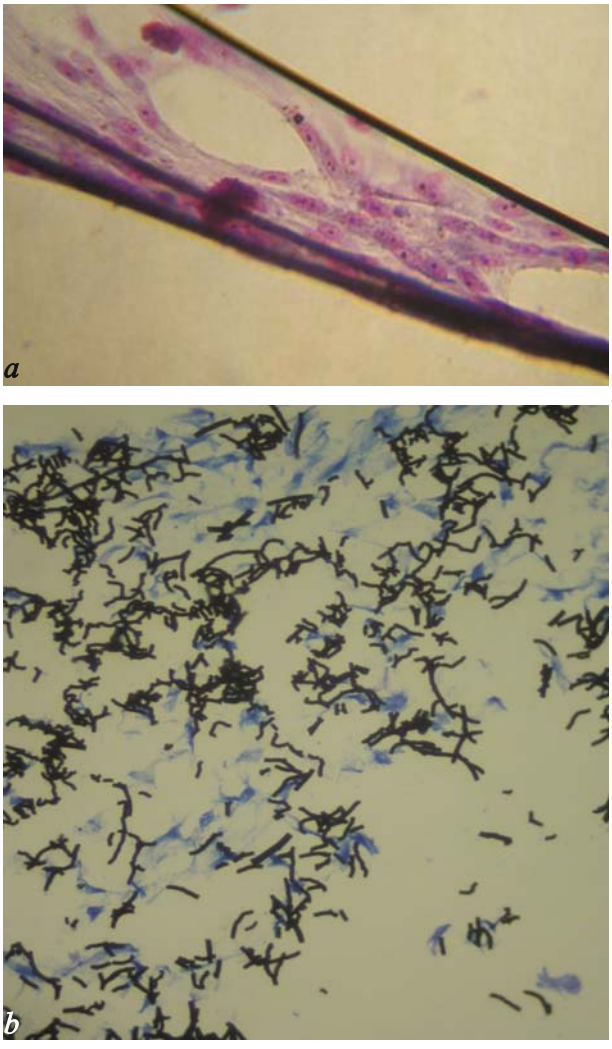

Fig. 3. Microscopy of 3D CCD structure with human bone marrow stromal cells after 10 days in culture. *a*) membranes between carbon fibers formed by stromal cells (azur-eosin staining, ×200), *b*) histological section of 3D structure (15μ thickness, azur-eosin staining, ×100).

On day 2 in culture, the level of AB reduction by human stromal cells was 9939±1904 AUF. Further culturing of stromal cells in the carbon matrix was associated with an increase in AB reduction level to 15,384±2148 and 19,794±3376 on days 7 and 10, respectively (Fig. 2). AB is an integral parameter of activity of redox enzymes in cells, and increased level of AB reduction during culturing reflects the increase in the number of cells, *i.e.* their proliferation [11].

After 10 days in culture, 3D structures containing human stromal cells were fixed and examined by histological methods (Fig. 3).

Histological sections of these matrices populated with stromal bone marrow cells after 10 days of static culturing contained cells with normal structure. In some sections, stromal cells formed bridges and membranes consisting of 2-3 cells between the neighboring supporting fibers (Fig. 3, *a*).

The processes and bodies of stromal cells bound the neighboring fibers with the formation of a cellular-carbon structure; cells located in sites of fiber crossings had the maximum area of cell contact with carbon substrate. Stromal cells were observed on both the outer and inner surfaces of the structure and in the depth of carbon material (Fig. 3, *b*). The surface of the structure was coated with an interrupted cell layer consisting of 1-3 layers of flattened cells with clear-cut nucleus and light cytoplasm contacting with each other and with carbon fibers. In the depth of the carbon matrix stromal cells were equally, though sparse distributed. Hence, the location of flattened stromal cells relative to the structure of the carbon carrier attested to realization of their adhesion properties and proliferation capacity during 3D culturing.

Thus, carbon catalytic deposits obtained by gas deposition method using $FeCl_3 \times 6H_2O$ as the catalyst are a biocompatible material for human bone marrow stromal cells promoting adhesion, proliferation, and migration of cells within the 3D carrier, and therefore can be used for tissue engineering.

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