HUMAN OSTEOBLAST-LIKE MG 63 CELLS IN CULTURES ON NANOFIBROUS PLGA MEMBRANES LOADED WITH NANODIAMONDS

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Abstract

Using an electrospinning technique, we constructed composite nanofibrous membranes containing a copolymer of L-lactide and glycolide (PLGA, ratio 85:15) and 33 wt% of nanodiamond particles. he number of initially adhering human osteoblast-like MG 63 cells on day 1 after seeding, their spreading and subsequent growth were similar on both types of membranes. However, higher cell numbers on day 3 and 7 after seeding and a larger cell spreading area were found in the cells in the control polystyrene cell culture dishes. Nevertheless, the composite PLGA-ND membranes provided relatively good support for colonization with bone-derived cells; thus this material is promising for bone tissue engineering.

Keywords: *nanofibers, nanoparticles, nanotechnology, electrospinning, bone tissue engineering*

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Introduction

Electrospinning is a promising method for creating many types of nanofibers, which can have a broad spectrum of applications, including tissue engineering.

Nanofibers, especially in the form of nanofibrous scaffolds, have become promising cell carriers for advanced tissue replacement. This is mainly due to the fact that these scaffolds closely mimic the structure of the fibrous component of the native extracellular matrix. In comparison with conventionally-used flat or microstructured cell growth supports, nanostructured carriers improve the cell-matrix interaction, e.g. by adsorption of cell adhesion-mediating molecules in an appropriate geometrical (spatial) conformation, which enables good accessibility of specific sites on these molecules to cell adhesion receptors. In addition, nanostructured substrates can be advantageously applied particularly in bone tissue engineering. The reason is that these substrates preferentially adsorb vitronectin, which is recognized mainly by osteoblasts [1,2].

In the present study, we have constructed nanofibrous membranes by electrospinning of poly (lactide-co-glycolide) (PLGA) mixed with nanodiamond (ND) particles as potential growth supports for bone cells. We therefore investigated the adhesion and proliferation of human osteoblast-like MG 63 cells in cultures on these materials. In our earlier studies, PLGA proved to be an appropriate material for constructing porous and fibrous scaffolds for bone tissue engineering [3,4]. In comparison with pure polylactic acid (PLA), the PLGA copolymer is less brittle, and in comparison with pure polyglycolide (PGA), it is more slowly degradable (i.e., less prone to hydrolytic degradation). It thus provides a more stable support for bone tissue regeneration, which is a relatively long-term process in human beings, especially among the aged. The ND particles were added to reinforce the nanofibrous scaffolds and to create an additional nanostructure on the fiber surface in order to enhance the scaffold bioactivity. In our earlier studies, nanocrystalline diamond films provided excellent growth support for bone-derived cells [5,6].

Materials and Methods

Preparation of the nanofibrous membranes

The nanofibrous membranes were prepared from a copolymer of L-lactide and glycolide (PLGA, ratio 85:15, PURASORB PLG 8531, Purac Biomaterials, Germany). The copolymer was dissolved in a mixture of methylene chloride (Sigma-Aldrich, Cat. No. D6 510-0) and dimethyl formamide (Sigma-Aldrich, Cat. No. D15 855-0) at a concentration of 2.3 wt%. The ratio of the two solvents was 2:3. The nanofibrous membranes were then prepared by electrospinning in a Nanospider ™ machine (Elmarco, Czech Republic), using a vertically positioned spike-like electrode (voltage 24.6 kV, working distance between the electrodes: 145 mm). Some nanofibrous membranes were created in a combination of PLGA with nanodiamond (ND) powder, kindly provided by Prof. Stanislaw Mitura, Technical University of Lodz, Lodz, Poland [7]. In 1 ml of PLGA solution, 0.007 g of the ND powder was added and homogeneously dispersed by sonication (LABSONIC U-2000, B. Braun Biotech, Germany, time of sonication 5s). The concentration of ND in PLGA diluted in methylene chloride and dimethyl formamide was therefore 0.7 wt%, and in the pure PLGA after evaporation of the solvents, this concentration reached almost 33 wt%.

Cell culture on the nanofibrous membranes

The nanofibrous membranes were cut into square samples 1x1 cm in size, fixed in CellCrown inserts (Scaffdex, Finland), sterilized by gamma irradiation, inserted into polystyrene 24-well cell culture plates (TPP, Trasadingen, Switzerland; well diameter 1.5 cm), rinsed overnight with Dulbecco's Modified Eagle Minimum Essential Medium (DMEM; Sigma, U.S.A.). The membranes were then seeded with human osteoblast-like MG 63 cells (European Collection of Cell Cultures, Salisbury, UK) in a density of 17,000 cells/cm¸ and in 1.5 ml of DMEM medium supplemented with 10% foetal bovine serum (Gibco). The cells were cultured for 1, 3, or 7 days at 37°C in a humidified atmosphere of 5% of CO_2 in the air. The polystyrene culture well was used as a reference material. For each experimental group and time interval, five samples were used.

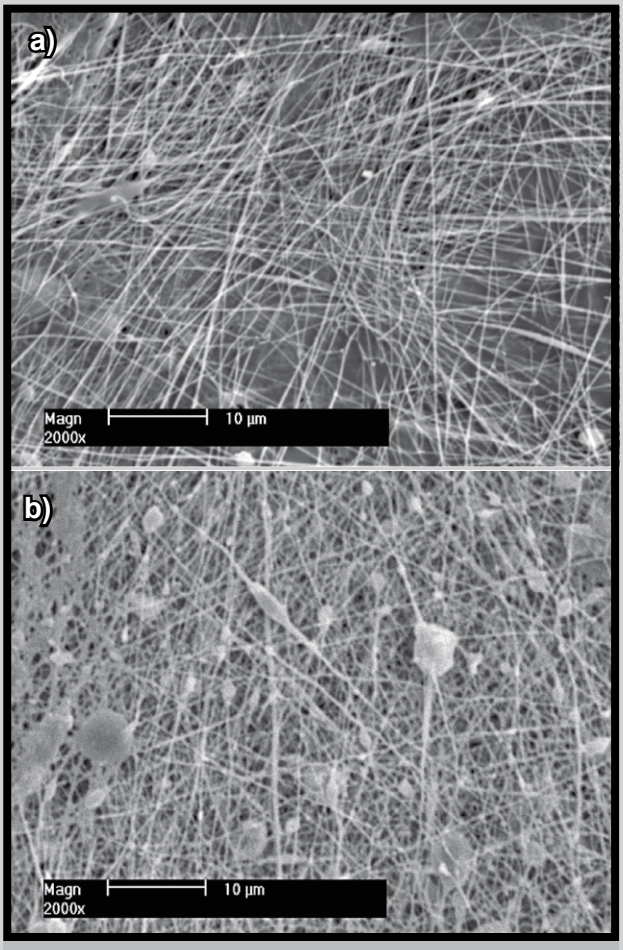

FIG. 1. Morphology of a pure (a) and nanodiamond-enriched (b) PLGA nanofibrous membrane. Scanning electron microscope XL30CP (Phillips Electron Optics GmbH, Kassel, Germany), objective magnification 2000x, bar = 10 µm.

Evaluation of cell adhesion and growth on the nanofibrous membranes

On days 1, 3 and 7 after seeding, the samples were rinsed in phosphate-buffered saline (PBS). The cells on one sample per experimental group and time interval were fixed by 70% cold ethanol (-20°C) and stained with a combination of Texas Red C_2-maleimide fluorescent membrane dye (Molecular Probes, Invitrogen, Cat. No. T6008; concentration 20 ng/ml in PBS) and Hoechst # 33342 nuclear dye (Sigma, U.S.A.; 5 µg/ml in PBS). The morphology and distribution of the cells on the membrane surface were then evaluated on pictures taken under the Nikon Eclipse Ti-E microscope equipped with a Nikon DS-Qi1 MC digital camera and NIS-Elements AR software, version 3.10.

On day 1 after seeding, the size of the cell spreading area was also measured using Atlas Software (Tescan Ltd., Brno, Czech Republic). For each experimental group, 141 to 349 cells were evaluated.

The four remaining samples were used for evaluating the cell number. The cells were rinsed with PBS, released with trypsin-EDTA solution (Sigma, Cat. No. T4174; incubation 5 minutes at 37°C) and counted in a Bürker hemocytometer under an Olympus IX 51 microscope.

Statistics

The quantitative results were presented as a mean ± standard error of mean (SEM). Statistical significance was evaluated using the ANOVA, Student-Newman-Keuls method. Values of $p \leq 0.05$ were considered as significant.

Results and Discussion

Morphology of the nanofibrous membranes

Both pure PLGA and composite PLGA-ND membranes formed a fine cobweb-like layer on the polypropylene fabrics which served as a collector for the nanofibrous membranes. As revealed by scanning electron microscopy, both types of membranes were composed of mostly straight and randomly oriented fibers (FIG. 1). In the PLGA-ND membranes, the fibers were thicker (diameter 270 ± 9 nm) and the pores among them were smaller (area 0.46 ± 0.02 µm^2) than in the pure PLGA membranes (diameter 218 ± 4 nm, area 1.28 ± 0.09 µm^2).

Cell adhesion and growth on nanofibrous membranes

The adhesion and subsequent growth of MG 63 cells was similar on both pure and ND-loaded PLGA membranes. On the first day after seeding, the number of initially adhering cells on the pure PLGA membrane was 11,300 ± 1,200 cells/cm^2, and on the PLGA-ND membranes it was 10,951 ± 1,027 cells/cm^2. The highest average number of initially adhered cells was observed on the control polystyrene dishes (14,000 ± 1,038 cells/cm^2). However, as revealed by ANOVA, this value was not statistically different from the values obtained on the two nanofibrous membranes (FIG. 2).

Also on the third day of the experiment, the cell numbers on both pure and ND-loaded PLGA membranes were similar (36,100 ± 2,200 cells/cm^2 and 37,000 ± 2,500 cells/cm^2, respectively). However, on control polystyrene dishes, the cell number was significantly higher, reaching 61,900 ± 2,100 cells/cm^2. This trend continued towards the 7th day after seeding, when the cells on both pure and ND-loaded PLGA membranes again reached similar values (167,700 ± 4,500 cells/cm^2 and 169,900 ± 6,100 cells/cm^2, respectively).

The cell spreading area, measured on day 1 after seeding, was on an average larger in the cells on PLGA-ND membranes (477 ± 16 µm^2) than in the cells on pure PLGA membranes (442 ± 15 µm^2), but this difference was not statistically significant. However, both these areas were significantly smaller than those of the cells on the bottom of control polystyrene wells (1,057 ± 33 µm^2). This may be due to the relatively thin diameter of both PLGA and PLGA-ND

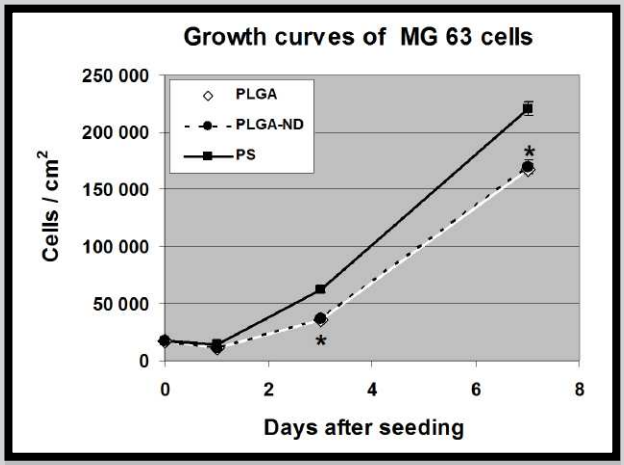

FIG. 2. Growth dynamics of human osteoblast-like MG 63 cells on pure PLGA nanofibrous membranes (PLGA), composite PLGA-nanodiamond membranes (PLGA-ND) and control cell culture polystyrene dishes (PS) from day 1 to 7 after seeding. Mean ± S.E.M. from 72 measurements taken from 4 samples for each time interval. ANOVA, Student-Newman-Keuls Method. Statistical significance: * $p \leq 0.05$ in comparison with PS.

fibers (on an average 218 and 270 nm, respectively), and also their random orientation. In a study performed on NIH 3T3 fibroblasts in cultures on PLGA meshes [8], the cell spreading areas on meshes with fiber diameters of 140 nm and 760 nm were significantly smaller than on continuous PLGA films, and were equal to the value on these films only at a fiber diameter of 3,600 nm. Increasing fiber orientation (from random to parallel) also contributed to enlarging the cell spreading area [8].

Nevertheless, the cells on the PLGA-ND membranes were similar in shape to the cells on the pure PLGA membranes and on the polystyrene dishes, i.e. polygonal or spindle-like, and were distributed homogeneously on the samples. Their number rose continuously with the time of the experiment, and at the end of the experiment these cells were able to create a confluent layer (FIG. 3). These results suggest that the cells on the PLGA nanodiamond membranes behaved physiologically without noticeable signs of cell damage.

Conclusion

Using an electrospinning technique, we constructed fibrous membranes (average fiber diameter 218-270 nm) made of pure PLGA and PLGA with 33 wt% of nanodiamond. Both pure PLGA and composite PLGA-nanodiamond membranes enabled attachment, spreading and subsequent proliferation of human osteoblast-like MG 63 cells in cultures on these materials, and this cell behavior was similar on both types of membranes. Therefore, our composite PLGA-nanodiamond scaffolds are promising materials for bone tissue engineering.

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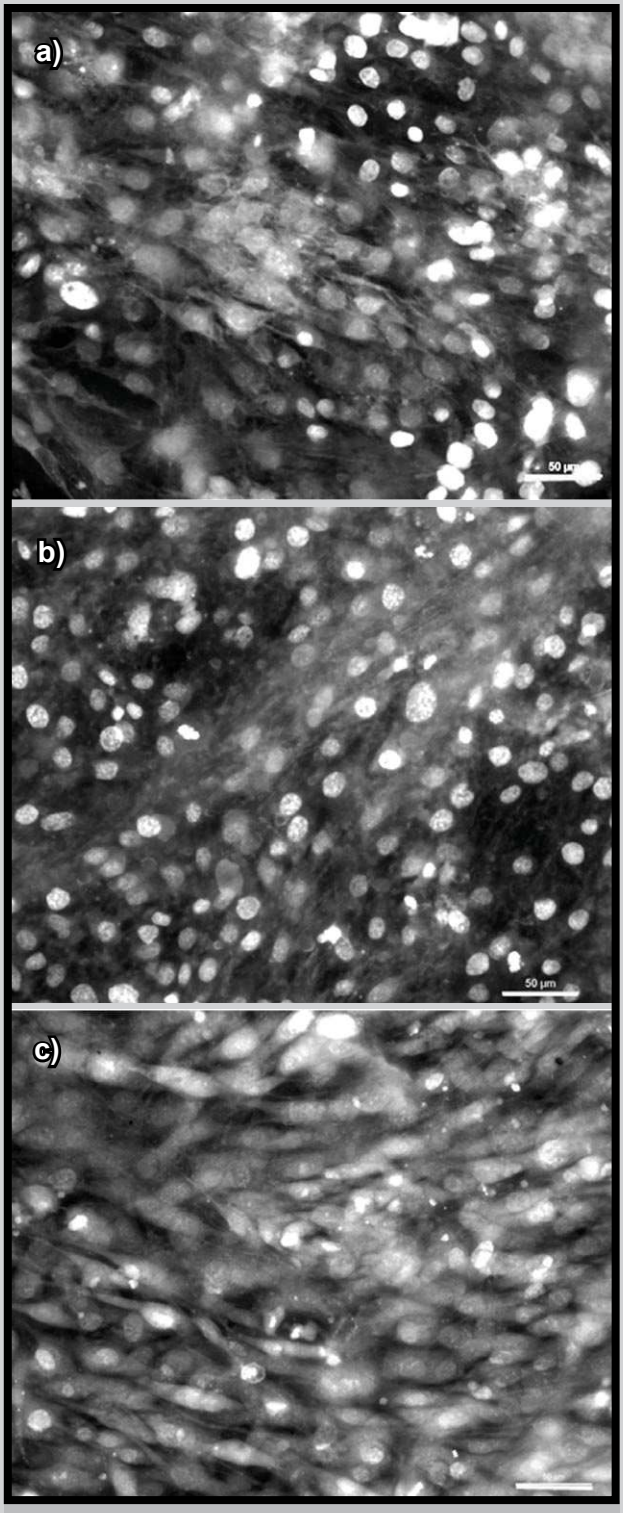

FIG. 3. Morphology of human osteoblast-like MG 63 cells on day 7 after seeding on a pure PLGA membrane (a), composite PLGA-nanodiamond membrane (b) and polystyrene culture well (c). Cell membrane and cytoplasm were stained with Texas Red C_2-Maleimide, and the cell nuclei with Hoechst #33342. Nikon Eclipse Ti-E microscope, obj. 20x1.5; Nikon DS-Qi1 MC digital camera, NIS-Elements AR software, version 3.10. Summarization of Z-sections with deconvolution, bar = 50 µm.