Encapsulation of bone marrow stromal cells in alginate microbeads

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Introduction

Tissue engineering is key component of regenerative medicine, since integrated use of isolated cells, biomaterial scaffolds and bioreactors enables the best biophysical regulation of *in vitro* cell differentiation and tissue assembly (Lavik 2004). Despite many advances in recent years, researchers in this field still face significant challenges in regenerating specific tissues or organs, related to the cellular component of engineered tissue, the biomaterial composition and configuration, as well as to the environmental factors needed to induce differentiation signals into surgically transplantable formats.

Challenges related to the cellular component of an engineered tissue include cell sourcing, expansion, differentiation, as well as regulatory and production issues, such as sterility, safety, storage, shipping, quality control and scale-up. The use of mesenchymal cells derived from bone marrow (herein reffered to as bone marrow stromal cells - BMSC) represents a feasible approach to address many of these issues. Namely, within the bone marrow stroma are undifferentiated cells with high proliferative activity and with potential to differentiate into a number of mesenchymal cell lineages including cartilage, bone, muscle, adipose tissue, and tendon, as well as to support hematopoiesis *in vitro*, when cocultured with hematopoietic progenitors. Since BMSC maintain their biosynthetic activity in older individuals and can be easily harvested and quickly and reproducibly expanded *ex vivo*, over the past decade these cells became a subject of intensive investigations in cell therapy and regenerative medicine (Bianco 2001).

The other important consideration associated with developing a functional tissue-engineered graft is the biomaterial support used. The scaffolds should ideally provide a 3-D template for cell attachment and extracellular matrix deposition, mechanical integrity untill new tissue is formed and biodegradation at a rate matching the rate of tissue assembly. Cell encapsulation is an important biotechnology in tissue engineering, since it promotes tissue regeneration by facilitating the localized retention of entrapped cells, as well as by controlling the release of therapeutic agents to the host. Alginate is a biomaterial that has been broadly investigated for cell immobilization, due to its biocompatibility, biodegradability and ease of processing into different shapes. However, research was mostly aimed at immunoisolatory and biochemical processing applications (Melvik 2004) and far less is known on alginate as a matrix for tissue formation. Therefore, in this work, we have investigated potentials for immobilization of murine BMSC on alginate supports aimed to establish the alginate-bioreactor culture system for chondrogenic and/or hematopoiesis-suportive tissue progression. In the initial studies the procedure for isolation of stromal cells from murine bone marrow was established and culture-expanded mesenhymal cells characterized. Further on, two immobilization methods were investigated and compared, the cell entrapment within alginate microbeads by using electrostatic droplet generation and simple cell adsorption onto alginate gel surfaces. The stability of alginate in culture and its suitability as cell support material were tested under both static conditions and in packed bed bioreactors.

Material and methods

Cell isolation and expansion. To isolate BMSC, 8- to 10-week old male CBA mice (Breeding Facilities of the Institute for Medical Research, Military Medical Academy, Belgrade, Serbia) were used. Pooled femoral bone marrow cell suspensions were prepared in expansion medium (Dulbecco's Modified Eagle Medium-DMEM supplemented with 10% Fetal Bovine Serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, all from Sigma, St. Luis, MO, and 1 ng/ml human recombinant bFGF, R&D Systems, Minneapolis, MN). Cells were then plated in culture flasks at a density of 0.5-1 x 10^6 cells per cm^2 and cultured at 37^oC in a humidified atmosphere with 5% CO_2 in air for a period of 7-10 days, replacing the culture medium every 3 days. BMSC were selected on their ability to adhere to the tissue culture plastic, and non-adherent cells were removed with the medium during refeeding. After 7-10 days of culture, when BMSC became about 80% confluent, the cells were detached by using 0.25% trypsin/1mM EDTA and replated at a density of 20-25 x 10^3 cells/cm^2 in culture flasks with the expansion medium for an additional period of 10-15 days, replenishing the medium as above. After this period, at about 80-90% confluence, this second passage cells were deteched with trypsin/EDTA and were then either encapsulated in alginate microbeads produced by electrostatic droplet generation or seeded onto alginate gels.

CFU-F (Colony Forming Unit – Fibroblastic) assay. Pooled bone marrow cell suspensions were plated at 5 x 10^6 cells per 9.5 cm^2 and cultured for 48 h at 37^oC in a humidified atmosphere with 5% CO_2 in air. The non-adherent cell fraction was then removed and the cultures incubated an additional 6 days. Cultures were then fixed with cold methanol, stained with Giemsa and the number of CFU-F colonies representing more than 50 cells determined.

Alginate microbeads generation. Sodium alginate powder (medium viscosity, Sigma, St. Luis, MO) was dissolved in WFI water at a concentration of 2% w/w and then mixed with a suspension of BMSCs in culture medium to obtain final concentrations of 1.5 % w/w alginate and 1 or 5 x 10^6 cells/ml. Alginate microbeads were produced by electrostatic droplet generation (Bugarski et al 2004). In brief, cell/alginate suspension was extruded through a positively charged blunt stainless steel needle (24G, 6.4 kv applied potential), at a constant flow rate of 25.2 ml/h using a syringe pump (Razel, Scientific Instruments, Stamford, ST). Resulting droplets were collected in a gelling bath (1.5 % $CaCl_2$) and after 30 min of gelation the alginate microbeads were used in further experiments. The same procedure was used to produce acellular alginate microbeads, used in studies of cell adsorption by adding 1 ml of cell suspension with 1 x 10^6 cells per 1 ml of alginate microbeads.

Alginate discs formation. In order to form 1 mm thick calcium-alginate crosslinked hydrogel discs in 6-well plates, 1.5 ml of 3% alginate solution was added to each well, followed by 2% $CaCl_2$. After jellification, the $CaCl_2$ solution was replaced with DMEM media and the plates were stored at 4^oC until cell seeding. BMSC suspensions were seeded on alginate gels by direct application of 1.5 ml with 4-8 x 10^4 cells on top of each gel.

Static culture conditions. Alginate microbeads with entrapped and seeded BMSC, as well as alginate discs with seeded BMSC were cultivated in flat-bottomed 6-well plates at 37^oC in a humidified atmosphere with 5% CO_2 in air for up to 30 days, with medium exchange every 3 days. The culture media comprised DMEM, 10% FCS, pen/strep, 50 μg/ml ascorbic acid, 10 nM dexamethasone and 100 ng/ml mouse recombinant IGF-I (R&D Systems, Minneapolis, MN). Cultures were routinely examined using phase contrast microscopy. Viability of immobilized BMSC was determined by means of trypan-blue exclusion test or the MTT assay after alginate carriers were dissolved in 0.05 M citrate solution.

Bioreactor cultivation. Packed bed bioreactors were custom made (Faculty of Technology and Mettalurgy, Belgrade) as two glass cylinders with sintered glass plates at the ends, fit into a piece of silicone tubing to form a chamber of 1 ml in volume. Bioreactor was connected to a peristaltic pump and a medium reservoir using silicone tubing that ensures gas change in the culture medium. Bioreactor system with BMSC contained 12 ml and was perfused at a rate of 0.18 ml/min for 5 weeks with medium exchange of 40% twice a week. Culture medium for these experiments was DMEM, supplemented with 10% FCS, pen/strep, 50 μg/ml ascorbic acid, 10 nM dexamethasone and 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN).

Results and Discussion

The initial studies were focused on the establishment and standardization of an efficient and reproducible procedure for the isolation of BMSC, as well as describing the characteristics of culture-expanded BMSC. To quantify bone marrow mesenchymal progenitor cells, CFU-F assay was used in which BMSC from whole marrow are isolated by their adherence to plastic, wherein the cells grow as clonal populations derived from a single precursor, as foci with fibroblast-like morphology (Figure 1). Using this assay we showed significant differences in the yield of mesenchymal progenitor cells from different mouse inbred strains, choosing CBA mice as a model due to the highest number of bone marrow derived CFU-Fs.

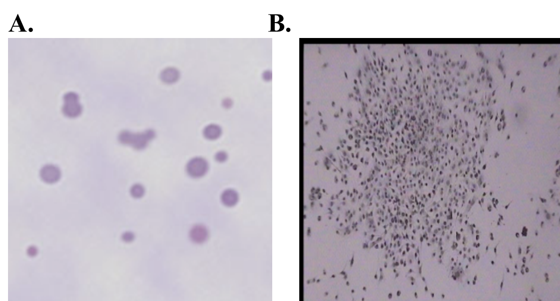

Figure 1: CFU-F (Colony Forming Unit – Fibroblastic) assay, standard *in vitro* method to quantify bone marrow stromal progenitor cells (A); one CFU-F colony (B).

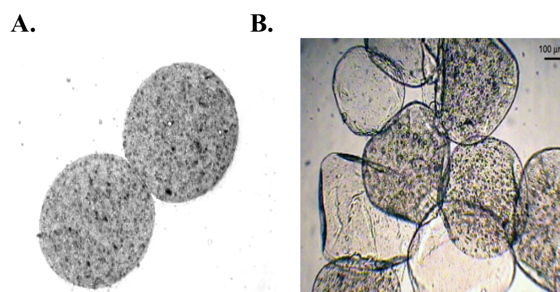

Figure 2: Alginate microbeads with immobilized BMSC - on day 29 of cultivation in static dishes (A); after 5 weeks of bioreactor cultivation (B).

Electrostatic droplet generation was previously shown to be a suitable technique for controlled production of alginate microbeads down to 200 μm in diameter based on the use of electrostatic forces and was also successfully applied for immobilization of microbial, insect and mammalian cells (Bugarski et al 2004). In this study we demonstrated that cell entrapment in microbeads by electrostatic droplet generation is also an efficient technique for immobilization of BMSC, providing high cell yields at preserved viability and uniform distribution (BMSC viability was app. 97% vs 95% before and after extrusion experiment, respectively). Under the experimental conditions used, microbeads of an average diameter of 680 ± 100 μm were obtained in which BMSC remained viable over 4 weeks of cultivation, but failed to differentiate into cartilaginous tissue. The alginate microbeads maintained consistency and spherical shape during the examined period (Figure 2A), although slight bead swelling and surface alterations manifested by the increasement of the average pore size were observed during the cultivation time. BMSC were retained within the microbeads and no cells were detected in the culture medium.

We also investigated the possibility for BMSC adsorption onto alginate surface, since cell adsorption onto surfaces of biomaterial supports can provide direct exposure of the cells to the

medium and consequently efficient mass transport. In both cultures with either microbeads or discs, the cells remained loosely attached and round, over10 days of cultivation, thus revealing that BMSC do not readily adsorb on Ca-alginate gel. This finding imply that alginate surface should be modified in order to improve adhesion properties, such are the reactions with polyaminoacids resulting in positively charged coatings of the alginate gel, more suitable for cell attachment.

We next tested the potentials of alginate microbeads in packed bioreactor systems with continuous perfusion of culture medium. The alginate microbeads with immobilized BMSC, at the concentration of 5×10^6/ml, maintained consistency and spherical shape over 5 weeks of bioreactor cultivation (Figure 2B), and the immobilized cells remained viable at approximately constant concentration, but showing no significant proliferation during the cultivation period. Accordingly, most of the alginate microbeads remained separated and only occasionally loosely bonded groups were observed, due to low extracellular matrix deposition. Since some of the microbeads appeared void of cells, taken together these results imply that the starting cell concentration used (5 x 10cells/ml) was too low to result in tissue formation. This result is in agreement with the findings that biodegradable fibrous scaffolds seeded with chondrocytes at low densities collapsed after 12 days in culture and confirm the hypothesis that there is a minimal cell concentration required for cell survival and extracellular matrix synthesis (Vunjak-Novaković 1998).

Conclusions

Results of this study demonstrated that alginate is suitable for cultivation of bone marrow stromal cells and as a biocompatible material of natural origin, easily processed in various forms, it has significant potential as a support for engineered tissues. However, the data also imply that further studies of size, shape and surface properties of alginate gels in conjunction with bioreactor design are needed, along with the optimization of cultivation conditions in terms of efficient delivery of more differentiation-specific regulatory molecules to the immobilized cells, to enable engineering of variously designed transplants.

Acknowledgement

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