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A perfusion bioreactor system capable of producing clinically relevant volumes of tissue-engineered bone: In vivo bone formation showing proof of concept

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Abstract

In an effort to produce clinically relevant volumes of tissue-engineered bone products, we report a direct perfusion bioreactor system. Goat bone marrow stromal cells (GBMSCs) were dynamically seeded and proliferated in this system in relevant volumes (10 cc) of small sized macroporous biphasic calcium phosphate scaffolds (BCP, 2–6 mm). Cell load and cell distribution were shown using methylene blue block staining and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was used to demonstrate viability of the present cells. After 19 days of cultivation, the scaffolds were covered with a viable, homogeneous cell layer. The hybrid structures became interconnected and a dense layer of extracellular matrix was present as visualized by environmental scanning electron microscopy (ESEM). ESEM images showed within the extracellular matrix sphere like structures which were identified as calcium phosphate nodules by energy dispersive X-ray analysis (EDX). On line oxygen measurements during cultivation were correlated with proliferating GBMSCs. It was shown that the oxygen consumption can be used to estimate GBMSC population doubling times during growth in this bioreactor system. Implantation of hybrid constructs, which were proliferated dynamically, showed bone formation in nude mice after 6 weeks of implantation. On the basis of our results we conclude that a direct perfusion bioreactor system is capable of producing clinically relevant volumes of tissue-engineered bone in a bioreactor system which can be monitored on line during cultivation and show bone formation after implantation in nude mice.

Keywords: Bioreactor; Bone marrow cells; Flow perfusion; Online measurement; Bone tissue engineering; In vivo

1. Introduction

Spinal fusion is considered to be one of the most challenging applications for bone graft substitutes, since even autologous bone has a relatively high rate of failure. All of the current procedures still show nonunion rates varying from 7% to 30% [1]. Therefore, tissue engineering of bone by combining bone marrow stromal cells (BMSCs) with a suitable ceramic carrier could provide an interesting alternative, because this technique has the intrinsic potency to overcome the reported disadvantages of autologous bone grafts. The proof of concept of bone tissue engineering has been shown both ectopically [2,3,7] and orthotopically in rodent studies [4–7]. Few studies demonstrate this technique in large animal models ectopically [8], orthotopically [9,10], and even fewer compared the functioning ectopically and orthotopically [11]. However, studies comparing tissue-engineered bone to autologous bone grafts in a clinically relevant model, or in controlled studies in primates have not been reported. Although this technique is promising there are still some problems that have to be solved in order to be

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clinically applicable. Osteogenic constructs are often produced by isolating osteoprogenitor cells from a marrow aspiration biopsy that are multiplied in tissue culture flasks and seeded on and in a three-dimensional scaffold [12,13]. For large-scale production, however, this process has some serious drawbacks. The flasks are limited in their productivity by the number of cells that can be supported by a given area, while repeated handling for culture maintenance makes the process labor-intensive and susceptible to human error or initiative. Moreover, the microenvironment of the cells is not readily monitored and controlled which might result in sub-optimal culture conditions [14]. Another challenge complicating the clinical application is the available amount of a tissue-engineered product. Clinically relevant amounts of hybrid construct (defined as a combination of a biomaterial and bone marrow stem cells) for spinal surgery vary depending the approach from 4–6 cm^3 for an anterior inter body fusion (AIF) to 15 cm^3 or more when applying a posterolateral fusion (PLF) [15]. Production of these amounts of hybrid construct is complicated because of potential mass transfer limitations with respect to the supply of oxygen and medium components. It is well known that mass transfer limitations involved during in vitro culturing of 3D constructs result in limited amount of cell growth into the 3D construct [16,17]. For example, deposition of mineralized matrix by stromal osteoblasts cultured into PLGA constructs reached a maximum penetration depth of 240 μm from the top surface [18]. Rat marrow stromal cells seeded on PLGA scaffolds and cultured in spinner flasks demonstrated enhanced proliferation and expression of osteoblastic markers compared to statically cultured constructs [19]. However, only external mass-transfer limitations can be reduced in spinner flasks or stirred tank bioreactors. Bioreactors that perfuse medium through scaffolds allow the reduction of internal mass-transfer limitations and the exertion of mechanical forces by fluid flow [20]. Cultivation of osteoblast like cells [21] and rat bone marrow stem cells on 3D constructs in perfusion bioreactors have shown to enhance growth, differentiation and mineralized matrix production in vitro [22–24]. Only few studies show in vivo bone formation of hybrid constructs cultivated in perfusion bioreactors in rodents [25]. In an effort to produce clinically relevant volumes of tissue-engineered bone products, we report a direct perfusion bioreactor system which can drastically reduce the amount of space and handling steps involved and increase the volumes of tissue-engineered product. Furthermore this system allows the on-line monitoring of oxygen consumption during seeding and cultivation of goat BMSCs (GBMSCs) on ceramic scaffolds up to 19 days. We chose a small sized macroporous biphasic calcium phosphate scaffold that could potentially fill every shape of defect and showed osteoinductive potential in goats [26] but not in nude mice [27].

2. Material and methods

2.1. Bioreactor and bioreactor system

A direct perfusion flow bioreactor was used as described in Fig. 1. The bioreactor comprised an inner and outer housing, which were configured as coaxially disposed, nested cylinders. The inner housing was designed as a rigid basket from polycarbonate in which the scaffolds were kept press-fit during cultivation. The basket had a perforated lid and a perforated bottom and was placed in the medium flow path for axial flow through the basket. The bioreactor system comprised a bioreactor, a sterile fluid pathway (made of γ-sterilized PVC tubing, which had low gas permeability) that includes a medium supply vessel, a pump, an oxygenator and a waste vessel.

The fluid pathway contained a temperature sensor and two dissolved oxygen sensors, which were placed at the medium inlet and outlet of the bioreactor. The whole bioreactor system was placed in a temperature-controlled box (incubation unit), which was kept at 37 °C. These incubation units lack a gas-controlled atmosphere and to supply the cells with oxygen and carbon dioxide an oxygenator was developed. The oxygenator comprised a closed chamber containing a gas-permeable silicon tube. The gas environment in the chamber was kept at a constant level of 21% O_2 and 5% CO_2 and medium was pumped through the gas-permeable tube at a flow rate of 4 ml/min. This system enables a medium flow through the bioreactor of a constant pH and a constant oxygen concentration.

2.2. Initial cell culturing of GBMSCs in tissue culture flasks

Goat BMSCs aspirates were isolated from the iliac crest and cultured as described in detail [13]. Culture medium comprised of α-MEM supplemented with 15% FBS, antibiotics, 0.1 mM L-ascorbic acid-2-phosphate, 2 mM L-glutamine and 1 ng/ml basic fibroblast growth factor (Instruchemie, The Netherlands). Cells were cultured at 37 °C in a humid atmosphere with 5% CO_2. At the end of the first passage (P1), the cells were cryopreserved. Within 12 months, the cryopreserved cells were thawed and replated in tissue culture flasks. When cells were near confluence, the cells were washed with phosphate buffered saline (PBS), enzymatically released by means of a 0.25% trypsin-EDTA solution and replated at a density of 5000 cells/cm^2. After one additional passage, cells were enzymatically released as described before, resuspended in culture medium and transported into a seeding vessel which was attached to the seeding loop of the bioreactor system described in Fig. 1.

2.3. Scaffolds

Biphasic calcium phosphate scaffolds (BCP, OsSatura™, IsoTis, The Netherlands) were made of 36% macroporous (pores > 100 μm) biphasic calcium phosphate. The total porosity of these scaffolds was 59% (average interconneced pore