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Formation of cartilage matrix proteins by BMP-transfected murine mesenchymal stem cells encapsulated in a novel class of alginates

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Abstract

Proliferation and differentiation of wild-type, BMP-2 and BMP-4 transfected cells of C3H10T1/2, a mouse mesenchymal stem cell line that can differentiate into chondrocytes, were studied under monolayer (2D-) and encapsulation (3D-) conditions. Cells were encapsulated in a novel class of alginate. The alginate was of clinical grade (CG) because of complete removal of mitogenic and cytotoxic contaminants by chemical means. Compared to commercial alginates used so far for encapsulation it was characterized by ultra-high viscosity (UHV; viscosity of a 0.1% w/v solution of about 20 cP). In contrast to monolayer cultures, proliferation of cells was prevented when the cells were encapsulated in UHV/CG alginate at the same suspension density. As revealed by immunohistochemistry and quantitative RT-PCR, transfected and wild-type monolayer cells showed synthesis of type I collagen after transfer into differentiation medium, while culture in an alginate scaffold resulted in an upregulation of type II collagen and other hyaline cartilage proteins. BMP-4 transfected cells produced considerably more type II collagen than BMP-2 transfected and wild-type cells. BMP-4 transfected cells were also characterized by type I collagen production up to Day 10 and exhibited transient alkaline phosphatase activity levels that were much higher than the peak values observed for the other two cell lines. The coincidence of the ALP peak values with downregulation of type I collagen in BMP-4 transfected cells suggested that C3H10T1/2 cells differentiate into chondrocytes via a chondroprogenitor-like cell. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Cartilage; Stem cells; Chondrocytes; Alginate; Real time RT-PCR; BMP

1. Introduction

Recently, many efforts have been undertaken to repair articular cartilage lesions by transplantation of autologous chondrocytes cultured and expanded under in vitro conditions. A major problem of human cells in culture is the phenomenon of dedifferentiation. Thus, after a few days in monolayer cultures, chondrocytes change their appearance to a fibroblast-like morphology and lose their biochemical and functional properties. Instead of hyaline cartilage-specific type II collagen and other hyaline-specific glycosaminoglycans, type I collagen is mainly synthesized [1]. Matrix molecules are released into the medium and do not aggregate to an appropriate extracellular matrix. However, redifferentiation and formation of an extracellular matrix occurs when the cells are encapsulated in a three-dimensional matrix. This is essential for the successful generation of tissue-engineered cartilage. Among the polymers tested so far, alginate has been and will continue to be one of the most important scaffold materials. Alginate cross-linked with Ca^{2+} or Ba^{2+} has been used successfully to encapsulate cells and to maintain their function in tissue culture [2–4]. Alginate is biodegradable. It is degraded by enzymatic pathways to its two monomeric subunits, mannuronic and guluronic acids [5–7]. Furthermore, the ability

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to mold Ca^{2+} alginate gels to produce defined shapes creates the opportunity to fabricate patient-designed cartilage transplants. These and other characteristics are advantageous for a polymer support matrix of cell and tissue transplantation. However, when commercial alginates (cross-linked with Ca^{2+} or with Ba^{2+}) were implanted into rodents or other animals, heavy foreign body reactions were observed within a few weeks (for review articles, see [2]). The reason for this is that commercial alginate contains at least 10–20 mitogenic and cytotoxic impurities [8–10]. Biocompatibility is not only an obligatory requirement for in vitro construction of transplantable vital tissue structures with cell carriers and for granting medical approval, but also for elimination of interferences of toxic contaminants with the biochemical and biophysical signals that regulate the development of the highly complex extracellular matrix [11].

Therefore, in this communication we used ultra-high viscosity alginate of clinical grade cross-linked with divalent cations as a scaffold matrix. This alginate was obtained by extraction and several purification steps from fresh algal material that removed the impurities without degradation of the polymeric guluronic/mannuronic acid chains [12]. When implanted in rodents or baboons, this product did not evoke any significant foreign body reaction [9]. Similarly, transplantation of encapsulated parathyroid allogeneic tissue segments in rodents showed a proper function (i.e., parathormon release associated with normocalcemia) over more than 1 yr (Bohrer et al., manuscript in preparation). Despite these successes, the question remains whether encapsulation of suspended cells (such as chondrocytes) survives and functions satisfactorily in a matrix made up of ultra-high viscosity alginate cross-linked with divalent cations (for a detailed discussion of the problems, see [13]). For the first approach, we employed this alginate to the encapsulation of C3H10T1/2, a mouse mesenchymal stem cell line. This permanent cell line, when cultured in a favorable environment, can undergo chondrogenesis [14,15]. According to Caplan [16], stem cells are superior for tissue engineering than chondrocytes for several reasons. Among other things, they have the decisive advantage over primary cells that high cell numbers can be produced in a reproducible manner [17]. In addition, C3H10T1/2 cell lines that stably transfected with human BMP-2 and BMP-4 vectors have also been established [14,18]. BMPs (bone morphogenetic proteins) are members of the transforming growth factor-β (TGF-β) superfamily and are involved in the development of cartilage and bone [19–22]. Thus, the study of encapsulated BMP-transfected and wild-type cells allows to elucidate the interplay between the alginate matrix and the differentiation processes of these cells in the absence of any mitogenic and cytotoxic impurities.

2. Materials and methods

2.1. Cell culture

Puromycin-resistent, BMP-2 and BMP-4 transfected and expressing C3H10T1/2 cells were obtained from G. Gross (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). C3H10T1/2 is a murine mesenchymal progenitor cell line permanently transfected with cDNAs encoding the human bone morphogenetic proteins BMP-2 and BMP-4 [14]. Transfected and non-transfected cells were routinely grown in 650 ml polysterene tissue culture flasks (Greiner, Nürtingen, Germany). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM, Sigma, Deisendorf, Germany) supplemented with 2 mM L-glutamine, 10% (v/v) fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), 50 μM 2-mercaptoethanol (Sigma, Deisendorf, Germany) and 100 U/ml/100 μg/ml penicillin/streptomycine (Biochrom, Berlin, Germany). The culture medium of the transfected cells contained additional 5 μg/ml puromycin (Sigma, Deisendorf, Germany). Cultures were incubated in a humidified incubator at 37°C and 5% CO_2. After 3 days, the cells were nearly confluent. Cells were harvested by treatment of the monolayer with 4 ml trypsin/EDTA (0.5 g/l trypsin, 0.2 g/l EDTA dissolved in phosphate buffered saline, PBS) for 2 min at 37°C. After centrifugation at 170 g for 10 min, the pellet was washed with PBS and resuspended in 10 ml PBS. The number of cells was determined electronically by using the Casy® cell analyzer (Schärfe, Reutlingen, Germany). The cells were centrifuged again and resuspended in culture medium at a suspension density of 1×10^6 cells/ml. For proliferation studies cells were placed in standard 24-well polysterene tissue culture dishes. For immunohistochemistry the cells were seeded on CELLocates® (Eppendorf, Hamburg, Germany). After 3 days culture at 37°C and 5% CO_2 the medium was replaced by differentiation medium. This medium consisted of a culture medium to which 10 mM β-glycerophosphate (Sigma, Deisendorf, Germany) and 50 μg/ml ascorbic acid (Sigma, Deisendorf, Germany) were added. Every 3 or 4 days, half of the differentiation medium was replaced by fresh medium. Experiments were performed over a period of 17 days. Every 2–4 days cell proliferation and differentiation stage were examined.

2.2. Alginate encapsulation

Alginate was extracted from the inner stipes of the kelp *Laminaria pallida* and purified according to the protocol of Hillgärtner [12] (see also [23]). The ultra-high viscosity alginate was of clinical grade (termed UHV/CG alginate; [9,10]). Before use the alginate was sterilized and then dissolved in sterile, endotoxin-free

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