Development of porous collagen beads for chondrocyte culture

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Abstract A method for the preparation of bioresorbable collagen beads with an open porous structure is presented. These beads were prepared from collagen-alginate composite beads by removal of the alginate component. These collagen beads were suitable for rapid proliferation of chondrocytes in a dynamic, spinner culture system. Histology and immuno-histology showed that biochemical markers of chondrocytes are present during this cell proliferation, indicating that there was control of phenotype and that cell de-differentiation had not occurred. Unlike other 3-D scaffolds that have been used, the cells were amplified from very low cell densities and were

able to proliferate freely without loss of phenotype.

Keywords Collagen beads · Alginate · Cell culture · Chondrocytes · Tissue engineering

Introduction

The cartilaginous tissues in the knee exhibit a very limited capacity to repair, and then only after surgical intervention. For articular cartilage, any repair, for example after microfracture or mosaicplasty, is typically with mechanically inferior fibrocartilage and is unlikely to provide lasting repair (Hunziker 2001; Gilbert 1998). For meniscal cartilages, any repair is usually restricted to the vascular zone at the peripheral edge after fixation by sutures on equivalent devices (King 1936).

Tissue engineering and cell therapy offer emerging approaches towards effective repair of damaged articular tissues in the knee. For articular cartilage, autologous chondrocyte implantation has shown promise as a repair technology (Brittberg et al. 1994; Henderson 2005), while analogous approaches may prove possible for assisting in meniscal repair or replacement (Izuta et al. 2005). A key aspect of any tissue engineering or cell therapy approach is the availability of a sufficient quantity of the patient's cells after

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expanding the numbers from a small biopsy sample. Cell culture in monolayers provides a convenient and an effective method for expanding cell numbers, but during cell culture the cell phenotype is generally lost, and the biochemical characteristics of the extracellular matrix that is produced are changed (von der Mark et al. 1977). Thus, for example, articular cartilage chondrocytes lose their rounded phenotype and assume a fibroblastic like appearance while producing type I collagen rather than the type II collagen that is characteristic of this hyaline tissue. In previous studies, it has been shown that good chondrocyte proliferation can be achieved in spinner culture while the phenotype is also substantially retained, possibly due to mechanical forces present in the tissue culture environment (Fronzoza et al. 1996; Bouchet et al. 2000; Thissen et al. 2005).

In the present study we present a method for preparation of porous collagen beads for use in spinner culture for the expansion of chondrocytes, with examination of the cell growth and type of matrix generated on and within the porous collagen bead system. The use of collagen is attractive since this fibrous protein is known to serve as the natural scaffold for chondrocytes. However, most studies have examined only two-dimensional growth on collagen templates under static conditions (Kleinman et al. 1981) or more recently collagen coatings on non-degradable synthetic microcarriers (Grohn et al. 1997; Malda et al. 2003), which do not mimic the physiological conditions of cells in vivo.

Materials and methods

Bead production

Porous beads of collagen type I or collagen type II were prepared by mixing solutions of collagen (4.7–7.2 g l⁻¹ in 0.02M PBS) with alginate (0.3% to 1.4% w/v) (Hsu et al. 2000). Bovine hide type I collagen and bovine articular cartilage type II collagens were prepared using 1 g l⁻¹ pepsin digestion in 100 mM acetic acid adjusted to pH 2.5 with HCl as previously described (Miller and Rhodes 1982). Alginate, from *Macrocystis pyrifera*, was obtained from Merck. The mixture was

added drop-wise into 1.5% CaCl₂ (pH 7.2) to form spherical gel beads. At the same time, an airflow was applied to help break up the liquid flow from the syringe as droplets. Typically, a blunt ended # 19 needle was used, but it was observed that reducing the needle diameter could lead to smaller beads. The collagen-alginate beads were washed in 1.1% CaCl₂ (pH 7.2) and 0.1% CHES (2-(*N*-cyclohexylamino)ethane-sulfuric acid), and were stabilised either by immersing in a Na-CHES buffer (0.15 M NaCl, 1.1% w/v CHES, pH 7.2), containing 0.05% w/v L-lysine for 10–15 s as described previously (Hsu et al. 2000), or in Na-CHES buffer containing 0.05% or 0.1% glutaraldehyde for 16 h. The alginate within the stabilised gel beads was liquefied with a 50 mM sodium citrate solution, pH 7.2, washed for 16 h with Na-CHES buffer and re-suspended in PBS buffer. The resulting beads were incubated at 37°C for 2 h. Beads with diameters from around 100–1,500 µm were obtained depending on the selection of the rates of drop-wise addition and the rate of airflow. The collagen structure and distribution in the beads was examined by TEM using a JEOL 2000FX microscope after fixation in 4% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 20 mM CaCl₂, followed by post fixation in 1% OsO₄, progressive dehydration in a series of graded ethanol solutions and cured in Epon 812.

Cell isolation and growth

Fresh chondrocytic cells were isolated from 3 separate animals using *circa* 1 mm³ pieces of fresh ovine meniscal or articular cartilage by digestion (2 ml/g tissue) using 10% trypsin (MP Biomedicals), for 1 h at 37°C, followed by bacterial collagenase (354 U/ml *Clostridium histolyticum*, type IAS, Sigma, St Louis MO) and hyaluronidase (760 U/ml bovine testicular type IV-S, Sigma, St Louis MO), for 16 h at 37°C. Released cells were collected through a 70 µm filter, and washed and counted prior to seeding. Cells were seeded at an initial density ranging from 5 × 10⁴ to 5 × 10⁵ cells/150 mg (wet weight) collagen beads in 50 ml DMEM/F12 10% FBS containing 100 mg l⁻¹ penicillin and streptomycin at 37°C with 5% CO₂ in

125 ml spinner bottles in triplicate. Cell seeding was performed with intermittent stirring for 2 min every 30 min, initially at 25 rpm for 3 h, then 45 rpm for 1.5 h. This was followed by continuous stirring at 45, 50, 55 to a final 60 rpm at 15 min intervals. Cultures were supplemented with daily additions of ascorbate (Sigma, St Louis, MO) at a final concentration of 50 mg l⁻¹ for 10 d prior to matrix analyses. Cell seeding efficiency was examined at 24 h by counting 3 separate aliquots using a haemocytometer. Cell viability was assessed using a Live/Dead (Green/Red) viability assay where calcein AM and ethidium homodimer-1 (EthD-1) were used to distinguish live (green) and dead (red) cells respectively (Molecular Probes, Eugene, OR). Cell phenotype and matrix production was monitored using a variety of histochemical and immunohistochemical markers, including standard Haematoxylin & Eosin, Alcian Blue at pH 2.5 and pH 5.8, and Masson Trichrome and specific antibodies against collagens types I, II, III and VI, and to keratan sulfate. Prior to staining with antibodies, cultured cells on beads were washed in warm PBS, and then pre-fixed in 50% v/v methanol for 10 min, 70% v/v methanol for 10 min (twice), 70% v/v ethanol for 10 min, and then rinsed in PBS. A range of antibodies was used to assess the type of matrix: goat anti-collagen type II (Southern Biotechnology, Birmingham, AL), goat anti-collagen type I (Southern Biotechnology, Birmingham, AL), mouse monoclonal anti-collagen type VI (1D8-F8/Col6) (Werkmeister et al. 1993) and mouse monoclonal anti-keratan sulphate (5D4, Seikagaku Corp., Tokyo, Japan) using the protocol described by Caterson et al. (1983). Beads were incubated with antibodies for 1 h, washed and then reacted with corresponding FITC-conjugated secondary antibodies, rabbit anti-goat IgG (Southern Biotechnology, Birmingham, AL) or sheep anti-mouse Ig (Silenus, Melbourne, Australia). As controls in all studies, primary antibodies were omitted and replicate slides were stained with only secondary FITC-labelled antibodies.

Results and discussion

Cell culture using microcarriers as three dimensional scaffolds are now accepted as a method of

choice for cell expansion of cell lines and primary cultures. Indeed a number of carrier substrates have been developed primarily based on synthetic surfaces like modified dextran (CytodexTM 1, CytodexTM 2), plastic, or synthetic polymers like modified PLGA (Thissen et al. 2005). In addition a number of these surfaces have been modified by coating with collagens (RapidCellTM C beads) or gelatine (CytodexTM 3) to enhance cell attachment (Overstreet et al. 2003). Gelatin microbeads can also be readily prepared (Cultispher[®]) with defined particle diameters (Iwamoto et al. 2002), and with varying degradation rates (Glattauer et al. 2004). Collagen microbeads are an alternate microcarrier system that provides a natural three dimensional environment to proliferate primary cells. Collagen beads can be prepared from crude collagen extracts of bovine corium using liquid N₂, followed by dehydration and cross-linking (Dean et al. 1989) or can be further purified with pepsin (CellagenTM beads). Other forms of microbead scaffolds include alginate polymer encapsulation (Majmudar et al. 1991) or alginate beads that have been cross-linked with gelatin (Kwon and Peng 2002) or coated with collagen (Grohn et al. 1997).

The collagen beads in the current study are formed by making an intermediate bead that incorporates an alginate phase that was subsequently removed. This system allows for preparation of porous collagen beads using purified type I and type II collagens. The porosity is sufficient to allow cell infiltration and tissue ingrowth. In addition, these porous beads can be cross-linked to various degrees to allow control of collagen persistence and regulation of resorption if the beads are used directly in implants. The size of the beads was dependent on the concentration of collagen and alginate, the rate of flow of the mixture into CaCl₂ and the rate of airflow. Bead sizes could be readily controlled from 100 µm to 1500 µm (Fig. 1). Coating with L-lysine avoided microsphere disintegration (Hsu et al. 2000), while the mechanical strength and the durability of the collagen beads could be further augmented by cross-linking with glutaraldehyde (GA), as has been shown for a range of biomedical devices (Ramshaw et al. 1995). In the present study either 0.05% or 0.1% GA was used. In other studies using gelatine beads, GA crosslinking using con-

Fig. 1 Light micrograph of porous collagen beads obtained after alginate removal. **A:** Type I collagen bead approximately 1.5 mm in diameter, made using 4.7 g l^{-1} alginate and 1.4% type I collagen solutions. **B:** Type I collagen bead approximately 0.3 mm in diameter, made

using 7.2 g l^{-1} alginate and 0.3% type I collagen solution. **C:** Type II collagen bead approximately 0.7 mm in diameter, made using 6.6 g l^{-1} alginate and 0.3% type II collagen solution. Graticule images are included to identify bead diameters

centrations from 0.005% to 0.10% was found to extend the stability of beads from days up to >4 months (Glattauer et al. 2004).

Examination of beads by immuno-histology (Fig. 2A) showed the porous nature of the beads that arises from the alginate removal. Examination of the collagen using transmission electron microscopy (Fig. 2B) showed that the soluble, individual collagen molecules used in the solution for bead preparation had formed into ordered banded fibrils, analogous to those present in native tissues (Chapman and Hulmes 1984). The conversion of soluble collagen into fibrils is probably assisted during bead preparation by the incubation of the beads at 37°C after prior removal of alginate. TEM shows that a small amount of residual alginate may be present as the fine, dark staining aggregates (Fig. 2B).

Articular and meniscal cartilage have a limited capacity to self repair mainly due to the lack of vascularity and the low metabolic and proliferative capacity of the localised chondrocytes that are scarce and confined within a well defined matrix (Hunziker 2001; King 1936; Buckwalter and Mankin 1997). Cell-based repair or autologous chondrocyte implantation (ACI) is becoming an acceptable surgical procedure to repair articular cartilage damage (Brittberg et al. 1994; Henderson et al. 2005). Nonetheless, there are still issues regarding the degree of dedifferentiation of the cells that could compromise the reproducibility and regeneration of a true hyaline cartilage (Hunziker 2001; Brittberg 1999; Temenoff and Mikos 2000). Meniscal chondrocyte implantation is not a current surgical procedure and is restricted by the source of cells and low

Fig. 2 Porous collagen beads obtained after alginate removal. **A:** Laser confocal micrograph using anti-collagen type I antibody to demonstrate the distribution and abundance of collagen type I (white) and the internal

channels (black) within the bead preparation. **B:** Transmission electron micrograph of beads prepared using type I collagen showing the formation of banded collagen fibrils. Bars: 2A = $200 \mu\text{m}$, 2B = $5 \mu\text{m}$

yield of cells (Izuta et al. 2005). The preparation and expansion of autologous chondrocytes is therefore challenging in these surgical procedures. Isolation of cells from meniscal tissue typically produces very low numbers of cells (Nakata et al. 2001). In the present study, from 3 menisci, best cell yields ranged from 3.9 to 5.3×10^5 cells/g tissue, which was only around 10% of the yields we have obtained from articular tissue (Thissen et al. 2005); in some experiments, the meniscal cell yields were up to 10-fold lower. Meniscal cell expansion used cells derived from 3 separate isolations and in each case was performed in triplicate, and at two concentrations. These were, a relatively low density (5×10^4 cells/0.15 g beads/spinner flask) and a higher cell density (5×10^5 cells/0.15 g beads/spinner flask) using unfixed porous collagen beads prepared as above. Cell seeding efficiency was typically high, with at least 85–95% cell attachment over the initial 24 h period. For all cell preparations it was observed that the cells proliferated at a fast rate once in log phase. For all three isolates, the replicate experiments with a cell seeding of 5×10^5 cells, proliferation rates around 20-fold were achieved over the 12 days in culture. With the lower cell seeding, there was more variation ranging from 5- to 12-fold proliferation rates. This compares favourably with conventional monolayer cell cultures where 4-fold proliferation rates were observed over 12 days.

Cells rapidly infiltrated the porous bead as shown with Haematoxylin and Eosin staining (Fig. 3A) or with Masson trichrome staining (Fig. 3B). Alcian Blue staining, at both pH 2.5 and pH 5.8 (Fig. 3C), showed that the new matrix within the collagen beads contained high levels of glycosaminoglycans (GAG) associated with newly synthesized proteoglycans. The predominant proteoglycan present in cartilaginous tissues is aggrecan. Beyond the N-terminal hyaluronic acid binding domain of the proteoglycan core protein, the aggrecan core protein contains a region containing predominantly keratan sulphate side chains, while further to the C-terminal chondroitin sulfate chains are predominant (Caterson et al. 1983). The keratan sulphate composition of aggrecan is typically higher in newly forming cartilage (Sorrell and Caterson 1989). In the present study, immuno-histology using an anti-keratan sulphate antibody confirmed the distribution of these GAGs as strong but patchy within areas where there were high density of cell clusters (Fig. 4A). Keratan sulphate staining was not observed in monolayer cultures of the same cells after 12 days in culture (data not shown).

New collagen deposition can be readily determined using type specific monoclonal antibodies for collagens that are not constituents of the original bead. For example, with collagen type I beads, collagen type II staining could be examined. Thus, collagen type II staining was strong

Fig. 3 Light micrographs of non-crosslinked type I collagen beads after 12 d in culture with meniscal chondrocytes. **A:** Haematoxylin and Eosin staining showing cell infiltration and proliferation within the porous beads. **B:** Masson Trichrome staining showing moderate to strong new

connective tissue formation within bead channels and between cell clusters. **C:** Alcian Blue at pH 5.8 staining showing strong patchy localization of GAG in areas of new matrix between cell clusters. Bars = 100 μ m

Fig. 4 Immuno-histology of non-crosslinked type I collagen beads after 12 d in culture with meniscal chondrocytes. **A:** Staining using anti-keratan sulphate antibody showing strong patchy localization. **B:** Staining using anti-type II

collagen antibody showing even distribution within the new matrix. **C:** Staining using anti-type VI collagen antibody showing localization at the leading edge of the growing tissue. Bars = 100 μ m

within the new matrix and was evenly distributed around the proliferating cells (Fig. 4B). Like articular cartilage, collagen type II is characteristic of meniscal cartilaginous tissues. Unlike articular cartilage, the meniscus also contains other types of collagens, including collagen type I. In the tissue formed with cultured meniscal cells, collagen type I was also present as expected, although it was difficult to negate the background staining due to the nature of the endogenous type I collagenous beads. When type II collagen beads were used (Fig. 1C), collagen type I was detected in the newly formed matrix at comparable levels to collagen type II that was detected on collagen type I beads (Fig. 4B). No differences were observed between the type of matrix produced in cells cultured on collagen type I or II beads. Immuno-histology also showed patchy minute amounts of collagen type III (data not shown). Type VI collagen was observed at a low level throughout the bead, although the strongest staining was localized to the outer regions of the bead where new tissue and mitotic activity was evident (Fig. 4C). This collagen is characteristic of newly forming tissues (Werkmeister et al. 1993) and is the main collagen component of the chondron structure that surrounds chondrocytes and has been postulated to be important in cell anchoring and matrix cell signaling (Poole et al. 1992). No quantitation of matrix proteins was performed in the present study. In previous

studies using cultured articular chondrocytes on non-resorbable (Cytodex) beads (data not shown), we have shown that RT-PCR results correlated well with observed histology and immuno-histology profiles. These collagen types, along with the proteoglycan production are consistent with the native cellular environment of the meniscus, and suggest that meniscal chondrocytes can be appropriately expanded in culture to allow autologous transplantation similar to the current articular cell transplantation protocols (Brittberg et al. 1994; Henderson et al. 2005; Thissen et al. 2005).

These studies have shown that using these newly developed porous collagen beads in a dynamic culture environment allows for the ready expansion of cartilage cells from small biopsy samples to give large numbers of cells with controlled phenotype, suitable for use in tissue engineering or cell therapy strategies of repair of damaged meniscal cartilage. The cells can be readily released from these beads by enzymatic digestion, for example by collagenase. Alternatively, the choice of collagen for the beads means that they are both biocompatible and biodegradable, so cell isolation is not necessarily needed and the cell-collagen composite can be introduced into the defect being repaired, either directly or in conjunction with a delivery vehicle such as collagen gel (Werkmeister et al. 2002; Chiang et al. 2005).

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