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Cellular Photoencapsulation in Hydrogels

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Culture of Cells for Tissue Engineering, edited by Gordana Vunjak-Novakovic and R. Ian Freshney

1. BACKGROUND

The goal of tissue engineering is to regenerate tissue and organ structures to replace those lost from trauma, congenital abnormalities, or disease [Mooney and Mikos, 1999]. One strategy for tissue engineering encompasses seeding cells by photoencapsulation within a hydrogel scaffold. The scaffold is designed to promote desired cell function and tissue development while physically protecting the nascent

tissue after its implantation in vivo. Hydrogels are a class of biomaterial scaffolds that have shown potential for numerous tissue engineering applications.

1.1. Hydrogels

Hydrogels are formed by cross-linking water-soluble polymer chains to form a water-insoluble polymer network [Brannon-Peppas, 1994]. Cells may be encapsulated during the cross-linking process to create cell-hydrogel constructs for drug delivery and tissue engineering applications. Hydrogels have unique properties that make them potentially useful for tissue engineering, such as high water content for nutrient and waste transport, elasticity, and the ability to encapsulate or immobilize cells in a three-dimensional environment in situ. The properties of a hydrogel can be altered by manipulating polymer chemistry and cross-linking density. The distance between cross-links, or cross-linking density, directly influences the pore size of a hydrogel and related physical properties such as water content and mechanical strength. A scaffold with a high cross-linking density and a smaller pore size will imbibe less water and exhibit stronger mechanical properties compared to a hydrogel with a lower cross-linking density and a larger pore size.

Cross-linking density and pore size also influence cell behavior and tissue development. Researchers have shown that chondrocytes have increased extracellular matrix production in hydrogels with larger pore sizes. Therefore a balance must be found in the hydrogel formulation for optimal scaffold physical properties for a desired application with optimal cell function and tissue development. Hydrogels may be designed to remain stable or degrade over time, and their degradation properties can be controlled to match the rate of tissue development or matrix formation. Polymers that form the hydrogel can be chemically altered to incorporate growth factors or cell binding sites in order to promote cell proliferation, extracellular matrix production, or cell differentiation. Aside from cell encapsulation and tissue engineering technologies, numerous applications for hydrogels exist, including biosensors, dentistry, surgery, and drug-delivery systems. This chapter will focus on methods for isolating cells and encapsulating them in photopolymerizing hydrogels, with an emphasis on cartilage tissue engineering.

1.2. Methods for Forming a Hydrogel

There are numerous chemical options and methods for forming hydrogels under mild conditions that are compatible with cell encapsulation and tissue engineering applications. Both synthetic and naturally derived hydrogels have been applied to cell encapsulation. Naturally derived polymers that can form hydrogels include collagen, fibrin, agarose, hyaluronic acid, chondroitin sulfate, and chitosan [Buschmann et al., 1992; Ye et al., 2000; Silverman et al., 1999; Madihally and Matthew, 1999]. These natural hydrogels often have interesting biological properties that help promote tissue development. The physical properties and cross-linking of naturally derived hydrogels are often more difficult to control, compared to synthetic materials, leading in some cases to mechanically weak scaffolds. Mechanical properties of hydrogels pose a challenge for musculoskeletal tissue

engineering because of their inability to withstand physiological loading. Synthetic polymers can form hydrogels with highly controlled cross-linked structures. For example, polyethylene glycol (PEG), polyvinyl alcohol, and polypropylene fumarate are synthetic polymers that have been modified to create cross-linked gels with controlled porosity [Drumheller and Hubbell, 1994; Behravesh et al., 2002; He et al., 2000].

Numerous methods may be used to form cross-links between polymer chains and create a hydrogel by covalent, ionic, or physical cross-links (van der Waals forces, hydrogen bonds). The polymerization or cross-linking process can be triggered by radiation, temperature changes, addition of a chemical cross-linker, or ionic agents. For many of these methods, once cross-linking is induced, the process cannot be stopped or accelerated. This lack of control of the cross-linking process and the subsequent difficulties in clinical application led to the development of photopolymerization to encapsulate cells in hydrogels for cartilage tissue engineering.

2. PRINCIPLES OF METHODOLOGY: PHOTOPOLYMERIZATION FOR CELL ENCAPSULATION

Photopolymerization is a method to covalently cross-link polymer chains to form a hydrogel. A photopolymerization occurs when a photoinitiator and polymer (with groups sensitive to the initiating species) are exposed to a light source specific to the photoinitiator species. The reaction is rapid, allowing for fast curing of a liquid to a cross-linked, water-swollen polymer network at room or body temperature. This technique allows for enhanced control over the gelation process compared to gel formation by physical or ionic interactions. Photoinduced gelation provides spatial and temporal control during scaffold formation, even permitting shape manipulation after injection and during gelation in vivo. Photopolymerizing hydrogels have been used in a wide variety of biomedical applications and have the potential to create a significant impact in tissue engineering [Burdick et al., 2002]. Photopolymerizations are utilized in the field of dentistry for applications ranging from sealants for caries prevention to root canal procedures and in the fields of drug delivery and tissue engineering [Tarle et al., 1998; Anseth et al., 1994].

Photopolymerizing polymer networks with a range of physical and mechanical properties have been developed, and their function as tissue engineering scaffolds has been studied. For example, Anseth and colleagues have examined photopolymerizing polyanhydrides for bone tissue engineering and polyvinyl alcohols and polyethylene oxide (PEO) for cartilage tissue engineering [Young et al., 2000; Poshusta and Anseth, 2001; Bryant et al., 1999; Burkoth and Anseth, 2000]. Hubbell and colleagues developed novel degradable photopolymerizing hydrogels based on PEO [Sawhney et al., 1996]. These polymers have been applied to drug delivery and cell encapsulation and have been studied as lung sealants and for the prevention of postoperative adhesions [Sawhney et al., 1996; Lyman et al., 1996; Hill-West et al., 1994]. Our research has demonstrated the feasibility of cell photoencapsulation for cartilage tissue engineering, and the key methods are presented in this chapter [Ye et al., 2000; Lyman et al., 1996; Elisseeff et al., 1999].

3. PREPARATION OF MEDIA AND REAGENTS

3.1. Transport Medium

Either cold phosphate-buffered saline (PBSA) supplemented with antibiotics or DMEM (Dulbecco's modified Eagle's medium with high glucose) with antibiotics.

3.2. Collagenase

DMEM containing 0.2% collagenase and 5% fetal bovine serum.

3.3. DMEM-FB-PS

DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin. and 100 μg/ml streptomycin.

3.4. Papain

Papain 125 μg/ml in 0.1 M phosphate buffer, 10 mM cysteine, 10 mM EDTA, pH 6.3.

4. TISSUE HARVEST AND CELL ISOLATION

When designing a tissue engineering system, the source of the cells that are seeded on the biomaterial scaffold will significantly impact on the quality of engineered tissue and must therefore be chosen carefully. As we are studying tissue engineering in the musculoskeletal system, chondrocytes (the cells that comprise cartilage) and bone marrow-derived mesenchymal stem cells (capable of differentiating into cartilage) are two potential cell options for incorporation in the scaffold. Isolation of chondrocytes is essentially a two-step procedure: harvesting the tissue and isolating the cells from the tissue. Previously, most authors performed sequential digestion of cartilage with selected enzymes, including collagenase, hyaluronidase, and trypsin [Sah et al., 1991]. More recently, collagenase alone has been found to be adequate for cartilage digestion.

4.1. Chondrocytes

In current autologous chondrocyte transplantations, isolated chondrocytes are expanded by serial passage in monolayer culture because of the limited quantity of cartilage tissue that can be harvested from a patient. However, the expansion in monolayer culture causes the dedifferentiation or loss of chondrocyte-specific gene expression. Chondrocytes propagated in monolayers are marked by a decrease in their ability to produce cartilage-specific proteins such as collagen type II and aggrecan. Loss of the chondrocytic phenotype during monolayer expansion may be linked to the variable results of chondrocyte transplantation for cartilage injury.

Protocol 9.1. Chondrocyte Isolation from Bovine Knee Joint

Reagents and Materials

Sterile

- ❑ Transport medium, refrigerated (See Section 3.1)

- ❑ Dissection instruments
- ❑ Collagenase, 0.2% (See Section 3.2)
- ❑ Plastic tube, 50 ml
- ❑ Nylon filter, 70-μm mesh

Nonsterile

- ❑ Hemocytometer
- ❑ Trypan Blue

Protocol

(a) Harvest cartilage from the knee joint of 5- to 8-week-old bovine calves under aseptic conditions.
(b) Transfer to the tissue culture laboratory in transport medium.
(c) Dissect the cartilage free from connective tissue or bone under sterile conditions.
(d) Mince into small pieces (1–3 mm^3) with a scalpel.
(e) Rinse the tissue several times with cold PBSA.
(f) Transfer to a preweighed digestion vessel and weigh the tissue. A 50-ml tube works well for tissue samples less than 300 mg.
(g) Incubate the tissue in 0.2% collagenase for 14–16 h at 37 °C under 5% CO_2 on an orbital shaker rotating at approximately 75 rpm.
(h) Resuspend, using a pipette. Filter the resulting cell suspensions through a 70-μm nylon filter.
(i) Wash the cells three times with PBSA to remove collagenase, any matrix debris, or undigested particles.
(j) Count the cells with a hemocytometer, assessing cell viability by Trypan Blue dye exclusion.

4.2. Mesenchymal Stem Cells

Bone marrow-derived mesenchymal stem cells (MSCs) are another cell option for musculoskeletal tissue engineering. They are isolated based on their ability to adhere to the culture dish while nonadherent hematopoietic cells are removed by media change. MSCs are capable of differentiating into multiple cell types to form cartilage, bone, muscle, and fat. Methods to isolate these cells are presented in Protocol 9.2.

Protocol 9.2. Bone Marrow-Derived Stem Cell Isolation and Expansion

Reagents and Materials

Sterile or Aseptically Prepared

- ❑ Femurs or iliac crest of three- to three-and-a-half-year old castrated male goats
- ❑ DMEM-FB-PS (See Section 3.3)
- ❑ MSCGM: mesenchymal stem cell growth medium (See Sources of Materials)
- ❑ FBS: fetal bovine serum

- ❑ Trypsin-EDTA: 0.025% trypsin in 0.01% EDTA
- ❑ DMSO
- ❑ Syringe, 10 ml, with heparin, 6000 U
- ❑ Syringe needles, 16 and 21 gauge
- ❑ Tissue culture flasks, 75 cm^2, 175 cm^2

Protocol

(a) Aspirate bone marrow from the femurs or iliac crests of three- to three-and-a-half-year-old castrated male goats into 10-ml syringes with 6000 U heparin.
(b) Make single-cell suspensions by passing the marrow through 16- and 21-gauge needles three times.
(c) Resuspend the cells in DMEM-FB-PS.
(d) Wash the marrow samples twice in mesenchymal stem cell growth medium before suspension in fresh MSCGM.
(e) Count the number of mononuclear cells with a hemocytometer.
(f) Plate in 75-cm^2 tissue culture plastic flasks at a density of approximately 1.2×10^5 mononuclear cells/cm^2.
(g) Change the culture medium after 4 days and then every 2–3 days thereafter until confluence (12–14 days).
(h) When cells are near confluent, passage the cells with trypsin-EDTA for 5 min at 37 °C and replate in 75-cm^2 or 175-cm^2 flasks at 5000 MSCs/cm^2.
(i) Freeze MSCs in liquid nitrogen at 5×10^6–1×10^7 cells/ml in 50% MSCGM, 40% FBS until needed.

5. CELL PHOTOENCAPSULATION

Now that the cells are isolated and, if necessary, expanded, they can be encapsulated in the hydrogel scaffold. As discussed above, photopolymerization is a fast and efficient method for encapsulating cells, but care must be taken to ensure cell survival in the hydrogel (Fig. 9.1). In particular, a cytocompatible photoinitiator, which produces the radical that is responsible for polymerization and hydrogel formation, must be chosen. A concentration of photoinitiator may be determined that is not toxic for the cells yet allows the polymerization reaction to proceed efficiently. For each new cell type that we photoencapsulate, the toxicity of the photoinitiator is evaluated and if cytotoxicity is observed, the concentration is modified or a new initiator is chosen. There are numerous methods to monitor cell viability including Trypan Blue dye exclusion, fluorescent live-dead cell assay (e.g., with diacetylfluorescein and propidium iodide), MTT [Plumb et al., 1989], and WST-1 [Ukeda et al., 2002; Huhtala et al., 2003]. MTT and WST-1 assays are based on the use of a chemical compound that is converted by mitochondrial enzymes to a dye that can be monitored by a spectrophotometer. As augmentation of enzymatic activity leads to an increase in dye production, there is a correlation with the dye absorbance and the number of metabolically active cells. An example of a protocol for WST-1 analysis to determine photoinitiator toxicity is provided in Protocol 9.3.

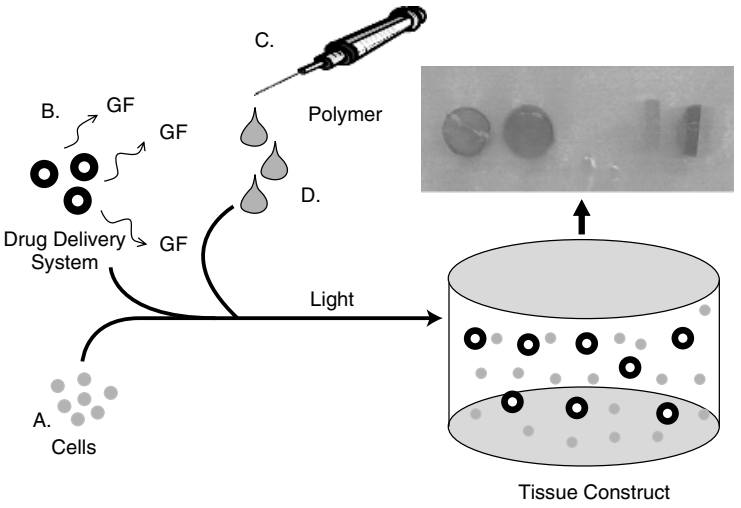

Figure 9.1. Schematic diagram of the photoencapsulation process. Cells are isolated (A) and combined with a drug delivery vehicle (B), if desired, and polymer (C). The cell-polymer liquid is placed in a mold or injected into an animal. The mixture is then exposed to light (D) to cause a photopolymerization and form a cell-laden hydrogel as pictured.

Protocol 9.3. WST-1 Analysis of Photoinitiator Toxicity

Reagents and Materials

Sterile

- ❑ Cells under study plus materials for trypsinization (See Protocol 9.2)
- ❑ Growth medium, e.g., Section 3.3
- ❑ PBSA
- ❑ Initiator stock solution (i.e., 100 mg/ml Irgacure D2959)
- ❑ WST-1 solution
- ❑ Tissue culture plate, 24 well

Nonsterile

- ❑ Light source
- ❑ Multiwell spectrophotometer (ELISA plate reader)

Protocol

(a) Prepare desired cell type to be studied at a concentration of 5×10^5 cells/ml medium.
(b) Add 400 μl medium to each well of a 24-well tissue culture plate.
(c) Add 100 μl cell suspension to each well.
(d) Add 5 μl initiator stock solution.
(e) Incubate for 30 min.
(f) Expose plates to light for photoinitiator activation (i.e., for Irgacure D2959 365 nm 4 mW/cm^2 for 0, 3, 6, 10 min).

(g) Incubate for 48 h.
(h) Aspirate medium from each well and rinse with 1–2 ml phosphate-buffered saline (PBSA)
(i) Add 500 μl medium to each well (including three extra cell-free wells for controls).
(j) Add 50 μl WST-1 solution to each well, incubate at 37 °C, 5% CO_2 for 4 h, and then measure absorbance at 440 nm on a multiwell spectrophotometer (ELISA reader).

Protocol 9.4. Polymer-Chondrocyte Preparation with Photoinitiated Hydrogels

Reagents and Materials

Sterile

- ❑ PEGDA: polyethylene glycol diacrylate
- ❑ PBSA with antibiotics (See Section 3.1)
- ❑ Photoinitiator: Irgacure D2959
- ❑ Cylindrical molds

Nonsterile

- ❑ UV light, 365 nm

Protocol

(a) Prepare the hydrogel solution by mixing 10% w/v PEGDA in sterile PBSA with antibiotics.
(b) Add the photoinitiator to the PEGDA solution and mix thoroughly to make a final concentration of 0.05% w/v.
(c) Immediately before photoencapsulation, resuspend chondrocytes in the solution to make a concentration of 2.0×10^7 cells/ml and gently mix to make a homogeneous suspension.
(d) Transfer 100 μl cell-polymer-photoinitiator suspension into cylindrical molds with a 6-mm internal diameter and expose the suspension for 5 min to long-wave, 365-nm UV light at 4 mW/cm^2.
(e) Remove the hydrogels from the molds and incubate them in separate wells of 12-well plates with the appropriate medium.
(f) Change the culture medium twice a week.

6. ENGINEERED TISSUE ANALYSIS

After cellular photoencapsulation and in vitro or in vivo incubation, the quality of tissue that develops in the material must be evaluated. In the case of cartilage, two major matrix components, type II collagen and proteoglycan, should

be analyzed. In addition, the cellular content should be monitored to determine whether cell death or proliferation occurred and the gene expression (mRNA levels) of cartilage-specific markers may be evaluated. Protocols 9.5 through 9.7 describe procedures to evaluate cartilage tissue production in photopolymerizing hydrogels.

Protocol 9.5. Biochemical Analysis of Cartilage Hydrogels

Reagents and Materials

Nonsterile

- ❑ Papain, 125 μg/ml (See Section 3.4)
- ❑ Hoechst 33258
- ❑ Dimethylmethylene Blue dye
- ❑ *p*-Dimethylaminobenzaldehyde
- ❑ Chloramine-T
- ❑ Balance, mg range
- ❑ Tissue grinder

Protocol

(a) Remove construct from culture medium, lightly blot dry, and obtain the wet weight. Lyophilize for 48 h under vacuum and obtain dry weight.

(b) Crush the dried construct with a tissue grinder.

(c) Digest each specimen in 1 ml papain solution for 18 h at 60 °C. Digested construct may be stored in the freezer for future analysis.

(d) Determine the DNA content (ng of DNA/mg dry weight) with Hoechst 33258 [Kim et al., 1988].

(e) Estimate glycosaminoglycan (GAG) content by measuring the amount of chondroitin sulfate using dimethylmethylene blue dye [Farndale et al., 1986].

(f) Determine total collagen content by measuring the hydroxyproline content of the specimens after acid hydrolysis and reaction with *p*-dimethylaminobenzaldehyde and chloramine-T, using 0.1 as the ratio of hydroxyproline to collagen [Woessner, 1961].

Protocol 9.6. Gene Expression Analysis by RT-PCR of Cartilage Hydrogels

Reagents and Materials

Nonsterile

- ❑ TRIzol reagent
- ❑ Chloroform
- ❑ RNeasy mini kit
- ❑ Superscript amplification system
- ❑ Gel electrophoresis system with 2% agarose gel in TAE buffer

- ❑ Microcentrifuge tubes
- ❑ Tissue grinder
- ❑ Vortex mixer

Protocol

(a) Harvest constructs or explanted tissues and immediately grind with a tissue grinder that is RNase free in 1.5-ml microcentrifuge tube containing 200 μl TRIzol reagent.
(b) Add 800 μl more TRIzol reagent to the microcentrifuge tubes and leave for 10 min at room temperature.
(c) Add 200 μl chloroform, vortex, and incubate the tube for 5 min.
(d) Centrifuge the tube for 15 min at 4 °C at 12,000 g.
(e) After centrifugation, transfer upper transparent aqueous layer to a collecting tube of RNeasy mini kit (Qiagen) and add equal volume of 70% ethanol.
(f) Perform the next steps of the manufacturer's protocol of the RNeasy mini kit.
(g) Make cDNA, using random hexamers with the Superscript amplification system per the manufacturer's instructions.
(h) Amplify 1-μl aliquots of the resulting cDNA in a total 50-μl volume at annealing temperatures optimized for cartilage-specific phenotypic markers (type II collagen, aggrecan, link protein, COMP, type IX collagen, etc).
(i) Analyze each PCR product by separating 4 μl amplicon and 1 μl loading buffer in a 2% agarose gel in TAE buffer. Compare the relative levels of band intensity of the gene of interest to those of the internal control of housekeeping gene.

Protocol 9.7. Histologic Analysis of Cartilage Hydrogels

Reagents and Materials

Nonsterile

- ❑ Paraformaldehyde, 4%
- ❑ Ethanol, 70%
- ❑ Reagents for embedding
- ❑ Safranin-O/Fast Green stain
- ❑ Histostain-SP kit

Protocol

(a) Observe the hydrogels by inverted light microscopy during incubation. In general, the cells may be observed and their distribution in the gel can be monitored. Also, opacity that develops in the gel, indicative of matrix formation, can be observed over time.
(b) After the required culture period, harvest the constructs and fix overnight in 4% paraformaldehyde at 4 °C.
(c) After the overnight incubation, transfer the samples to 70% ethanol until embedded in paraffin. Section to 5 μm.

(d) Stain with Safranin-O/Fast Green to assess the presence of proteoglycans.
(e) Immunostain with the antibodies of interest (type II collagen, type I collagen, aggrecan, link protein, etc) with the Histostain-SP kit, following the manufacturer's protocol.

7. PHOTOENCAPSULATION OF BOVINE CHONDROCYTES FOR CARTILAGE TISSUE ENGINEERING

Our previous research has focused on encapsulating chondrocytes in PEG-based hydrogels both in vitro and in vivo to engineer cartilage-like tissue [Elisseeff et al., 1999, 2000]. Recent studies have demonstrated the importance of cartilage-specific tissue architecture. In particular, the superficial, middle, and deep zones of cartilage each have unique genetic and biochemical characteristics that contribute to the structural and functional properties of the tissue. We studied the relevance of depth variation in tissue-engineered cartilage with a bovine chondrocyte model system. We were interested in recreating the zones of articular cartilage in a photopolymerizing hydrogel system capable of forming complex, multilayered structures for cartilage tissue engineering. Chondrocytes from varying depths (superficial, middle, and deep) differ in proliferation and expression of matrix markers on plating for amplification and matrix production after encapsulation in the hydrogel. The goal of this study was to prove that chondrocytes isolated from the three layers would differ in gene expression patterns and matrix formation after being encapsulated in a photopolymerizing hydrogel.

7.1. Design

Cartilage slices were removed from three (upper, middle, and lower) zones of articular cartilage of young bovine legs. Histology and biochemical composition of the cartilage slices were analyzed to confirm that they had been obtained from the proper zone. Gene expression of chondrocytes in monolayer culture and matrix formation in photopolymerizing hydrogels were evaluated. Cell viability and maintenance of cell viability from each respective layer were evaluated with the Live/Dead viability kit. After 3 weeks, the constructs were harvested for gene expression, biochemical, and histologic examination including immunohistochemistry for type II collagen.

7.2. Methods

Cartilage Layers

Articular cartilage was isolated from the patellofemoral groove and distal femoral chondyles of 5- to 8-week-old bovine legs (Research 87, Marlboro, MA). The top 10%, central 20%, and lower 10% of the excised tissue were removed to isolate the superficial (S), middle (M), and deep (D) layers (Fig. 9.2, See Color Plate 4A).

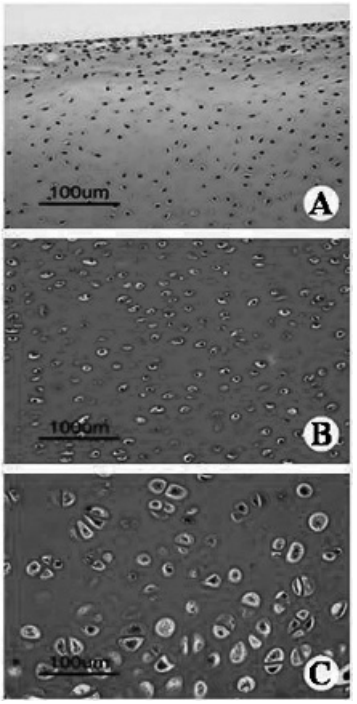

Figure 9.2. Histologic sections of the superficial, middle, and deep zones of juvenile bovine cartilage. The intensity of extracellular matrix staining and cell size increase with depth of zone: superficial (A), middle (B), and deep (C) zones. [Adapted from Kim et al., 2003] (See Color Plate 4A).

Cell Isolation

Chondrocytes from the individual layers were isolated by digestion of the cartilage chips in 0.2% collagenase overnight (See Protocol 9.1). Number and size of isolated cells was determined with a Z2 Coulter Particle Size and Number Analyzer.

RT-PCR

RNA was isolated from chondrocytes with the RNeasy Mini Kit. cDNA was synthesized with random hexamers. Cartilage-specific primers included type II collagen and aggrecan with β-actin as a housekeeping gene (See Protocol 9.6).

Cell Encapsulation

Polyethylene oxide-diacrylate was dissolved in PBSA to make a 10% w/v solution to which photoinitiator (0.05% Irgacure D2959) was added. The polymer solution was combined with a cell pellet to make a final concentration of 2×10^7 cells/ml. Approximately 100 µl of thoroughly mixed cell suspension in polymer solution was placed in an 8-mm cylindrical mold under a UVA lamp (365 nm $\sim$4 mW/cm^2) for 5 minutes. The resulting polymerized gel was removed from the mold, placed in complete DMEM (See Section 3.3), and incubated for 3 weeks.

Biochemical Characterization

Wet weights (ww) and dry weights (dw, after 48 h of lyophilization under vacuum) were obtained from constructs from each group ($n = 3–4$). The dried constructs were crushed with a tissue grinder and digested in 1 ml of papain, and the amounts of DNA, GAG, and total collagen were measured (See Protocol 9.5).

7.3. Results

Analysis of histology and biochemical composition confirmed that the cartilage slices had been obtained from the specific zone (superficial, middle, and deep; See Fig. 9.2, Color Plate 4A). The superficial layer exhibited minimal staining for GAG and type II collagen and smaller cells compared to the middle and deep zones. Chondrocytes from each zone differed in gene expression in monolayer and in matrix synthesis in three-dimensional culture (Figs. 9.3, 9.4). The gene expression of the cartilage-specific markers differed among the cells from different zones (See Fig. 9.3). Type II collagen expression of the superficial-zone chondrocytes was notably lower than the middle- and deep-zone chondrocytes. The aggrecan expression in freshly isolated cells had no remarkable differences among the zones. A slight decrease in aggrecan expression was observed in all groups on plating.

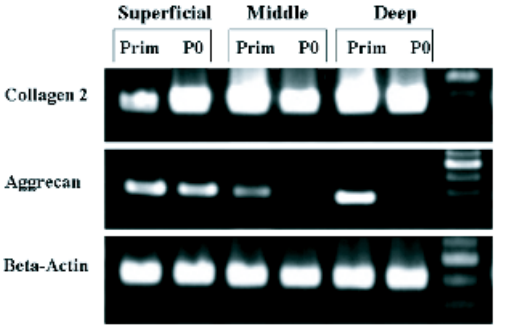

Figure 9.3. Gene expression profiles for cartilage-specific proteins in cells isolated from the different zones of cartilage.

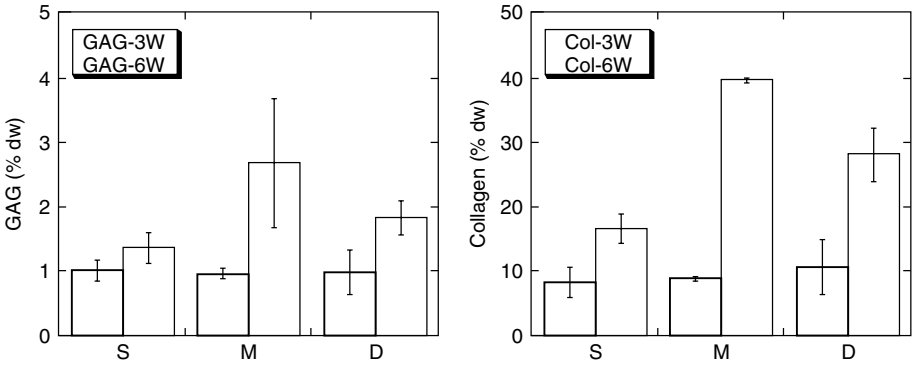

Figure 9.4. Biochemical analysis (GAG and total collagen) of zone-specific constructs incubated for 2 and 6 weeks.

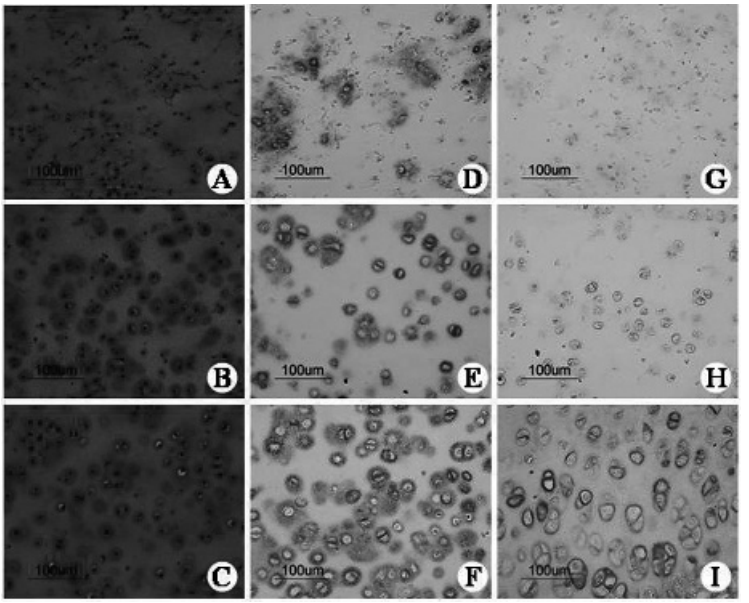

Figure 9.5. Histology of a multilayered PEGDA hydrogel encapsulated with different zone chondrocytes for different layers: Safranin-O staining (A–C) and immunohistochemistry for type II collagen (D–I) (200×, meter bar = 100 μm). Each layer of the constructs showed findings similar to those in native cartilage (A, D—upper; B, E—middle; and C, F—lower zones). Negative controls without primary antibody had no positive signals (G—upper zone, H—lower zone). I) Positive control with a cartilage slice of lower zone. [Adapted from Kim et al., 2003] (See Color Plate 4C.).

After 3 weeks in culture, the histologic differences between hydrogel constructs prepared with chondrocytes from the three zones were similar to the respective differences between the three zones in native articular cartilage. Superficial-zone cells maintained their relatively small size after the encapsulation in hydrogel, and accumulated less extracellular matrix staining, compared to the hydrogel-encapsulated deep cells. (See Fig. 9.5 and Color Plate 4C).

In native bovine cartilage, there is a 22% increase in GAG (%dw) from the S to the D layer and a 10% decrease in collagen content (graph not shown). The compositions of hydrogels cultured for 2 and 6 weeks with chondrocytes from the S, M, and D layers were significantly different from each other, and the changes were consistent with the zonal origin of chondrocytes (See Fig. 9.4). GAG content (%dw) in constructs containing chondrocytes from the D zone was 45% higher than in constructs containing S chondrocytes. Collagen production showed a similar trend, and the amount of collagen (%dw) of D hydrogels was 56% higher compared to S hydrogels.

Previous studies showed that articular chondrocytes retain the metabolic features characteristic of their zones of origin even after they are isolated from cartilage and cultured in suspension [Aydelotte et al., 1988; Aydelotte and Kuettner, 1988]. Biochemical assays showed that these metabolic differences between chondrocytes

from different zones were maintained when chondrocytes were photoencapsulated and cultured in a hydrogel (See Fig. 9.4). The environment of the hydrogel is similar to that of the native tissue in that the chondrocytes are isolated with little cell-cell contact, which encourages them to synthesize extracellular matrix [Elisseeff et al., 1999., 2000, 2002; Anseth et al., 2002]. The differences in biosynthetic activity, with the deep-zone cells significantly exceeding the superficial-zone cells in terms of matrix synthesis, are in line with the study by Wong and his colleagues [Wong et al., 1996]. They investigated the zone-specific biosynthetic activity in mature bovine articular cartilage and found significant differences between the biosynthetic activities of chondrocytes in deep and superficial zones. The photopolymerization system can therefore efficiently encapsulate cells, and the differences in chondrocyte biology and engineering can be maintained and may potentially be used to create more complex tissue-engineered cartilage structures.

8. PHOTOENCAPSULATION OF GOAT BONE MARROW-DERIVED MESENCHYMAL STEM CELLS FOR CARTILAGE TISSUE ENGINEERING

Bone marrow-derived mesenchymal stem cells are another cell source for cartilage tissue engineering. MSCs have numerous advantages including a relatively easy clinical harvest, large potential for expansion, and possible allogeneic cell application in orthopedics. We studied the ability of MSCs to survive photoencapsulation and differentiate into chondrocytes and produce cartilage-like tissue.

8.1. Design

MSCs were photoencapsulated in hydrogels and cultured in three experimental groups: (1) 3 weeks with TGF-β1 (3wk + TGF), (2) 6 weeks with TGF-β1 (6wk + TGF), and (3) 6 weeks without TGF-β1 (6wk − TGF) ($n = 6–7$/group). Histologic, biochemical, and RNA analyses were performed to evaluate both the differentiation of MSCs into a chondrogenic phenotype and the accumulation of ECM products in the hydrogels.

8.2. Methods

Cell Isolation and Expansion

Bone marrow from the femurs of three- to three-and-a-half-year-old castrated male goats being sacrificed for other reasons was aspirated into 10-ml syringes with 6000 U of heparin and processed within 4 hours of harvest. The marrow samples were washed and centrifuged twice (1000 rpm, 1500 g for 10 min) in MSCGM before suspension in fresh MSCGM. The mononuclear cells were counted with a hemocytometer and plated in 75-cm^2 tissue culture plastic flasks at a density of approximately 1.2×10^5 mononuclear cells/cm^2. Culture medium was changed after 4 days and then every 2–3 days thereafter until confluence (12–14 days). Cells were passaged with 0.025% trypsin-EDTA for 5 min at 37 °C and replated

in 75-cm^2 or 175-cm^2 flasks at 5×10^3 MSCs/cm^2. MSCs were frozen in liquid nitrogen in 50% MSCGM, 40% FBS, 10% DMSO until needed. Cell viability after thawing was consistently above 92%. When needed, the frozen cells were thawed, plated in 75-cm^2 or 175-cm^2 flasks in MSCGM, and grown until confluent. Passage 3 cells were trypsinized, centrifuged, and resuspended in the hydrogel solution as described below.

MSC Photoencapsulation

The hydrogel solution was prepared by mixing 10% weight/volume (w/v) of PEGDA in sterile PBSA with 100 U/ml penicillin and 100 μg/ml streptomycin. The photoinitiator, Irgacure D2959, was added to the PEGDA solution and mixed thoroughly to make a final concentration of 0.05% w/v. In experimental groups (1) and (2), TGF-β1, 10 ng/ml, was added to the hydrogel solution. Immediately before photoencapsulation, MSCs were resuspended in the hydrogel solution to make a concentration of 2×10^7 cells/ml and gently mixed to make a homogeneous suspension. Seventy-five microliters l of cell-polymer-photoinitiator suspension was transferred into cylindrical molds with a 6-mm internal diameter and exposed for 5 min to long-wave, 365-nm UV light at 4 mW/cm^2. The hydrogels were then removed from their molds, washed once with sterile PBSA containing penicillin-streptomycin, and incubated in separate wells of 12-well plates.

In Vitro Cultivation

The hydrogels were incubated at 37 °C in 5% CO_2 on an orbital rocker at 70 rpm in 2 ml of chondrogenic medium with or without TGF-β1. Chondrogenic medium consisted of high-glucose DMEM, 100 nM dexamethasone, 50 μg/ml ascorbic acid 2-phosphate, 100 μg/ml sodium pyruvate, 40 μg/ml proline, 100 U penicillin, 100 μg/ml streptomycin, and 5 ml of ITS + premix in 500 ml of medium (insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 μg/ml), linoleic acid (5.35 μg/ml), and bovine serum albumin (1.25 μg/ml)) with or without 10 ng/ml of TGF-β1. Medium was changed every 2–3 days.

Histology and Immunohistochemistry

Throughout the experiment, the hydrogels were observed by inverted light microscopy at least twice a week and digitally photographed at the beginning and end of the culture period. Particular attention was given to observing encapsulated cells for signs of cell division. At the end of the culture period, two constructs per group were harvested for histologic and immunohistochemical studies. The hydrogels were fixed overnight in 2% paraformaldehyde at 4 °C and transferred to 70% ethanol until being embedded in paraffin according to standard histologic technique. Sections were stained with hematoxylin and eosin and Safranin-O/Fast Green. Immunohistochemistry was performed with the Histostain-SP kit (Zymed

#95–9743), following the manufacturer's protocol. Rabbit polyclonal antibodies to collagen I and collagen II (Research Diagnostics Inc.) and mouse monoclonal antibodies to aggrecan and link protein (Hybridoma Bank, University of Iowa) were used as the primary antibodies.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from three constructs per group and from goat MSCs of the same passage cultured in monolayer with the RNeasy Mini Kit. To extract the total RNA the constructs were homogenized in 1.5-ml microcentrifuge tubes containing 200 μl of RLT buffer from the RNeasy Mini Kit, using a tissue grinder (See Protocol 9.6). After complete homogenization, 400 μl more of the RLT buffer was added to the microcentrifuge tubes and the suspension was further homogenized with the QIAshredder column. The homogenates were transferred to columns from the RNeasy Mini Kit after an equal volume of 70% ethanol had been added. RNA was isolated, following the manufacturer's protocol. The RNA was reverse transcribed into cDNA, using random hexamers with the Superscript amplification system per the manufacturer's instructions. One-μl aliquots of the resulting cDNA were amplified in a total 50-μl volume at an annealing temperature of 58 °C (collagen type II was annealed at 60 °C) for 35 cycles, using the Ex Taq™ DNA Polymerase Premix. PCR primers (forward and backward, 5′ to 3′) were as follows: collagen I, 5′-TGACGAGACCAAGAACTG-3′ and 5′-CCATCCAAACCACTGAAACC-3′; collagen II, 5′-GTGGAGCAGCAAGAGCAAGGA-3′ and 5′-CTTGCCCCACTTACCAGTGTG-3′; aggrecan, 5′-CACGCTACACCCTGGACT TG-3′ and 5′-CCATCTCCTCAGCGAAGCAGT-3′; β-actin, 5′-TGGCACCACACCTTCTACAATGAGC-3′ and 5′-GCACAGCTTCTCCTTAATGTCACGC-3′. Each PCR product was analyzed by separating 4 μl of the amplicon and 1 μl of loading buffer in a 2% agarose gel in TAE buffer. The relative levels of band intensity of the gene of interest were compared to those of the internal control of housekeeping gene.

Biochemical Characterization

Wet weights (ww) and dry weights (dw, after 48 h of lyophilization under vacuum) were obtained from constructs from each group ($n = 3–4$). The dried constructs were crushed with a tissue grinder and digested in 1 ml of papain, and the amounts of DNA, GAG, and total collagen were measured (See Protocol 9.5).

8.3. Results

Histology

Observations of the hydrogels immediately after photoencapsulation under inverted light microscopy showed rounded cells evenly dispersed throughout the constructs (data not shown). As the culture period extended for the groups cultivated

with TGF-β1, many single cells divided and produced small, multicellular aggregations with approximately two to five cells. Cell clusters were not seen in hydrogels cultured without TGF-β1.

Histologic study of fixed slides of the hydrogels showed that the experimental groups cultured with TGF-β1 were strongly positive for GAG compared to the other groups. Figure 9.6 shows histologic sections of the four groups stained with Safranin-O/Fast Green, which stains negatively charged glycosaminoglycans red and nuclei green. At Day 0 (Fig. 9.6A) only the light blue-green counterstain was present. The sections from the 3wk + TGF group (Fig. 9.6B) revealed an intense positive staining for GAG, particularly around the pericellular regions. The positive staining was enhanced in the 6wk + TGF group (Fig. 9.6C) and was strongly present in the intercellular matrix as well, indicating that the GAG had diffused throughout the PEGDA gels [Bryant and Anseth, 2002]. Conversely, in the 6wk − TGF section (Fig. 9.6D), only a small amount of GAG produced by spontaneous chondrogenic differentiation was seen in a few of the pericellular regions.

Immunohistochemical staining for aggrecan and link protein showed strong positive staining in the 6wk + TGF group but revealed negative or sporadic, weakly positive cells in the 6wk − TGF group (not shown). Staining for type I collagen was positive on both the 6wk + TGF and 6wk − TGF sections. Interestingly, type II collagen staining was also noted on sections from both 6wk + TGF and 6wk − TGF sections, which is in agreement with the gene expression results described below.

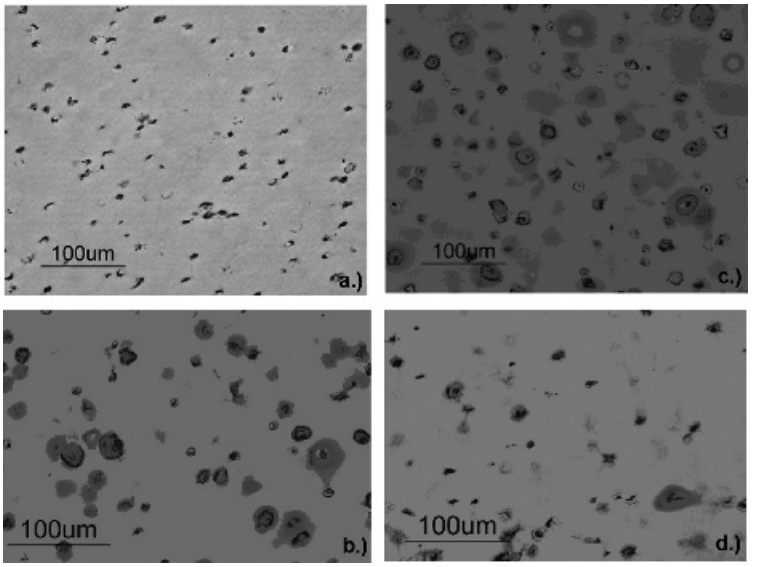

Figure 9.6. Paraffin embedded histologic sections of PEGDA-MSC hydrogels for Day 0 controls (A), group 1: 3wk + TGF (B), group 2: 6wk + TGF (C), and group 3: 6wk − TGF (D) stained with Safranin-O/Fast Green. Originally acquired at 200×. This dye combination stains GAG red and nuclei green. The scale bars are 100 μm. [Adapted from William et al., 2003] (See Color Plate 4B).

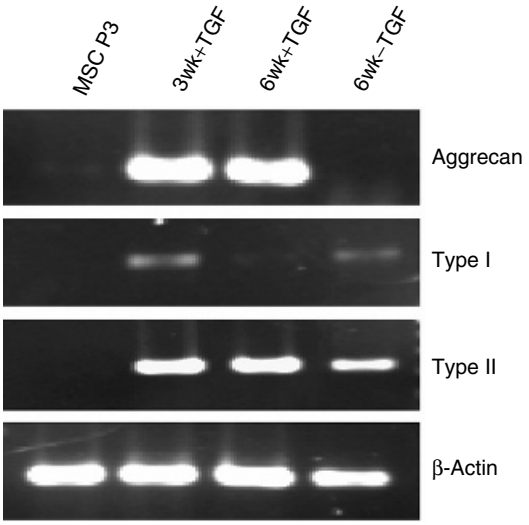

Figure 9.7. RT-PCR products for MSC passage 3 monolayer culture as control, 3wk + TGF, 6wk + TGF, and 6wk − TGF (left to right on gels). Primers used include aggrecan, type I collagen, type II collagen, and β-actin (from top to bottom of the gels). (Adapted from Williams et al., 2003).

RT-PCR

RT-PCR supported the histologic findings by demonstrating that the previously undifferentiated MSCs shifted their genetic expression during the culture period (Fig. 9.7). The expression of the aggrecan gene was almost entirely absent in monolayer controls, absent in the 6wk − TGF constructs, but strongly present in the 3wk + TGF and 6wk + TGF constructs. Type I collagen was not expressed in monolayer culture, almost absent in 6wk + TGF constructs, but weakly present in 3wk + TGF and 6wk − TGF constructs. Type II collagen was not expressed in monolayer culture of the MSCs, present in low quantities in 6wk − TGF, but strongly present in the 3wk + TGF and 6wk + TGF constructs.

Biochemical Analysis

The DNA content of the MSC constructs revealed a statistically significant increase to 1101 ng (±170 ng) of DNA/mg dw in the 6wk + TGF group from an initial value of 882 ng (±94 ng)/mg dw ($p = 0.036$) and a significant decrease to 681 ng (±43 ng)/mg dw in the 6wk − TGF group ($p = 0.028$).

Correlating with the RT-PCR and histologic findings, the hydrogels showed a significant increase in GAG and total collagen content (%dw) by detection of chondroitin sulfate and hydroxyproline at each time point when the constructs were cultured with TGF-β1 (Fig. 9.8B, C). The amount of GAG increased from 0 at Day 0 constructs to 1.4%dw in 3wk + TGF constructs ($p = 0.020$) and to 3.5%dw in the 6wk + TGF constructs ($p = 0.001$). The amounts of GAG in 6wk − TGF hydrogels (0.9%dw) were comparable to those in Day 0 controls ($p > 0.05$). Collagen contents (%dw) increased with time of culture in all experimental groups: to 2.3%dw in 3wk + TGF ($p = 0.001$); to 5.0%dw in 6wk + TGF ($p = 0.001$); and to 1.4%dw in 6wk − TGF ($p = 0.029$). Amounts of GAG and collagen in the cartilage-like tissue produced in this study are comparable to those

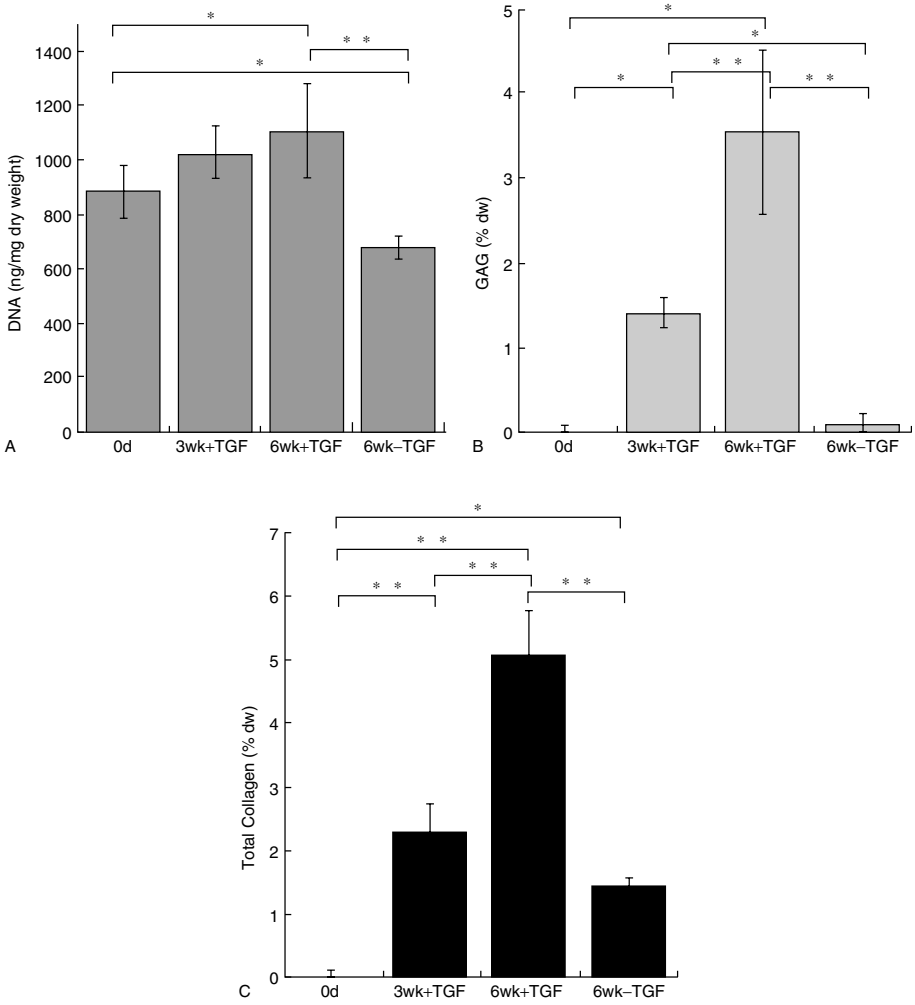

Figure 9.8. Results of biochemical assays for the Day 0 control, 3wk + TGF, 6wk + TGF, and 6wk − TGF hydrogels are depicted: DNA content (ng DNA/mg dry weight) (A), GAG content (% dry weight of construct) (B), and total collagen content (% dry weight of construct) (C). $*p < 0.05$, $**p < 0.001$. (Adapted from Williams et al., 2003).

in previous studies using cultured chondrocytes in PEGDM-based hydrogels and PGA mesh scaffolds [Bryant and Anseth, 2002; Freed and Vunjak-Novakovic, 1995]. Bryant and colleagues reported 0.4 ng of GAG/chondrocyte and 0.3 ng of collagen/chondrocyte produced after 4-week cultures of bovine chondrocytes in similar PEGDM-based hydrogel constructs. Our MSC constructs cultured in chondrogenic conditions for 6 weeks differentiate and produce 0.24 ng of GAG/cell and 0.34 ng of collagen/cell. Freed and Vunjak-Novakovic [1995] reported that a 6-week culture of approximately 1.0×10^7 bovine chondrocytes/mesh PGA construct produced 10% GAG/dw and 11–12% collagen/dw. Our 6-week PEGDA

hydrogel constructs, which contain approximately 1×10^6 MSCs/construct, produce about 3.5% GAG/dw and 5% collagen/dw. These rough comparisons suggest that MSCs can differentiate and produce similar amounts of ECM as native chondrocytes when photoencapsulated in a polyethylene glycol-based hydrogel. Interestingly, Barry and colleagues suggested that MSCs in an alginate gel culture system were capable of producing more GAG, as determined by ^{35}S-sodium sulfate incorporation, than native, isolated, and dedifferentiated articular chondrocytes [Barry et al., 2001]. The findings of our study indicate the robust biochemical productivity of MSCs in a photopolymerizing hydrogel and may have powerful implications for the development of a cartilage replacement therapy using MSCs.

Despite these encouraging results, our cartilage-like tissue is still approximately three- to fourfold lower in GAG (%dw) and 10 to 12-fold lower in collagen content (%dw) than native cartilage (10–15% dw and 55–85% dw, respectively) [Mow et al., 1992]. We also noted that chondrogenic differentiation and extracellular matrix production from the MSCs was not evenly distributed throughout the gels. The central area of the hydrogels showed less production of GAG and collagen on histologic sections. This contrasts with bovine chondrocytes photoencapsulated in similar hydrogels, which demonstrate an even distribution of GAG throughout the entire gels [Bryant et al., 1999].

A polyethylene glycol-diacrylate hydrogel provides a three-dimensional, nonadhesive environment for encapsulated cells. The DNA assay and microscopic observations in this study demonstrate that the MSCs could not only survive but could also divide in the presence of TGF-β1. Conversely, in the absence of TGF-β1, the number of encapsulated cells decreased with time in culture. Even though the findings of this study indicate that the PEGDA photopolymerizing hydrogel supports MSC survival, phenotypic differentiation, and accumulation of chondrogenic extracellular matrix, it seems clear, as evidenced by the results for the 6wk + TGF group, that cellular signaling driven by TGF-β1 is important for enhanced tissue development.

Interestingly, in the absence of TGF-β1, there is still a small degree of differentiation in the 6wk − TGF group, as indicated by several assays. Histology reveals sporadic cells producing small amounts of matrix staining with Safranin-O. The immunohistochemistry, RT-PCR, and collagen assays for the 6wk − TGF hydrogels demonstrate the presence of type I and II collagen proteins and RNA in this group. Indeed, the amount of total collagen produced in the 6wk − TGF constructs is similar to the amount of total collagen produced by the 3wk + TGF constructs ($p > 0.05$) but is lower than in the 6wk − TGF constructs ($p = 0.001$). However, little to no GAG, aggrecan, or link protein was produced in the 6wk − TGF group. The mechanisms that induce this partial differentiation are unclear. Previous studies have shown that cellular morphology might be associated with cell differentiation [Benya and Shaffer, 1982; Johnstone et al., 1998]. One possible explanation is that the rounded cellular morphology in our hydrogels, which is much different from the flattened morphology typical of a monolayer culture, aided the spontaneous chondrogenic differentiation of the MSCs as opposed to cellular division.

In summary, bone marrow-derived mesenchymal stem cells are attractive cells for tissue engineers and biologists. Hydrogels are a class of polymers used in tissue engineering that have many advantages including high, tissuelike water content. The creation of three-dimensional hydrogels by photopolymerization gives a great deal of spatial and temporal control to the engineer and can be adapted to a number of minimally invasive surgical techniques currently in clinical use. These in vitro studies suggest the potential use of MSCs with hydrogels for cartilage tissue engineering, and this technology could potentially expand the plastic and orthopedic surgeons' armamentarium for cartilage repair or augmentation in the future.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Arthritis Foundation and Johns Hopkins University for funding.

SOURCES OF MATERIALS

Item	*Supplier*
Ascorbic acid 2-phosphate	Sigma
Collagenase	Worthington
Dexamethasone	Sigma
Digital camera: DMX1200	Nikon
Dimethylene blue dye	
DMEM (Dulbecco's modified Eagle's medium with high glucose)	Invitrogen (GIBCO)
DMSO	Sigma
Ex Taq™ DNA Polymerase Premix	Takara Bio
FBS	Hyclone
Histostain-SP kit (#95–9743)	Zymed Laboratories
Inverted microscope: Eclipse TE200	Nikon
ITS^+ premix	BD Biosciences
Live/Dead viability kit	Molecular Probes
Monoclonal antibodies to aggrecan and link protein	Hybridoma Bank, University of Iowa
MSCGM	Clonetics, Cambrex
Nylon filter, 70 μm	Tekmar-Dohrmann
Papain	Worthington Biomedical Corporation
Penicillin-100 μg/ml streptomycin	Invitrogen (GIBCO)
Photoinitiator, Igracure D2959	Ciba Specialty Chemicals
Polyethylene glycol-diacrylate (PEGDA)	Shearwater Corp.
Proline	Sigma
QIAshredder	Qiagen
Random hexamers	Invitrogen (GIBCO)
RNeasy mini kit	Qiagen

Item	*Supplier*
Safranin-O/Fast Green	Sigma
Sodium pyruvate	Invitrogen (GIBCO)
SPSS (version 10.0)	SPSS
Superscript amplification system	Invitrogen (GIBCO)
TGF-β1	Research Diagnostics
Tissue grinder: Pellet Pestle Mixer	Kimble/Kontes
TRIzol reagent	Invitrogen (GIBCO)
Trypsin-EDTA	Clonetics, Cambrex
UV light	Glowmark Systems or VWR
WST-1 solution	Sigma

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