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In Vitro Generation of an Osteochondral Construct Using Injectable Hydrogel Composites Encapsulating Rabbit Marrow Mesenchymal Stem Cells

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Abstract

Injectable, biodegradable hydrogel composites of crosslinked oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) were utilized to fabricate a bilayered osteochondral construct consisting of a chondrogenic layer and an osteogenic layer, and to investigate the differentiation of rabbit marrow mesenchymal stem cells (MSCs) encapsulated in both layers *in vitro*. The results showed that MSCs in the chondrogenic layer were able to undergo chondrogenic differentiation, especially in the presence of TGF-β1-loaded MPs. In the osteogenic layer, cells maintained their osteoblastic phenotype. Although calcium deposition in the osteogenic layer was limited, cells in the osteogenic layer significantly enhanced chondrogenic differentiation of MSCs in the chondrogenic layer. The greatest effect was observed when MSCs were encapsulated with TGF-β1-loaded MPs and cultured with osteogenic cells in the bilayered constructs. Overall, this study demonstrates the fabrication of bilayered hydrogel composites that mimic the structure and function of osteochondral tissue, along with the application of these composites as cell and growth factor carriers.

Keywords

mesenchymal stem cell; cartilage tissue engineering; hydrogel; cell encapsulation; drug delivery; growth factors; composite; biomimetic material

Introduction

Articular cartilage and subchondral bone are two distinct tissues that contribute to the unique and important functions of the articular joint. Subchondral bone serves as a mechanical support

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for the articular surface [1], while articular cartilage protects bone from high stresses and allows for low-friction movements within the joint [1]. Due to its complex structure and poor access to progenitor cells, articular cartilage has limited ability to regenerate once injured [2,3]. Thus, degenerative changes in cartilage and subchondral bone cause severe joint pain and progressive loss of joint function, which affects many people of different ages [3].

Recently, tissue engineering has become a promising strategy for tissue regeneration, by combining scaffolds, cells, and bioactive molecules [4]. In this approach, mesenchymal stem cells (MSCs) hold great promise because MSCs can be easily isolated from the bone marrow and expanded without losing their capacity to differentiate into cells of various mesenchymal lineages, including chondrocytes and osteoblasts [5]. Many studies have reported the successful generation of cartilage or bone tissue alone *in vitro* using MSCs [6,7].

However, due to the different properties of articular cartilage and subchondral bone, a bilayered architecture with proper cell and extracellular matrix distribution is desirable because it mimics the complex structure of osteochondral tissue and contributes to the function of the articular joint. Some attempts have been made to fabricate cartilaginous and bony layers independently before integrating the two components together by suturing or gluing [8,9]. However, implantation of these bilayered constructs has typically revealed poor integration between layers, which may cause long-term device failure. Consequently, investigators are exploring methods to construct well integrated bilayered scaffolds that can guide the chondrogenic and osteogenic differentiation of cells in different regions of the same scaffold during coculture. The challenge lies in maintaining the appropriate chondrogenic and osteogenic phenotype under a single set of cell culture conditions [8,10].

A degradable macromer, oligo(poly(ethylene glycol) fumarate) (OPF), has been developed in our laboratory and shown to be suitable as an injectable hydrogel carrier for cells and growth factors for both cartilage and bone tissue engineering [11,12]. OPF can be synthesized from fumaryl chloride and poly(ethylene glycol) (PEG). The fumarate double bonds in the macromer chains allow this material to crosslink to form a hydrogel network, and the hydrolysis of ester linkages results in the degradation of the crosslinked hydrogel [13]. Previous work in our laboratory has demonstrated the cytocompatibility of OPF hydrogels with various cell types, including MSCs [11,14,15]. For example, rat MSCs encapsulated in OPF hydrogels and cultured in osteogenic medium have been shown to be viable and to differentiate down the osteogenic lineage, as evidenced by calcium deposition after 4 weeks [15]. Gelatin microparticles (MPs) can be incorporated into this hydrogel during the time of crosslinking for controlled release of growth factors [16,17]. Previous studies have shown that MPs can be loaded with transforming growth factor β1 (TGF-β1), an important molecule for *in vitro* and *in vivo* chondrogenesis, through polyionic complexation and encapsulated in OPF hydrogels. The hydrogel composites containing TGF-β1-loaded MPs enable controlled release of the growth factor [16,17]. Additionally, rabbit MSCs encapsulated with TGF-β1-loaded MPs in OPF hydrogels showed an increase in gene expression of type II collagen and aggrecan, indicating enhanced chondrogenic differentiation [14]. Further, the cellularity of the constructs and the distribution of the MPs in the OPF hydrogels were confirmed qualitatively via both light microscopy and histology [14]. It should also be noted that OPF allows the fabrication of a multi-layered structure with good integration between layers using a multi-step crosslinking procedure, as evidenced by swelling and mechanical studies [18,19]. All these features suggested that OPF hydrogel composites could be applied for fabrication of osteochondral constructs.

The present study explored the fabrication of a bilayered OPF/MP composite consisting of a chondrogenic layer and an osteogenic layer; MSCs were encapsulated within these composites and MSC differentiation in both layers was investigated. Specifically, the study asked the

following questions: (1) whether MSCs can undergo chondrogenic and osteogenic differentiation in the respective layers of a bilayered hydrogel composite; (2) how cells in the osteogenic layer affect MSC chondrogenesis in the chondrogenic layer; and (3) how cells in the osteogenic layer, in combination with TGF-β1 loaded MPs, influence MSC chondrogenesis in the chondrogenic layer.

Materials and Methods

Experimental Design

The overall experimental design is shown in Figure 1. In order to develop a set of cell culture conditions that is suitable for both osteogenic and chondrogenic differentiation of MSCs, an osteogenic culture study and a chondrogenic culture study were first performed with single-layer OPF hydrogel composites.

In the osteogenic construct culture study, MSCs were first cultured in a tissue culture flask with osteogenic medium for various periods of time (0, 3 and 6 days) to induce osteogenic differentiation (groups OS0, OS3, OS6 in Figure 1). The precultured MSCs were then encapsulated in hydrogel composites with blank MPs and subsequently cultured in chondrogenic medium (CM) supplemented with (+) or without (−) β-glycerophosphate (β-GP); osteogenic differentiation was assessed. The purpose of this study was to examine whether the osteoblastic phenotype (induced by preculture) can be maintained within OPF hydrogel composites exposed to chondrogenic culture conditions with (or without) the osteogenic supplement β-glycerophosphate.

In the chondrogenic construct culture study, MSCs were encapsulated in OPF hydrogel composites containing either blank MPs (group BLK) or TGF-β1-loaded MPs (group TGF in Figure 1) to examine their effect on chondrogenic differentiation of encapsulated MSCs. Cell constructs were cultured in chondrogenic medium supplemented with β-GP.

The next step was an osteochondral construct culture study, where bilayered hydrogel composites consisting of a chondrogenic layer at top and an osteogenic layer at bottom were fabricated. More specifically, in the top layer, MSCs were encapsulated in OPF hydrogels with either blank MPs (BLK) or TGF-β1 loaded MPs (TGF), while in the bottom layer, OPF hydrogel composites with blank MPs were utilized to encapsulate either MSCs (MSC) or osteogenic precultured cells (OS). Four formulations of bilayered constructs [groups BLK (MSC), TGF(MSC), BLK(OS), TGF(OS) in Figure 1] were cultured in CM supplemented with β-GP for 28 days. Samples from the top and bottom layers were analyzed to evaluate chondrogenic and osteogenic differentiation of the cells, respectively.

OPF synthesis and characterization

OPF was synthesized from fumaryl chloride and poly(ethylene glycol) (PEG) with a nominal molecular weight of 10,000 g/mol (Sigma, St. Louis, MO) according to a previously established method [13]. Molecular weights of both the parent PEG and the resulting OPF were determined by gel permeation chromatography (GPC; Model 410; Waters, Milford, PA) using a refractive index detector (n=3). The purified macromer was stored at −20°C and sterilized prior to use by exposure to ethylene oxide for 14 h.

Gelatin microparticle fabrication

Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) and crosslinked with 10 mM glutaraldehyde (Sigma, St. Louis, MO) following established procedures [11]. After drying, MPs were sieved to obtain particles of 50–100 μm in diameter and sterilized with ethylene oxide for 14 h.

Rabbit marrow MSC isolation and preculture

Rabbit marrow MSCs were isolated from the tibias of 4 month old New Zealand white rabbits as previously described [14]. Briefly, after anesthesia, the rabbit tibia was punctured with a 16-gauge needle and 4~5 ml of bone marrow were aspirated through a sterile tube into a 10 ml syringe containing 5000 U of heparin. The bone marrow was filtered through a cell strainer (40 μm) and cultured in general medium (GM) containing Dulbecco's modified Eagle's medium (DMEM), 10% v/v fetal bovine serum (FBS; Gemini, Calabasas, CA), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin for 2 weeks. In order to reduce any interanimal variation, a pool of rabbit marrow MSCs from a total of six rabbits was mixed together, and then cryopreserved in medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen. Prior to use, MSCs were thawed at 37°C and expanded in T-75 flasks with GM up to passage three. For osteogenic preculture (groups BLK(OS) and TGF(OS) in Figure 1), cells were expanded in GM up to passage two, and then, for the third passage, cells were cultured in osteogenic medium (OM), which is DMEM supplemented with 10% v/v FBS, 50 mg/l ascorbic acid, 10mM β-glycerophosphate, 10^{-8} M dexamethasone, 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin (all from Sigma), for 3 or 6 days before encapsulation. Cells from a single pool of rabbit marrow were used in all three studies and the cells used for each study were from each expansion of cryopreserved cells.

Fabrication of single-layer hydrogel composites

Before encapsulation, sterile MPs were loaded with TGF-β1 (R&D Systems, Minneapolis, MN) by swelling in an aqueous solution of the growth factor at pH 7.4 for 15 h according to a previously reported method [20]. The volume of TGF-β1 loading solution needed for equilibrium swelling of the MPs (110 μl) was combined with 22 mg of MPs. The loading solution had a concentration of 3.6 μg TGF-β1/ml phosphate buffered saline (PBS; Gibco) to achieve a final loading of 600 ng TGF-β1/ml in the crosslinked scaffolds. This growth factor amount has been shown to promote the chondrogenic differentiation of rabbit MSCs encapsulated in hydrogel composites *in vitro* [14]. Blank MPs were loaded with PBS in a similar fashion.

For fabrication of a single-layer hydrogel composite, 0.1 g of sterile OPF and 0.05 g of sterile poly(ethylene glycol) diacrylate (PEG-DA; Nektar Therapeutics, Huntsville, AL) were first dissolved in 300 μl of PBS and mixed with swelled MP solution (either TGF-β1-loaded or blank). The mixture was then added to equal volumes (46.8 μl) of the thermal radical initiator solutions, 0.3 M ammonium persulfate (APS) and 0.3 M N,N,N′,N′-tetramethylethylenediamine (TEMED) in PBS. A cell suspension (6.7 million cells in 168 μl of PBS) was subsequently added to the polymer solution to achieve a concentration of 10 million cells/ml final suspension. After gentle mixing, the suspension was quickly injected into Teflon molds (6 mm diameter, 1 mm thickness), followed by incubation at 37°C for 8 min. In the osteogenic construct culture study, all hydrogel composites contained blank MPs, while in the chondrogenic construct culture study, hydrogel composites contained either blank MPs or TGF-β1-loaded MPs, as shown in Figure 1.

Fabrication of bilayered hydrogel composites

Bilayered hydrogel composites were fabricated via a two-step crosslinking procedure. The desired composition for the osteogenic (bottom) layer (Figure 1) was first injected into the bottom 1mm of Teflon molds (6 mm diameter, 2 mm thickness) and incubated for 4 minutes, allowing for partial crosslinking. Meanwhile, another polymer-cell suspension was prepared, and then injected into the partially filled Teflon molds to form the chondrogenic layer. The resulting bilayered constructs were then incubated at 37°C for 8 minutes to achieve crosslinking.

All hydrogel constructs were transferred into 12-well tissue culture plates. Each construct was cultured with 2.5 ml chondrogenic medium, which was DMEM supplemented with ITS+ Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.35 μg/ml linoleic acid and 1.25 μg/ml bovine serum albumin) (BD Biosciences, Franklin Lakes, NJ), 1 mM sodium pyruvate (Sigma), 50 mg/l ascorbic acid, 10^{-7} M dexamethasone, 250 mg/l fungizone, 100 mg/l ampicillin and 50 mg/l gentamicin. In some of the groups, CM was supplemented with β-glycerophosphate, as indicated in Figure 1. The medium was changed every 3 days. In the osteogenic and chondrogenic construct culture studies, the hydrogel composites were cultured up to 14 and 21 days, respectively, while in the osteochondral construct culture study, samples were cultured up to 28 days.

At the time points (day 0, 7, 14, 21 and 28), samples were removed from culture medium, rinsed in PBS and collected for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4) and for biochemical assays (n=4). Bilayered samples were dissected with a blade to separate the chondrogenic layer and osteogenic layer; samples from each layer were stored for analysis.

Real time PCR

Samples from the chondrogenic construct culture study and the top layer of the osteochondral construct culture study were subjected to RT-PCR analysis to quantify MSC chondrogenic differentiation. Total RNA was extracted and reverse-transcribed to cDNA as described previously [14]. The final cDNA was then subjected to real time PCR (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA) to determine the expression of genes for collagen type II, aggrecan and collage type I.

Gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method as described previously [21, 22]. Briefly, all gene expression data were normalized to the expression of a house-keeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) and expressed as the fold ratio compared to baseline gene expression of a control group at day 0. In the chondrogenic construct culture study, the control group contained a total of four samples from the BLK and TGF groups immediately after encapsulation. Similarly, in the osteochondral construct culture study, four top-layer hydrogels containing MSCs were collected from all the groups at day 0 and served as a control. The sequences of the primers for GAPDH, type II collagen, aggrecan, and type I collagen were as follows [14]: GAPDH: 5′-TCACCATCTTCCAGGAGCGA-3, 5-CACAATGCCGAAGTGGTCGT-3′; type II collagen gene: 5′-AACACTGCCAACGTCCAGAT-3′, 5′-CTGCAGCACGGTATAGGTGA-3′; Aggrecan: 5′-GCTACGGAGACAAGGATGAGTTC-3′, 5′-CGTAAAAGACCTCACCCTCCAT-3′; type I collagen gene: 5′-ATGGATGAGGAAACTGGCAACT-3′, 5′-GCCATCGACAAGAACAGTGTAAGT-3′.

Biochemical assays

Samples from the osteogenic construct culture study and the bottom layer of the osteochondral construct culture study were analyzed for DNA, alkaline phosphatase (ALP) enzyme activity, and calcium content to characterize osteogenic differentiation of the cells. In the osteochondral construct culture study, a total of four bottom-layer samples from the BLK(MSC) and TGF (MSC) groups were collected after encapsulation and analyzed to represent day 0 values for both groups. Similarly, the BLK(OS) and TGF(OS) shared bottom-layer samples for biochemical assays at day 0.

At each time point, samples were homogenized with a pellet grinder (Fisher Scientific) and stored in 500 μl of ddH_2O. The homogenates were then stored at −20°C until analysis, when

they were subjected to three freeze-thaw-sonication cycles (30 min at −80°C, 30 min at room temperature, 30 min of sonication) for complete extraction of DNA from the cell cytoplasm.

DNA content was determined using the PicoGreen assay (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. ALP enzyme activity of each homogenized sample was measured by using an Alkaline Phosphatase Assay from Sigma (St. Louis, MO) as previously described [15]. After the DNA and ALP assays, each homogenized sample was mixed with an equal volume of 1 N acetic acid. Calcium content of the hydrogels was then determined using a calcium quantification assay (Diagnostic Chemicals; Charlottetown, PEI, Canada) as decribed previously [15].

Samples from the chondrogenic construct culture study and the top layer of the osteochondral construct culture study were subjected to DNA, glycosaminoglycan (GAG), and calcium assays to evaluate chondrogenic differentiation. In the chondrogenic construct culture study, a total of four samples from the BLK and TGF groups were collected after encapsulation and analyzed to represent day 0 values for both groups. Similarly, in the osteochondral construct culture study, the BLK(MSC) and BLK(OS) groups shared top-layer samples for biochemical assays at day 0, and the TGF(MSC) and TGF(OS) groups shared samples at day 0.

The analysis of GAG content required samples to be homogenized and digested in 500 μl of a proteinase K solution (1 mg/ml proteinase K, 10 μg/ml pepstatin A, and 185 μg/ml iodoacetamide in tris-EDTA solution (6.055 mg/ml Tris(hydroxymethyl aminomethane), 0.372 mg/ml EDTA, pH 7.6 adjusted by HCl); all from Sigma-Aldrich) at 56°C for 16 h. The homogenates were then frozen down and subjected to the same free-thaw-sonication cycles prior to analysis. DNA and calcium assays were performed on the lysate as described above. GAG content was determined using a biochemical assay, the dimethylmethylene blue dye (DMMB) assay (Sigma-Aldrich), as previously described [23].

Microplate readers (BIO-TEK Instrument, Winooski, VT) were utilized for all the absorbance/fluorescence measurements. Cell-free constructs were also fabricated, cultured, and analyzed with the samples. For all assays, fluorescence or absorbance of the cell-free hydrogels was subtracted from the values of experimental groups to account for fluorescence/absorbance caused by the scaffold material alone.

Statistical analysis

DNA, GAG, calcium contents, ALP activity and gene expression data are reported as means ± standard deviation. Repetitive ANOVA and Tukey's multiple comparison tests were used to determine possible significant differences ($p < 0.05$) between groups.

Results

Osteogenic construct culture study

In the osteogenic construct culture study, we examined whether osteoblastic phenotype (induced by preculture) can be maintained within OPF hydrogel composites in the subsequent chondrogenic culture conditions, with an osteogenic supplement. DNA, ALP and calcium results are presented in Figure 2(a–c). For all the treatments, DNA content significantly decreased from day 0 to day 7, but the values remained similar after day 7. At day 7, hydrogels with osteogenically precultured cells were found to have a higher DNA content than those with MSCs, and a significant difference was observed between OS0+ and OS6+ groups. However, by day 14, this difference among groups was no longer present.

ALP results revealed a significantly higher ALP enzyme activity at days 7 and 14 than at day 0 for all the treatments. However, there was no significant change in ALP activity between treatment groups.

At day 14, there was higher calcium deposition in the hydrogels containing cells that were precultured in osteogenic medium for three or six days (and subsequently cultured in CM with β-GP), compared to hydrogels containing cells expanded in general medium. Specifically, 8.29 ± 3.62 μg of calcium were deposited in the OS6+ samples (and 7.55 ± 7.84 μg in the OS3+ samples), compared to 0.38 ± 0.41 μg of calcium deposited in the OS0+ samples by day 14. The difference between the OS6+ and OS0+ groups was significant. Additionally, for MSCs precultured in osteogenic medium for 6 days, a significant difference in calcium content was observed at day 14; constructs cultured in chondrogenic medium with β-GP (OS6+) had much higher calcium deposition than those cultured without β-GP (OS6−).

Chondrogenic construct culture study

In the second study, we investigated whether MSCs will undergo chondrogenic differentiation when encapsulated in OPF hydrogel composites with blank MPs or TGF-β1 loaded MPs, and subsequently cultured in chondrogenic medium containing β-GP. The combined influence of β-glycerophosphate and TGF-β1 on scaffold mineralization was also evaluated.

Quantitative gene expression data for collagen type II, aggrecan, and collagen type I at each time point are depicted in Figure 3(a–c). The results revealed an increase in collagen type II and aggrecan gene expression over time for both BLK and TGF groups. Specifically, a significantly higher collagen type II gene expression compared to day 0 was seen in the group containing blank MPs at day 28, while in the group with TGF-β1 loaded MPs, significantly increased expression was observed at both day 14 and day 28 (and in the case of aggrecan gene expression, also at day 7). The presence of TGF-β1 significantly affected the gene expression of chondrogenic markers, as evidenced by significant differences between the BLK and TGF groups in collagen type II gene expression at day 28 and in aggrecan gene expression at day 14 and day 28.

For collagen type I gene expression, although the levels for TGF-β1 treated samples increased at day 7 and day 14 compared to day 0, gene expression subsequently decreased; the values on day 21 were found to be statistically equivalent to day 0. For the samples with blank MPs (no TGF-β1 treatment), there was no significant change in collagen type I gene expression over time.

Results for the biochemical assays, which quantified DNA, GAG and calcium content, are presented in Figure 4(a–c). For both BLK and TGF groups, there was a significant decrease in DNA content between day 0 and day 7, but the values on days 7, 14 and 21 did not show any significant difference. At day 7, BLK samples had a significantly higher DNA amount than TGF samples, but there was no difference in DNA content between the two treatments at days 14 and 21.

There was a trend of increasing GAG/DNA content for both BLK and TGF groups over the 21-day culture period; TGF samples had higher GAG/DNA values than BLK samples at each time point. However, statistical analysis revealed no significant difference in GAG/DNA content between groups at each time point during the culture period.

Little calcium deposition was observed for either BLK or TGF samples when cultured in the chondrogenic medium with β-GP. The values for each treatment were found to be below 1 μg at each time point and statistically equivalent to those at day 0.

Osteochondral construct culture study

After testing the effect of our set of culture conditions (specifically, CM + β-GP) on both MSC chondrogenic and osteogenic differentiation in single-layer composites, we developed a bilayered hydrogel composite consisting of a chondrogenic layer at top and an osteogenic layer at bottom. Chondrogenic and osteogenic differentiation of the cells in each respective layer, as well as the signaling between layers, were examined in this study.

RT-PCR analysis for gene expression of collagen type II, aggrecan, and collagen type I was performed on the top layer of the bilayered hydrogels to evaluate chondrogenic differentiation, as shown in Figure 5(a–c). For all the treatments, cells showed an increase in collagen type II and aggrecan gene expression over time. Significantly higher expression levels of both genes were found at days 14 and 28 (compared to day 0) in the TGF(OS) treatment group. Samples with osteogenic precultured cells in the bottom layer (indicated by (OS) in Figure 5) exhibited higher gene expression in both markers at every time point than those with MSCs in the bottom layer (indicated by (MSC) in Figure 5); this trend was observed both in the presence and absence of TGF-β1. Interestingly, although there was a significant difference in gene expression between BLK and TGF groups in the single-layer chondrogenic construct culture study, when the same formulations were co-cultured with MSCs encapsulated in the bottom layer, the difference in gene expression between BLK(MSC) and TGF(MSC) treatments at any time point was not significant (compare Figure 5(a–b) to Figure 3(a–b)). However, when osteogenic precultured cells were seeded in the bottom layer, they seemed to have a synergistic effect with TGF-β1 on chondrogenic differentiation of the cells in top layer, as evidenced by a significant increase in collagen type II gene expression at day 28 and in aggrecan gene expression at days 14 to 28 compared to all the other groups. Additionally, it should also be noted that samples without TGF-β1 loading on the top layer but with osteogenic cells in the bottom layer (group BLK(OS) in Figure 5) demonstrated a higher gene expression in the chondrogenic markers than the samples with TGF-β1 and MSCs at bottom (group TGF(MSC) in Figure 5).

Although there was no significant difference in collagen type I gene expression among formulations at each time point, the trend over time was different between MSC and OS treatments. The samples with MSCs at bottom showed an increase in collagen type I gene expression at later time points as compared to day 0, whereas when the bottom layer contained osteogenic cells, collagen type I gene expression decreased or remained unchanged over time.

DNA content of the top and bottom layers at each time point are shown in Figure 6(a) and Figure 7(a), respectively. For both layers, the results exhibited a decreasing trend over time with significant difference between day 0 and later time points. In the top layer, more DNA was seen in samples with OS cells at bottom than those with just MSCs at bottom. In particular, DNA content in the top layer of the BLK(OS) group was found to be significantly higher than that for the BLK (MSC) or TGF(MSC) group at day 28. Similarly, a higher DNA content was seen in the bottom layer for OS treatment groups compared to MSC treatment groups at each time point, either with or without TGF-β1. There was significantly more DNA in the bottom layer of BLK(OS) or TGF(OS) samples than BLK(MSC) constructs at day 7. No significant difference in DNA content of the bottom layer was observed at day 14 and 28 among formulations.

GAG content, indicative of chondrocytic phenotype, was measured for top layer hydrogels and is shown in Figure 6(b); GAG content was measured to further investigate chondrogenic differentiation of the encapsulated MSCs. For all four formulations, significantly higher GAG/DNA levels were observed at later time points (day 7, 14 and 28) compared to day 0. Significant differences in GAG/DNA values among groups were only found between the BLK(OS) group and both the BLK(MSC) and TGF(MSC) groups. However, this could be due to the artifact of

higher DNA content in the BLK(MSC) group compared to the other two groups at this time point. The results of calcium content in top-layer hydrogels are depicted in Figure 6(c). All the formulations had little calcium deposition (less than 1 μg) in the top layer and the results showed no differences among the groups.

For the bottom layer, ALP enzyme activity and calcium amount were measured to evaluate osteogenic differentiation of the cells, as shown in Figure 7(b) and (c). There was a general trend of increasing ALP activity over time for all the treatments. However, no significant differences were observed among the groups at any time point. A significant increase in ALP amount compared to day 0 was only observed for MSC treatment groups at day 28, likely because OS cells had a higher ALP level at day 0 than MSCs. Compared to the results obtained from the single-layer study [Figure 2(b)], the ALP values in this study were in a similar range.

Calcium content measurements indicated a generally low calcium deposition in the bottom layer of the bilayered scaffolds. At day 14, cells in BLK(OS) group produced 2.04 ± 2.74 μg calcium, which was a little higher than the other treatments. However, due to the large standard deviation, no statistical difference was found among groups or over time.

Discussion

Guiding both osteogenic and chondrogenic differentiation of MSCs in different regions of the same composite scaffold is challenging. We hypothesized that osteogenically precultured MSCs can maintain their phenotype in OPF hydrogel composites that are subsequently cultured with CM containing β-GP. Additionally, we hypothesized that this medium would support chondrogenic differentiation of MSCs encapsulated in the hydrogel composites. These hypotheses were first examined with an osteogenic construct culture study and a chondrogenic culture study using single-layer OPF hydrogel composites.

Bilayered hydrogel composites consisting of a chondrogenic layer and an osteogenic layer were then fabricated and cultured in CM containing β-GP. We specifically investigated: (1) the differentiation of MSCs in both layers of the bilayered hydrogel composites; (2) the effect of cells in the osteogenic layer on MSC chondrogenesis in the chondrogenic layer; (3) the effect of cells in the osteogenic layer combined with TGF-β1-loaded-MPs on MSC chondrogenesis in the chondrogenic layer.

Osteogenic construct culture study

In the osteogenic construct culture study, we developed a protocol for osteogenic differentiation, which involved preculture of MSCs in osteogenic medium in a tissue culture plate, encapsulation of the MSCs in OPF/MPs hydrogel composites and subsequent culture of cell constructs in a chondrogenic environment with β-glycerophosphate.

Our results revealed a decrease in DNA content from day 0 to day 7, which was consistent with previous studies using the same scaffold for chondrogenic and osteogenic differentiation [14, 15]. As previously reported, although the radical initiators were biocompatible at the concentration used in the present study, the encapsulation process may still result in cell death at early time points; cell number remains the same at later time points [14,15]. Alternatively, the decrease in DNA content could be due to the culture in the chemically defined, serum-free, chondrogenic medium. It has been shown that the serum-free condition is beneficial for MSC chondrogenic differentiation but not for cell proliferation [24]. During the process of chondrogenic differentiation, encapsulated MSCs may stop proliferating, resulting in a decrease in cell number. In the present study, after switching the cells to chondrogenic medium for 7 days, we found more DNA in the constructs with osteogenically precultured cells than in those with just MSCs, suggesting a more active role of the osteogenically precultured cells in

the chondrogenic environment. Additionally, during the preculture in the tissue culture plate before encapsulation, we also observed a faster proliferation rate of the cells in the osteogenic medium than that of cells in general medium (data not shown). Based on these observations, we speculate that, during osteogenic preculture, cells may produce growth factors that support cell survival and enhance proliferation.

It has been shown that osteogenic differentiation of MSCs includes three periods: proliferation, extracellular matrix maturation, and mineralization [25]. ALP is considered as an early marker for osteogenic differentiation; it is expressed after cell proliferation and is involved in the onset of extracellular matrix mineralization [25]. The significant increase in ALP activity at days 7 and 14 compared to day 0 suggested that cells continued osteogenic differentiation in the chondrogenic environment, after preculture. This could be attributed to the chemical compounds common to both chondrogenic medium and osteogenic medium, including dexamethasone and ascorbic acid. However, little difference in ALP activity among the groups was observed. In a previous study investigating rabbit MSC osteogenic differentiation in osteoblast-conditioned medium, the authors found similar levels of ALP produced by cells in a control medium compared to cells in conditioned or chemically supplemented medium. They inferred that MSCs have a natural predisposition to differentiate into the osteogenic lineage in the absence of any growth factors and cytokines [26]. Alternatively, the lack of difference in ALP activity among groups could be due to the difficulty in extracting ALP, a cell membrane-associated molecule, from hydrogels, as reported in a similar study involving rat MSC osteogenic differentiation in OPF hydrogels [15].

In contrast to the ALP results, calcium deposition, which is a late marker of osteogenic differentiation, was observed to have higher values in OS3+ and OS6+ samples than in either OS0+ samples or OS6− samples at day 14. This underscores the importance of both osteogenic preculture, and the addition of β-GP to the subsequent culture medium, on cell osteogenic differentiation. A previous review on sequential gene expression during osteoblast differentiation indicated that the onset of mineralization is associated with many factors besides ALP expression, such as co-expression of osteopontin and osteocalcin as well as the maturation of the extracellular matrix (ECM) [25]. Without osteogenic preculture, it is possible that the signals in chondrogenic medium with added β-glycerophosphate were not enough for MSCs to undergo osteogenic differentiation, or that these conditions led to a delay in matrix mineralization, resulting in significantly lower calcium deposition in OS0+ samples compared to OS6+ samples. Similarly, the significant difference in calcium amount between OS6+ and OS6− groups indicates that β-GP could have played an important role in providing further signals for maintaining osteoblastic phenotype and enhancing calcium deposition in chondrogenic medium.

Both ALP and calcium results confirmed that the osteoblastic phenotype was maintained in a chondrogenic environment with the osteogenic supplement β-GP. This protocol provides an exciting new method for using the same culture medium for both chondrogenic and osteogenic differentiation of MSCs in different portions of a scaffold material.

Chondrogenic construct culture study

It has been shown that when MSCs differentiate to chondrocytes, they start to express chondrocyte-specific marker genes such as collagen type II and aggrecan, along with downregulating the gene expression for collagen type I, which is produced by undifferentiated MSCs [21]. RT-PCR results from the chondrogenic construct culture study demonstrated general trends of increasing gene expression of collagen type II and aggrecan, and decreasing gene expression of collagen type I over time, which confirmed the chondrogenic differentiation of MSCs encapsulated in OPF composites and cultured in chondrogenic medium supplemented with β-glycerophosphate in the absence or presence of TGF-β1. Additionally, the effect of

TGF-β1 on MSC chondrogenic differentiation was found to be significant, as evidenced by the earlier appearance of a marked increase in gene expression of chondrogenic markers, as shown in Figure 3(a–b).

DNA results showed a trend of decreasing DNA content over time in all studies, as reported, for instance, in the chondrogenic construct culture study [Figure 4(a)]. Significantly lower DNA content was found in the TGF group compared to the BLK group at day 7, indicating a possible inhibitory effect of TGF-β1 on cell proliferation during the early portion of the culture period of the OPF hydrogel composites. Similar results were also seen in an osteogenic study by Roostaeian et al., in which the addition of TGF-β1 resulted in a 44% decrease in cellular growth of rabbit MSCs when compared to medium alone [27].

GAG production, associated with chondrocytic phenotype of the cultured cells, exhibited higher values for both groups at later time points than at day 0, which together with gene expression results confirmed chondrogenic differentiation, particularly in the presence of TGF-β1 [Figure 4(b), Figure 3]. However, GAG production was comparatively low and there was no statistical difference between groups; this may be because of the tight polymer network surrounding cells, which may have limited the deposition of matrix.

It should be noted that all the cell proliferation, gene expression and matrix production data in this study were comparable to those from a previous study where the same formulations were cultured in chondrogenic medium [28]. This study indicated that the addition of β-GP to chondrogenic medium did not significantly affect the chondrogenic potential of the medium.

Little calcium deposition was observed in the present study, where constructs were cultured in chondrogenic medium supplemented with β-GP, either in the presence or absence of TGF-β1. Although β-GP has been reported to have a stimulatory effect on osteogenesis and calcification, its function with respect to mineralization seems to require the presence of other signals, such as those present in hypertrophic chondrocytes. Coe et al. reported that the addition of 10 mM β-GP to hypertrophic chondrocytes resulted in stimulation of type X collagen synthesis and the onset of mineralization. In contrast, the addition of β-GP to non-hypertrophic chondrocytes failed to induce expression of type X collagen or to induce calcium production [29]. Alternatively, serum could also be an important factor that synergizes with β-GP to induce calcification. Calcium deposition was noted when MSCs were encapsulated in OPF hydrogels and cultured in osteogenic medium, which has similar components to the medium used in the present studies, but also has serum [15]. Although an osteogenic supplement, β-GP, was utilized as one of the medium components in the present study, the results indicated that the chemically defined, serum-free medium seemed to provide a favorable environment for chondrogenic differentiation, without eliciting calcium deposition.

Osteochondral construct culture study

Combining the results from the osteogenic and chondrogenic construct culture studies, we hypothesized that chondrogenic medium with β-GP would support both chondrogenic differentiation and osteogenic differentiation of MSCs in OPF/MP composites *in vitro*. In the osteochondral construct culture study, we fabricated bilayered composites consisting of a cartilage layer and a bone layer.

Chondrogenic differentiation of the MSCs in the top (chondrogenic) layer was evaluated via gene expression of chondrocytic markers, collagen type II and aggrecan, as well as a MSC-associated marker, collagen type I. As expected, the results showed upregulation of chondrogenic markers and downregulation of collagen type I during the culture period for all the formulations, which further confirmed the chondrogenic potential of the culture medium.

By comparing gene expression data from the MSC and OS treatment groups, it was apparent that the addition of osteogenic cells in the bottom layer of the composites enhanced the chondrogenic differentiation of the MSCs in the top layer. Samples without TGF-β1 in the top layer, but with osteogenic cells in the bottom layer [BLK(OS)], demonstrated either similar or higher gene expression of chondrogenic markers and a lower gene expression of collagen type I, compared to the samples with TGF-β1 loaded MPs in the top layer and MSCs in the bottom layer. Thus, it is likely that the osteogenic cells in the bottom layer of the BLK(OS) group produced chondro-inductive signals and had a positive effect on the differentiation of the cells in the top layer. In fact, studies in our laboratory have shown that rat MSCs cultured in osteogenic medium either express genes for or secrete many molecules of bioactive factors related to cartilage and bone formation, including TGF-β1, fibroblastic growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), bone morphogenetic protein 2 (BMP-2), and insulin-like growth factor 1 (IGF-1) [30,31]. These paracrine signals could have contributed to the chondrogenic differentiation of the cells in the top layer.

Similarly, bioactive molecules secreted by osteogenic cells in bilayered constructs may also explain the synergistic effect of OS cells and TGF-β1 on chondrogenic differentiation of MSCs in the top layer. Numerous studies have reported that the combination of TGF-β1 with other growth factors such as IGF-1, FGF-1, or BMP-2, has a synergistic effect on MSC chondrogenic differentiation *in vitro*, probably due to regulatory cross-talk between the signaling cascade of each growth factor [32–35].

In contrast, when MSCs were encapsulated in the bottom layer of the bilayered hydrogel, the effect of TGF-β1 on cell differentiation in the top layer was not as obvious as in the chondrogenic construct culture study. In that single-layer study, where the same formulations as the top layers of the osteochondral construct culture study were cultured, the TGF group showed significantly higher collagen type II and aggrecan gene expression than the MSC group. Since the same amount of TGF-β1 was loaded in the single-layer and bilayered constructs, the addition of a bottom layer with more MSCs (in the bilayered construct) could have resulted in each cell experiencing a lower TGF-β1 concentration as compared to the conditions in the single-layer hydrogel. A previous study has shown that with a lower dosage of TGF-β1, gene expression of collagen type II and aggrecan by MSCs declined in OPF/MP composites [14].

The incorporation of OS cells in the bottom layer affected not only MSC chondrogenic differentiation in the top layer, but also influenced cell number for both the top and bottom layers. As shown in Figure 7(a), more cells were present in the bottom layer of the hydrogels for OS groups than MSC groups at day 7, which was in accordance with the results obtained from the osteogenic construct culture study [Figure 2(a)]. The results of both studies indicated that after *in vitro* osteogenic preculture, the cells were more resilient than plain MSCs, and were able to survive the crosslinking procedure better and differentiate in OPF hydrogels. Additionally, the precultured cells in the bottom layer could have produced bioactive molecules that supported cell proliferation in the top layer through a paracrine effect, as speculated before. This was evidenced by higher DNA content in top layer hydrogels for OS treatment groups than MSC treatment groups at each time point [Figure 6(a)]. Similar results were also found in a study by Nakaoka et al., in which they demonstrated that proliferation and differentiation of chondrocytes were enhanced by soluble factors produced from osteoblasts in a co-culture system with both direct and indirect contact [36].

In fact, when comparing DNA results from the top and bottom layers to their corresponding single-layer culture results (chondrogenic and osteogenic construct culture studies, respectively), we found the values to be comparable. The results suggested that the increase in thickness of bilayered hydrogels did not affect cell growth, which further underscored the advantages of an OPF/MP crosslinked network for nutrient transport and cell growth.

GAG production in the top-layer hydrogels also exhibited similar values to those obtained in the single-layer study for the same formulations [Figure 4(b) and Figure 6(b)]. The increased GAG production compared to day 0 provided additional evidence of MSC chondrogenic differentiation. It is also important to see that no calcium was deposited in this layer under the influence of the osteogenic cells in the bottom layer [Figure 6(c)].

The continued osteogenic differentiation of the bottom-layer cells was confirmed by the increased amount of ALP over time and the presence of some calcium deposits in the constructs [Figure 7(b–c)]. Although numerous studies have reported that chondrocytes have a positive effect on the osteogenic differentiation of MSCs in co-culture systems [37, 38], similar values for ALP secretion and calcium deposition were observed in our bilayered study and single-layer study for either MSCs or precultured cells (Figure 2 and 6), suggesting a limited effect of the top-layer cells on the osteogenic differentiation of the bottom-layer cells. Since the MSCs in the top layer were undergoing chondrogenic differentiation into chondrocyte-like cells, the bioactive factors produced by these cells could be limited in amount and thus explain the limited effect of these cells on osteogenic differentiation. Additionally, TGF-β1 released from MPs in the top layer might have inhibited calcium deposition, explaining the difference between the calcium amount in the BLK(OS) group and the TGF(OS) group. Previous studies have also reported a similar inhibitory effect of TGF-β on osteogenic differentiation of both MSCs and osteoblasts differentiated from MSCs, as evidenced by reduced ALP activity and mineral deposition [39].

Conclusions

In the current work, we developed a set of culture conditions, which not only promote chondrogenic differentiation of MSCs encapsulated in OPF hydrogel composites, but which also maintain the osteoblastic phenotype of precultured MSCs encapsulated in the same hydrogel composites. A bilayered osteochondral construct containing a chondrogenic layer and an osteogenic layer was fabricated using OPF hydrogel composites containing gelatin MPs and MSCs. The culture of the bilayered constructs showed that MSCs were able to undergo chondrogenic differentiation in the cartilage layer. Although calcium deposition in the bone layer was limited, the osteogenic cells in this layer enhanced MSC chondrogenic differentiation in the cartilage layer. Most importantly, osteogenic cells in the bone layer exhibited a synergistic effect with TGF-β1 loaded MPs, promoting chondrogenesis in the cartilage layer, as evidenced by enhanced levels of gene expression for collagen type II and aggrecan. The present study demonstrated the fabrication of bilayered osteochondral constructs using biodegradable hydrogel composites for the co-delivery of growth factors and MSCs for cartilage tissue engineering.

Acknowledgments

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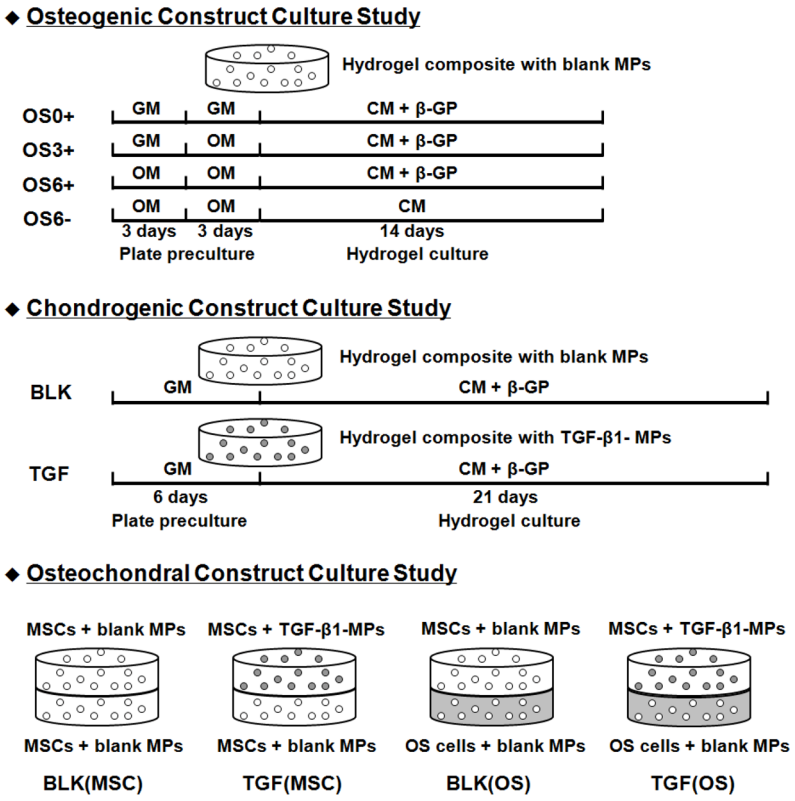

Figure 1.

A schematic representation of the overall experimental design. Four groups were included in the osteogenic construct culture study, namely OS0+, OS3+, OS6+ and OS6− groups. Two groups were included in the chondrogenic construct culture study, namely BLK and TGF groups. In the osteochondral construct culture study, four groups were included, namely BLK (MSC), TGF(MSC), BLK(OS), and TGF(OS) groups. CM: chondrogenic medium; GM: general medium; β-GP: β-glycerophosphate; MPs: gelatin microparticles; OM: osteogenic medium; OS: osteogenic; TGF-β1: transforming growth factor-β1.

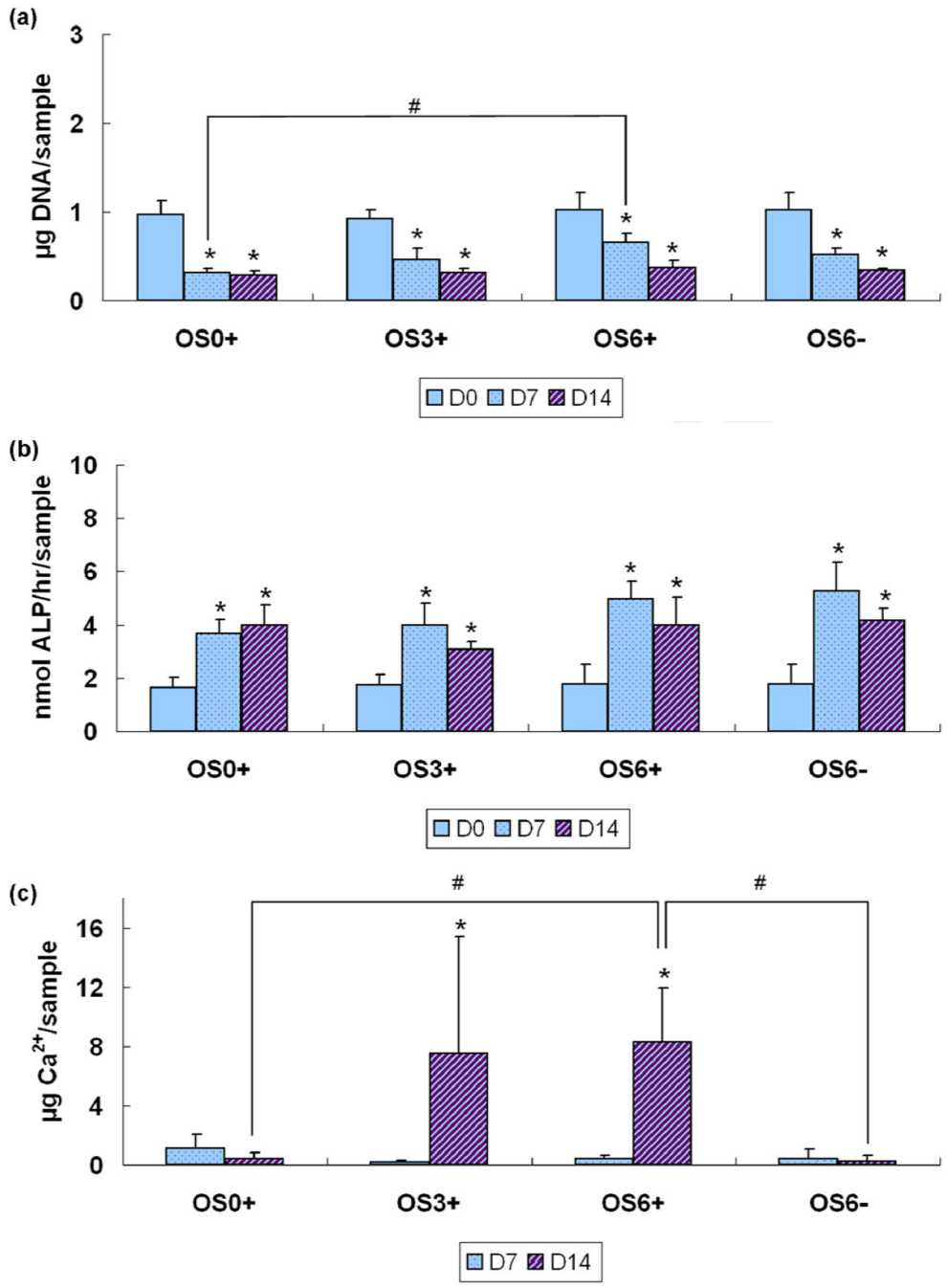

Figure 2.

Biochemical assay results for single-layer OPF/MP hydrogel composites encapsulating rabbit marrow MSCs (OS0), 3-day osteogenically precultured MSCs (OS3) or 6-day osteogenically precultured MSCs (OS6); all composites were cultured in chondrogenic medium supplemented with (+) or without (−) β-glycerophosphate. DNA content (a), ALP enzyme activity (b), and calcium content (c) of the samples are presented. Within a given group, a significant difference ($p<0.05$) compared to day 0 value (day 7 value for calcium assay) is noted with (*). Samples indicated with (#) had a significant difference from the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

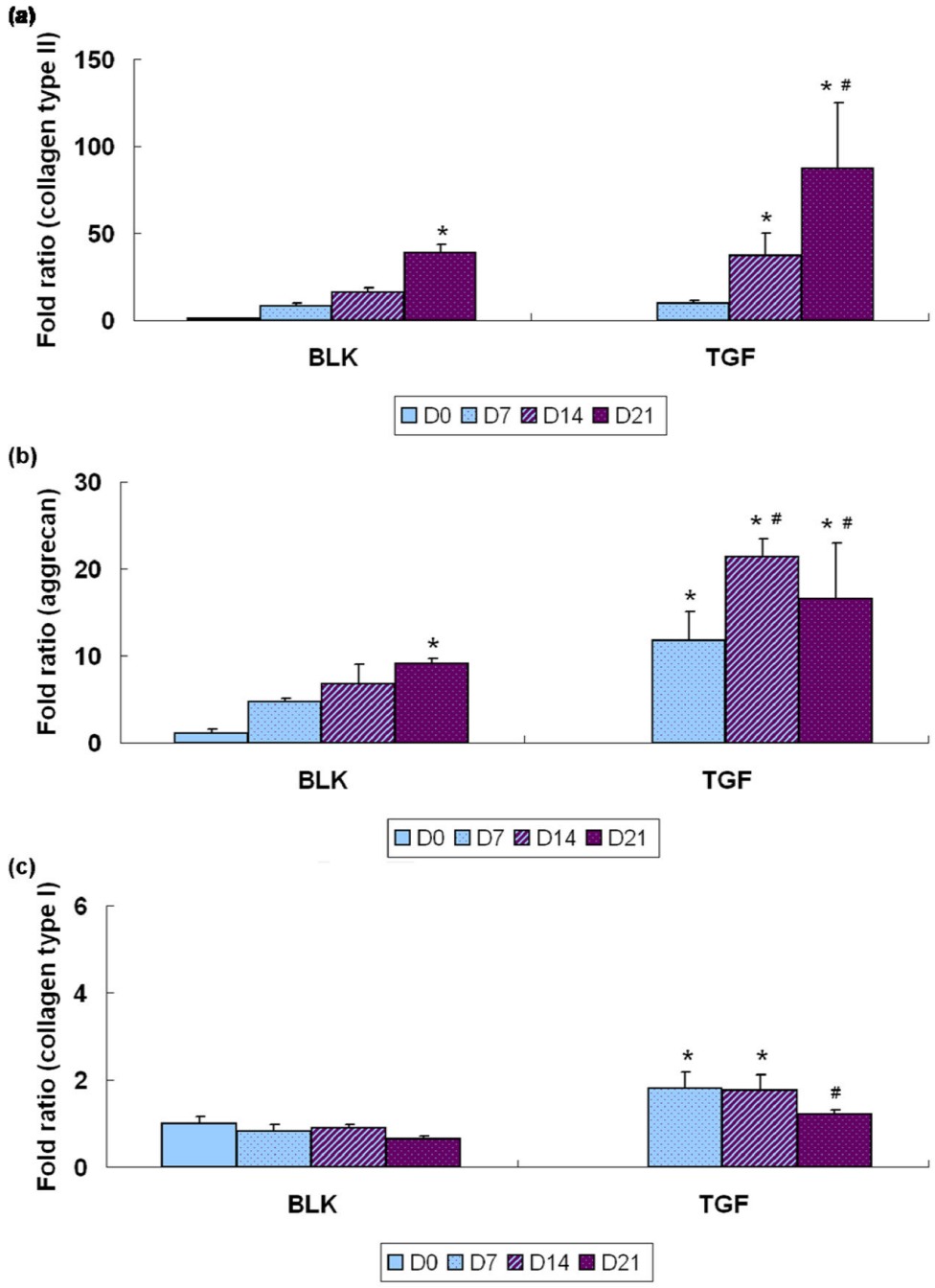

Figure 3.

Quantitative gene expression of collagen type II (a), aggrecan (b), and collagen type I (c) for hydrogel composites encapsulating rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF). Data are presented as a fold ratio after being normalized to GAPDH expression. The expression level of controls (Day 0, BLK group) is represented as one. Within a given group, a significant difference in gene expression ($p<0.05$) compared to day 0 value (control) is noted with (*). Samples indicated with (#) had a significantly higher gene expression than the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

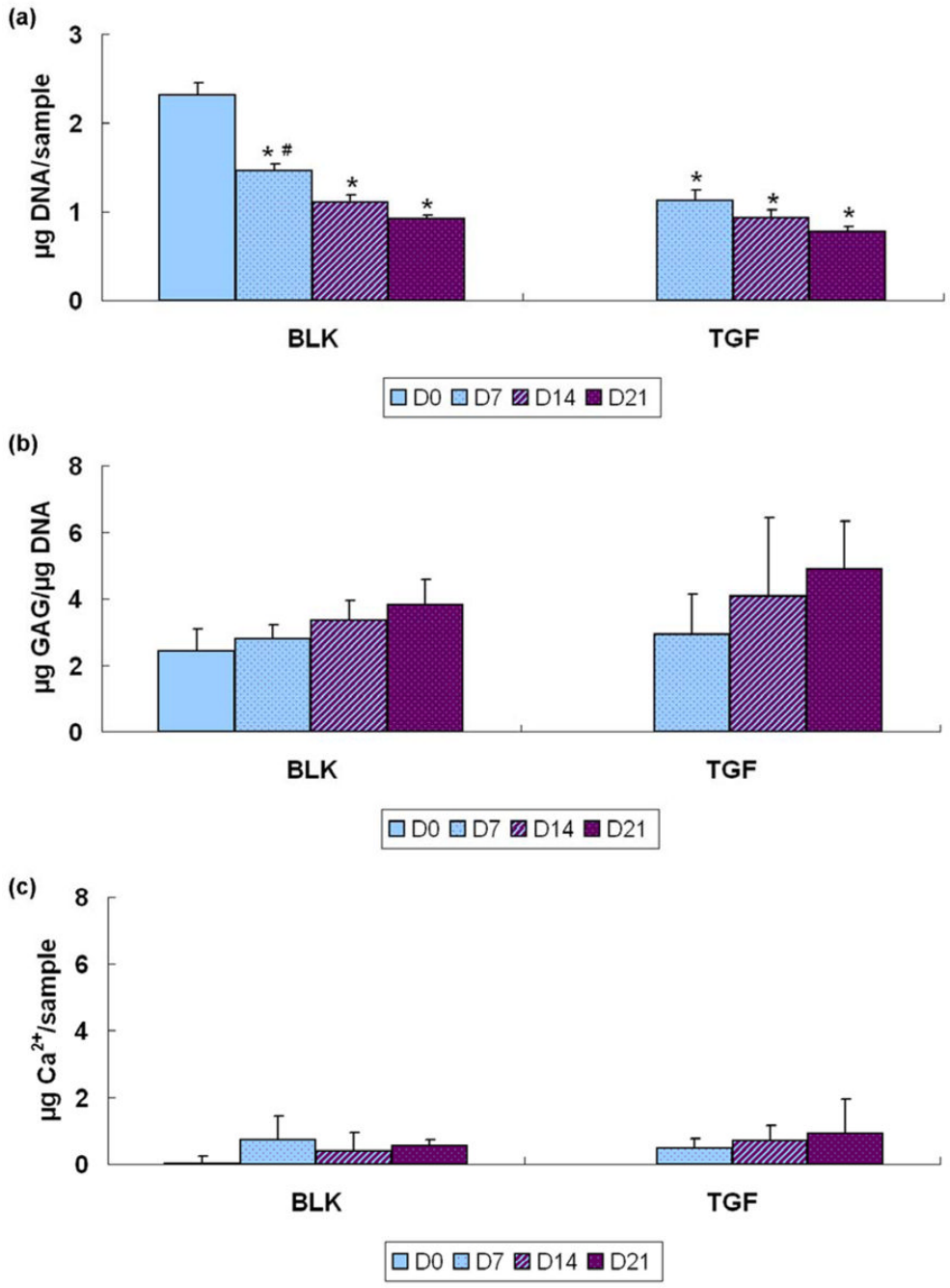

Figure 4.

Biochemical assay results for single-layer OPF hydrogel composites encapsulating rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF). DNA content (a), GAG content normalized to μg DNA (b), and calcium content (c) of the samples are presented. Within a given group, a significant difference ($p<0.05$) compared to day 0 value is noted with (*). Samples indicated with (#) had a significant difference from the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4. The BLK and TGF groups shared samples at day 0.

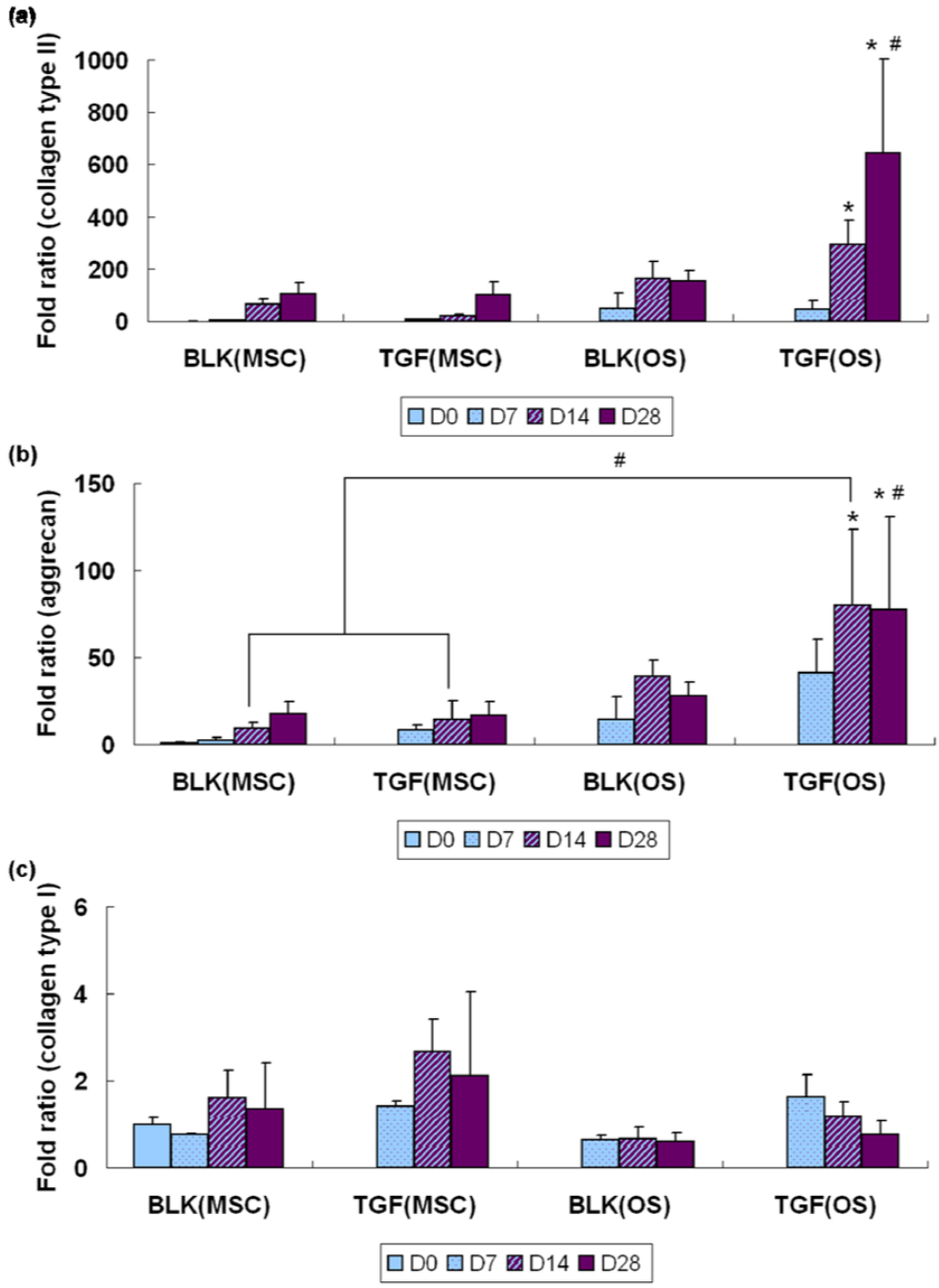

Figure 5.

Quantitative gene expression of collagen type II (a), aggrecan (b), and collagen type I (c) for the top layer of the bilayered OPF hydrogel composites. The top layer of each composite contained encapsulated rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF), which were co-cultured with a bottom layer consisting of an OPF hydrogel/blank MPs composite encapsulating MSCs (MSC) or osteogenic cells (OS). Data are presented as a fold ratio after being normalized to GAPDH values. The expression level of controls [Day 0, BLK(MSC) group] is represented as one. Within a given group, a significant difference in gene expression ($p<0.05$) compared to day 0 value (control) is noted with (*). Samples indicated with (#) had a significantly higher gene expression than the other

groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

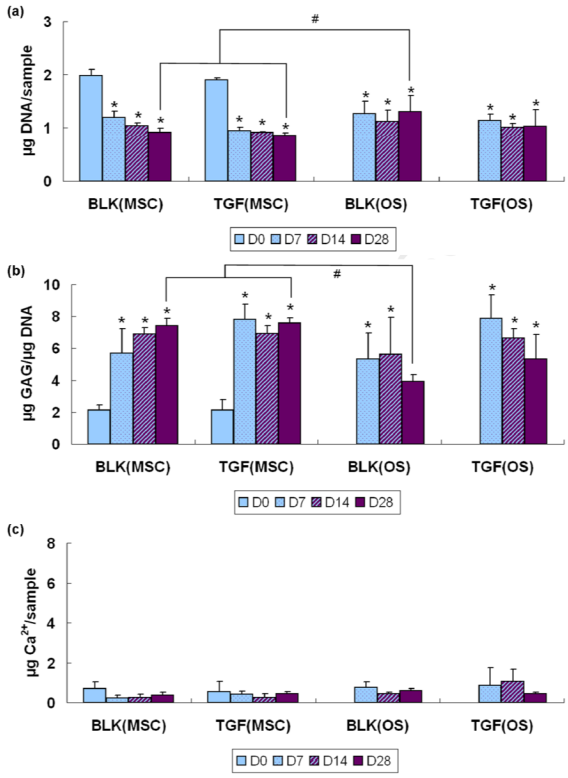

Figure 6.

Biochemical assay results for top layer of the bilayered hydrogels. The top layer of each composite contained encapsulated rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF), which were co-cultured with a bottom layer consisting of an OPF hydrogel/blank MPs composite encapsulating MSCs (MSC) or osteogenic cells (OS). DNA content (a), GAG content normalized to μg DNA (b) and calcium content (c) of the samples are presented. Within a given group, a significant difference in gene expression ($p<0.05$) compared to the day 0 value is noted with (*). Samples indicated with (#) had a significantly higher gene expression than the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4. At day 0, the BLK(MSC) and BLK (OS) groups shared top-layer samples for all biochemical assays; and similarly the TGF(MSC) and TGF(OS) groups shared samples.

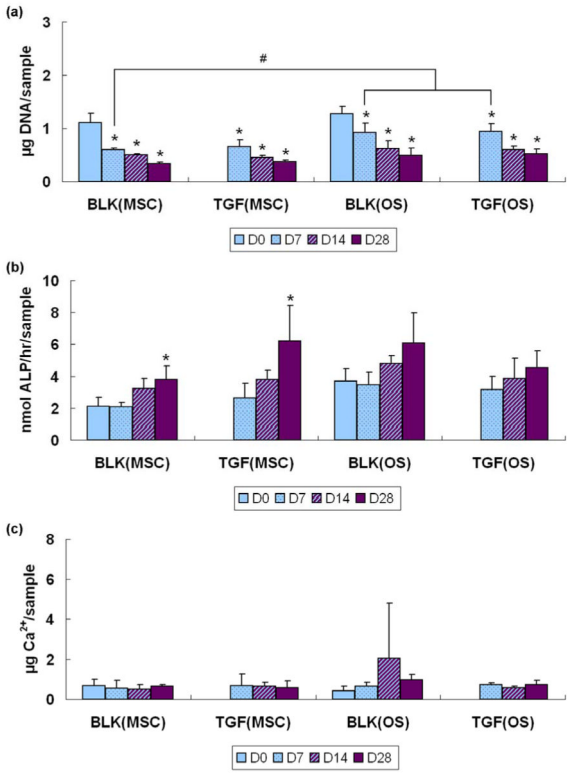

Figure 7.

Biochemical results for bottom layer of the bilayered hydrogels. The bottom layer of each composite consisted of an OPF hydrogel/blank MPs composite encapsulating MSCs (MSC) or osteogenic cells (OS); these were co-cultured with a top layer containing encapsulated rabbit marrow MSCs and blank MPs (BLK) or rabbit marrow MSCs and TGF-β1-loaded MPs (TGF). DNA content (a), ALP enzyme activity (b) and calcium content (c) of the samples are presented. Within a given group, a significant difference in gene expression ($p<0.05$) compared to the day 0 value is noted with (*). Samples indicated with (#) had a significantly higher gene expression than the other groups at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4. At day 0, the BLK(MSC) and TGF(MSC) groups shared bottom-layer samples for all biochemical assays; and similarly the BLK(OS) and TGF(OS) groups shared samples.