In vitro osteogenic differentiation of marrow stromal cells encapsulated in biodegradable hydrogels

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Received 1 October 2003; revised 10 March 2004; accepted 10 March 2004
Published online 25 May 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30064

Abstract: Novel hydrogel materials based on oligo(poly(ethylene glycol) fumarate) (OPF) crosslinked with a redox radical initiation system were recently developed in our laboratory as injectable cell carriers for orthopedic tissue engineering applications. The effect of OPF hydrogel material properties on *in vitro* osteogenic differentiation of encapsulated rat marrow stromal cells (MSCs) with and without the presence of osteogenic supplements (dexamethasone) was investigated. Two OPF formulations that resulted in hydrogels with different swelling properties were used to encapsulate rat MSCs (seeding density ~13 million cells/mL, samples 6 mm diameter × 0.5 mm thick before swelling) and osteogenic differentiation in these constructs over 28 days *in vitro* was determined via histology and biochemical assays for alkaline phosphatase, osteopontin and calcium. Evidence of MSC differentiation was apparent over the culture period for samples without dexamethasone, but there was large variability in calcium production between constructs using cells of the same source. Differentiation was also seen in samples cultured with osteogenic supplements, but calcium deposition varied depending on the source pool of MSCs. By day 28, osteopontin and calcium results suggested that, in the presence of dexamethasone, OPF hydrogels with greater swelling promoted embedded MSC differentiation over those that swelled less (43.7 ± 16.5 μg calcium/sample and 16.4 ± 2.8 μg calcium/sample, respectively). In histological sections, mineralized areas were apparent in all sample types many microns away from the cells. These experiments indicate that OPF hydrogels are promising materials for use as injectable MSC carriers and that hydrogel swelling properties can influence osteogenic differentiation of encapsulated progenitor cells. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 235–244, 2004

Key words: thermal crosslinking; PEG; hydrogel; marrow stromal cells; cell encapsulation; cellular differentiation; bone tissue engineering

INTRODUCTION

A promising tissue-engineering approach for restoration of orthopedic defects is the use of hydrogels as cell carrier materials to deliver cells directly to localized areas of injury. These hydrogel materials should be injectable, so constructs could be implanted minimally invasively, and biodegradable, to allow space for neotissue growth.[1] Previously, natural injectable materials, including fibrin[2,3] and alginates,[4,5] were examined as hydrogels for cell encapsulation. However, concerns with possible immune rejection, pathogen transfer, and availability of large quantities of natural materials have led others to study synthetic injectable materials as cell carriers.[1]

Synthetic materials can be easily mass-produced and, through altering synthesis parameters, their physical, mechanical, and degradative properties can be tailored for specific applications.[6] An example of a synthetic material possessing these advantages is oligo(poly(ethylene glycol) fumarate) (OPF), a novel oligomer recently developed in our laboratory.[7] Prior studies with OPF have shown that it is biodegradable[8,9] and that, depending on the choice of oligomer type and crosslinking molecules, the mechanical and degradative properties of these hydrogels can be altered in a controlled manner.[8,10,11] Recently, we showed that this system could be crosslinked under cytocompatible conditions to form gels with signifi-

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Contract grant sponsor: National Institutes of Health; contract grant number: R01 AR48156

Contract grant sponsor: Whitaker Foundation Graduate Fellowship (JST)

cantly different swelling properties.[9] Rat marrow stromal cells (MSCs) were then encapsulated in OPF hydrogels and cultured for 28 days in osteogenic media. Histology results indicated that calcified extracellular matrix (ECM) was formed over this time period and that mineralized areas were found throughout the hydrogel by day 28.[9]

Previous work with chondrocyte encapsulation in similar synthetic hydrogels indicated that swelling and degradative properties can influence the location of the ECM as well as the ratio of various ECM molecules produced by embedded cells.[12,13] Therefore, in this study, OPF-based hydrogels with different swelling properties were used to examine the effects of hydrogel material properties on MSC differentiation, as well as the location of the resulting ECM.

Because MSCs have shown the potential to differentiate into multiple cell types,[14] the use of OPF as a carrier for MSCs is envisioned to aid regeneration of a variety of orthopedic tissues. However, the experiments described here focus only on the osteogenic differentiation of encapsulated rat MSCs. The addition of glucocorticoids, such as dexamethasone, in cell culture media are known to induce osteogenic differentiation of MSCs in both rats and humans.[15–19] Thus, the impact of hydrogel material properties on *in vitro* osteogenic differentiation with and without the presence of osteogenic supplements was explored in this study. Specifically, the effects of two OPF formulations with significantly different fold swelling (OPF 10K and OPF 3K) on the differentiation of encapsulated rat MSCs cultured for 28 days *in vitro* with and without the addition of dexamethasone were determined via histology and biochemical assays for matrix molecules associated with bone.

MATERIALS AND METHODS

OPF synthesis and characterization

Two formulations of OPF were synthesized from poly(ethylene glycol) (PEG) of different nominal number average molecular weight: 10,000 g/mol (designated OPF 10K) or 3,300 g/mol (OPF 3K), following established procedures.[7,11] The OPF was then characterized with gel permeation chromatography (GPC; column 50–100,000-Da range; Waters Model 410, Milford, PA). The molecular weight of both the PEG starting material and the resulting OPF was determined (three samples of each type). The final product was stored at −20°C until use.

Marrow stromal cell (MSC) isolation and preculture

MSCs were isolated from male Wistar rats (~150 g) as previously described.[20] The MSCs were then seeded in T-75 flasks and precultured for 1 wk, with media changes after days 1 and 4 to remove nonadherent cells. Four-cell isolation procedures were performed, each time using three rats. Two of these isolations (I + 1 and I + 2) were precultured in media containing dexamethasone (DMEM high glucose; Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum (Gemini, Calabasas, CA), 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 50 mg/L ascorbic acid, 250 μg/L fungizone, 100 mg/L ampicillin, and 50 mg/L gentamicin (all from Sigma-Aldrich), whereas the other two isolations (I − 1 and I − 2) were precultured in identical media without dexamethasone.

MSC encapsulation and culture

After preculture, the MSCs were encapsulated in sterilized OPF 10K or 3K with poly(ethylene glycol) diacrylate (PEG-DA; Nektar Therapeutics, Huntsville, AL) as a crosslinking agent and 25 mM ammonium persulfate/N,N,N′,N′,-tetramethylethylenediamine (APS/TEMED) as thermal radical initiators, as described previously.[9] After the polymer solution was mixed with initiators, the cells were added at ~13 million/mL and the mixture was quickly injected in presterilized Teflon molds (6 mm diameter by 0.5 mm thick). After 8 min in an incubator, the crosslinked gels were aseptically transferred to 12-well plates, and 2.5 mL of the appropriate media were added. Over the following 3 h, the media were changed twice to ensure the removal of soluble hydrogel precursors.

During the 28-day culture period, the media were changed every 2–3 days. After 1, 7, 14, 21, and 28 days of culture, samples were removed for biochemical (n = 4, except day 28 OPF 3K without dexamethasone n = 3) and histological analysis (n = 3). Media were collected at every media change from the 28-day samples and stored at −20°C for later analysis for osteopontin secretion (n = 3). Samples containing cells from I − 1 and I + 1 were used for time points representing days 1, 7, 14, and 21, whereas samples containing cells from I − 2 and I + 2 were used for time points representing days 1, 7, 21, and 28.

In addition, OPF 10K and 3K hydrogels without cells were made as described above, except with the addition of phosphate-buffered saline (PBS; Gibco) instead of the cell suspension. These blank hydrogels, cultured in media with and without dexamethasone, were subject to biochemical analysis at the same time points as the samples containing MSCs.

Biochemical assays for MSC differentiation

At the given time points, the samples and hydrogel blanks were removed from culture, rinsed in PBS and homogenized with a pellet grinder (Fisher Scientific) in 500 μL ddH_2O. The samples were stored at −20°C until just before analysis, when they were subjected to three freeze-thaw cycles including sonication with ice for 30 min after each cycle. Except for the osteopontin assay, each sample (n = 3–4 per treatment per assay per time point) was run in triplicate. In

the case of osteopontin, each sample (n = 3 per treatment per time point) was run in duplicate.

Cell number

Homogenates underwent analysis for double-stranded DNA content using the PicoGreen assay (Molecular Probes, Eugene, OR) as per manufacturer's instructions.[21] The fluorescence of blank gels was subtracted to determine the amount of DNA/sample from the standard curve. Known numbers of MSCs from multiple isolations were also analyzed with this method to determine the amount of DNA/cell (3.46 pg/cell), which was used subsequently as a conversion to cell number for each sample.

Alkaline phosphatase (ALP)

The amount of alkaline phosphatase present in each homogenized sample was determined by using Sigma Diagnostic Kit 104 according to manufacturer's instructions.[21] Absorbance of the samples was adjusted with blank controls.

Osteopontin (OPN)

Media samples were analyzed for osteopontin content via a sandwich immunoassay (Assay Designs, Ann Arbor, MI) as per manufacturer's instructions. Media from samples both with and without MSCs were pooled to represent 1–7 days, 8–14 days, 15–20 days, and 21–28 days in culture.[21] Samples from blank hydrogels showed no sign of OPN content, so no adjustment to the absorbance of the remaining samples was necessary.

Calcium

Before analysis of calcium content for each homogenate, 0.5 N acetic acid (Fisher Scientific) was added to the samples, and they were placed on a shaker table overnight to dissolve mineral deposits. Calcium amount/sample was determined by using Sigma Diagnostic Kit 587, according to manufacturer's instructions.[21] The samples were adjusted with blank controls before calculation of the calcium content.

Histology

Histology specimens were prepared as described previously.[9] Briefly, they were fixed in 10% neutral buffered formalin (Sigma-Aldrich), serially dehydrated in ethanol, paraffin-embedded, and cut in 12-μm-thick cross sections (Microm, Walldorf, Germany). The sections were subsequently stained with Von Kossa reagent (5% w/v, Sigma-Aldrich) to visualize the mineralized matrix (black) and counterstained with Safranin-O (0.5% w/v, Sigma-Aldrich; cells and polymer appear red). Images were acquired with a light microscope (Eclipse E600; Nikon, Melville, NY) and attached video camera (3CCD Color Video Camera DXC-950P; Sony, Park Ridge, NJ).

Statistical analysis

Data from all studies were analyzed by using ANOVA and Tukey's multiple-comparison test or an unpaired Student's *t*-test ($p \leq 0.05$). The results for the DNA, ALP, and calcium assays were log-transformed before statistical analysis due to large variances in the data. Results are reported as means ± standard deviation.

RESULTS

Oligo(poly(ethylene glycol) fumarate) (OPF) synthesis and characterization

GPC analysis revealed that the OPF 10K was synthesized from PEG with number-average molecular weight (M_n) 13,650 ± 860 and weight-average molecular weight (M_w) 15,710 ± 970. The resulting oligomer had M_n of 24,250 ± 1750 and M_w of 54,260 ± 2000. For OPF 3K, the initial PEG had M_n of 4040 ± 210 and M_w of 4330 ± 220, whereas the oligomer showed M_n of 11,900 ± 1160 and M_w of 41,010 ± 2170.

Biochemical assays for marrow stromal cell (MSC) differentiation

For ease of comparison, in the following text, the treatments used in this study will be referred to as 10K− (OPF 10K hydrogel cultured without dexamethasone), 3K− (OPF 3K hydrogel cultured without dexamethasone), and 10K+ and 3K+ (corresponding hydrogels cultured with dexamethasone).

Cell number

Cell number/sample at each time point is depicted in Figure 1. Although a general trend of decreasing cell number/sample seems apparent, a statistically significant decrease in cell number was found only in the 10K+ day 28 samples compared with the rest of the time points for that treatment. In addition, at day 28, the 10K+ samples were determined to have significantly fewer cells than the other treatments.

Alkaline phosphatase (ALP)

ALP results over time are presented in Figure 2. Statistical analysis revealed significantly higher ALP

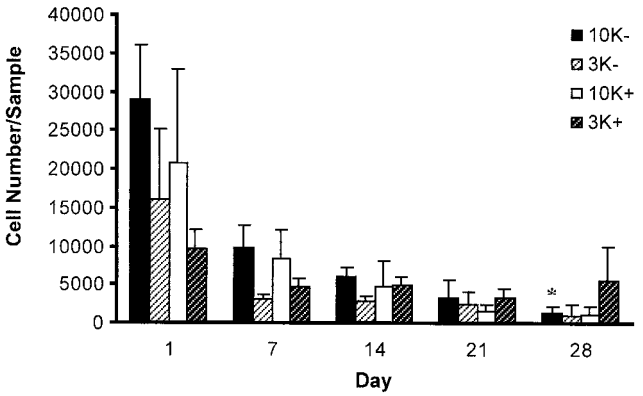

Figure 1. Cell number per sample for OPF-MSC constructs after various days in culture (± standard deviation, n = 3–4). *Indicates that, at day 28, 10K− samples had significantly fewer cells than the same sample type at the previous time points and fewer cells than the other treatments for this time point ($p \leq 0.05$).

values for the 10K+ and 3K+ samples at day 28 than the rest of the time points. There was no significant change in ALP for the nonsupplemented samples over the culture period. At day 1, the 10K− constructs showed significantly higher ALP levels than the 3K+.

Osteopontin (OPN)

Results of the OPN immunoassay are found in Figure 3. A statistically significant increase in total OPN secretion was seen with each time point for the 10K− samples, whereas there was no difference in total OPN over time for the 3K− treatment. OPN amounts at day 7 were significantly lower than at day 20 or 28 for the 10K+ constructs, but there was no significant increase in levels from days 14 to 28. Similarly, for the 3K+ samples, there was a significant increase in total OPN

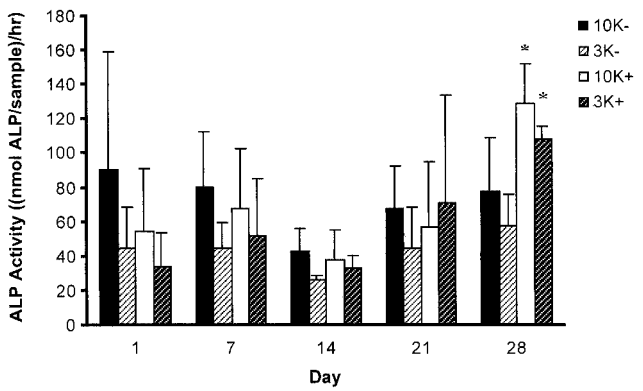

Figure 2. Alkaline phosphatase activity per sample for OPF-MSC constructs after various days in culture (± standard deviation, n = 3–4). *Indicates that, at day 28, 10K+ and 3K+ samples showed significantly higher ALP levels than for the same sample types at previous time points ($p \leq 0.05$).

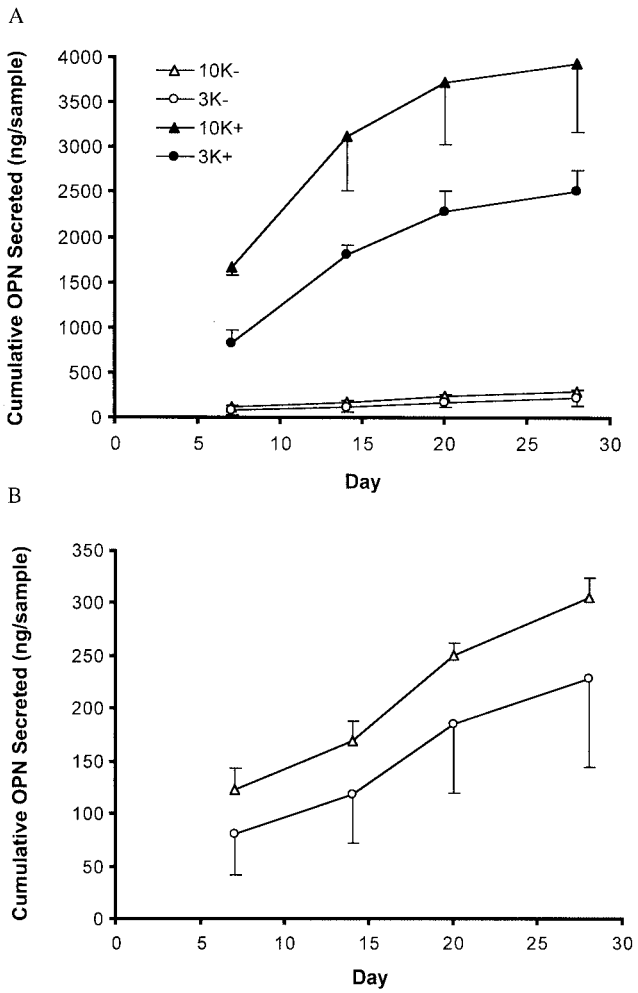

Figure 3. Cumulative osteopontin secretion per sample for OPF-MSC constructs over 28 days in culture (± standard deviation, n = 3). (A) All sample types. (B) Samples cultured without dexamethasone (10K− and 3K− only).

up to day 20, but the levels on days 20 and 28 were found to be statistically equivalent.

At all time points tested (days 7, 14, 20, and 28), the same statistical trends were found between treatments. There was no significant difference between the OPN levels of the 10K− and 3K− samples, and the nonsupplemented samples always exhibited lower OPN amounts than either the 10K+ or the 3K+. In addition, the 10K+ constructs showed greater OPN secretion than the 3K+ constructs at all time points.

Calcium

Calcium content of the constructs at various time points is presented in Figures 4 and 5. For both the 10K− and 3K− samples, there was significantly more calcium deposition at day 28 than at the rest of the time points. The calcium levels for the 10K+ constructs were significantly higher at days 21 and 28

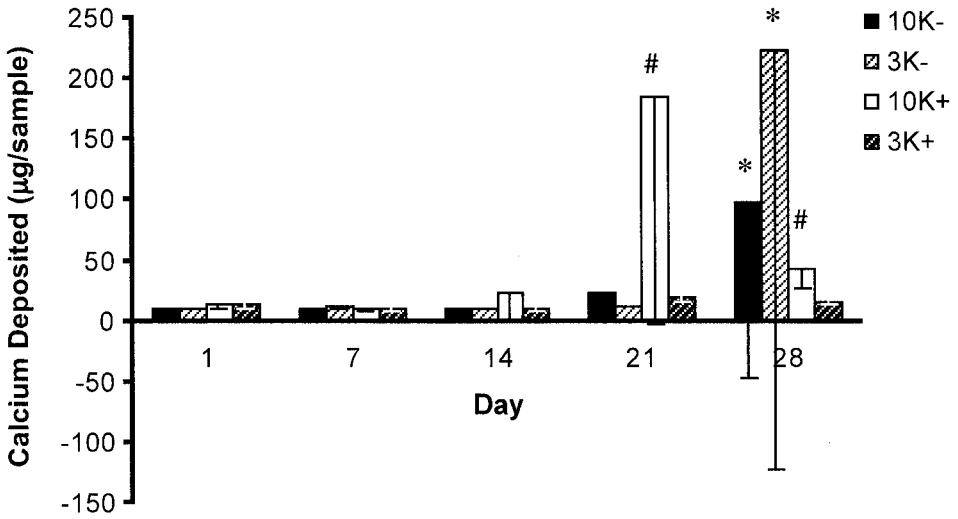

Figure 4. Calcium content per sample for OPF-MSC constructs after various days in culture (± standard deviation, n = 3–4). *Indicates that these constructs showed significantly higher calcium deposition than the same sample type on day 1 or day 7. #Indicates that the 10K+ samples exhibited more calcium accumulation than 10K+ constructs at days 1 and 7 and than 3K+ samples at days 21 or 28 ($p \leq 0.05$).

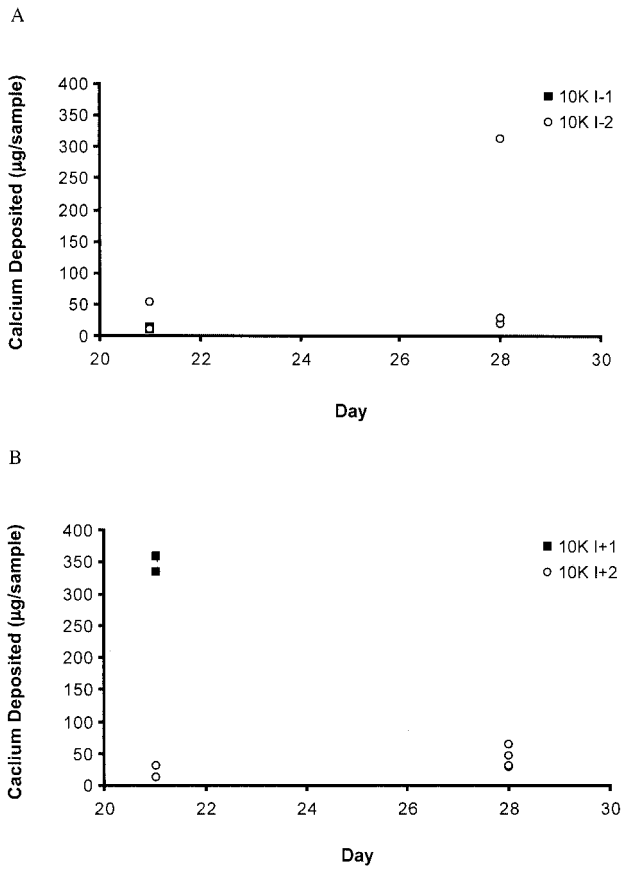

Figure 5. Calcium content per sample for 10K OPF-MSC constructs after 21 and 28 days in culture, depicted with reference to particular cell isolation. (A) Results from the two isolations cultured without dexamethasone. (B) Results from the two isolations cultured with dexamethasone.

than at days 1 and 7. In addition, there was a significant increase in calcium between days 14 and 21. However, there was no difference in calcium levels between days 21 and 28 for this treatment. There was also no significant change in calcium for the 3K+ constructs over the culture period.

The data were further examined at days 21 and 28, when significant calcium deposition had occurred for many sample types. At day 21, the 10K+ constructs were found to have produced more calcium than the other samples. There was no significant difference in calcium level between the 3K− and 3K+ samples at this time point. At day 28, there was no significant difference in calcium amount between the 10K− and 3K− samples, and there was no difference between levels for these samples and that of the 10K+. The 10K− produced more calcium than the 3K+ constructs, and the 3K+ calcium levels were significantly lower than for the 3K− samples. The 10K+ constructs exhibited a significantly higher calcium content than the 3K+ constructs at this time point.

Histology

Images showing representative cross sections from all treatments at days 7 and 21 of *in vitro* culture are found in Figure 6. Figure 7 depicts similar images from day 28. In these samples, the polymer (labeled P) is red-pink, nonmineralized cells are red, and mineralized matrix (M) appears brown-black (see online issue for color images). Arrows indicate the location of some of the cells found throughout the hydrogels. Histological processing resulted in tearing of certain

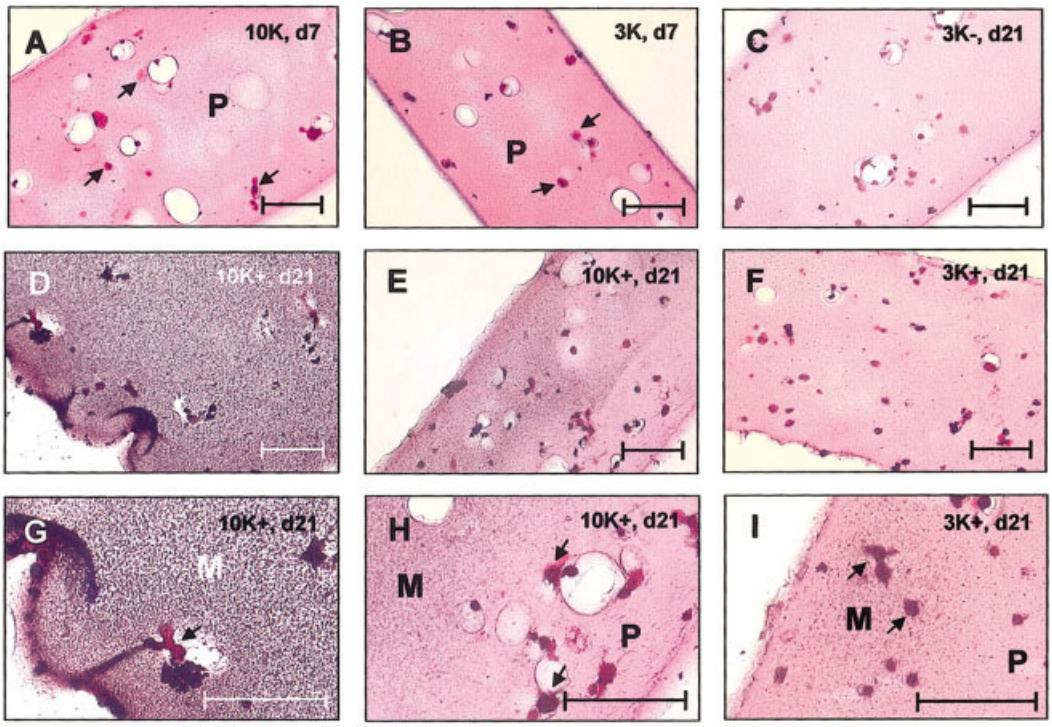

Figure 6. OPF-MSC constructs after 7 and 21 days of *in vitro* culture. Polymer is labeled P, mineralized matrix is labeled M, and arrows indicate examples of embedded cells. All 10K and 3K samples looked similar to (A) and (B), respectively, at day 7. (C) depicts 3K− hydrogels (I−1) at day 21. All 10K and 3K hydrogels with cells from either isolation cultured without dexamethasone (I−1 or I−2) appeared similar at this time point. (D) and (G) represent 10K+ hydrogels with cells from I+1 at day 21. (E) and (H) represent 10K+ hydrogels with cells from I+2 at day 21. (F) and (I) represent cells from either isolation (I+1 or I+2) used in 3K+ samples at day 21. (See Discussion section for definitions of abbreviations.) Original magnification for (A)–(F) is ×200 and for (G)–(I) is ×400. Scale bar represents 100 μm. [Figs. 6(A) and (F) appeared in modified form in Ref. 9.] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

samples, which appear as white areas in the images. In addition, folding of specimens containing mineralized matrix occurred during processing, resulting in wavy edges for these samples.

Qualitative evidence of mineralized matrix production can be seen the dark staining of many cells (and in some cases the entire hydrogel) for the samples with osteogenic supplements at day 21, compared with the red cells seen at day 7 [Fig. 6(A,B,D–F)]. In contrast, there is minimal appearance of mineral formation in samples cultured without supplements at this time point [Fig. 6(C)]. In addition, mineralized matrix is observed not only directly adjacent to the cells, but in areas microns away, for all sample types by day 28 (Fig. 7). Figure 7 also confirms the variability in calcium deposition for samples grown without dexamethasone, quantified previously in the calcium bioassay [Fig. 7(A–D)].

Although not apparent in these images, the hydrogels seemed to have more mineral in the center than at the edges. For the samples cultured without osteogenic supplements, there appeared to be bands of lighter and heavier mineral deposits in many of the more mineralized specimens [Fig. 7(D)]. This was most evident macroscopically during culture as swirls of more opaque areas, contrasting with areas that were more transparent. The blank gels remained transparent throughout culture. Mineral deposition seemed more homogenous throughout the supplemented samples, but in some cases (10K+, I + 2), a line of mineral appeared to be moving longitudinally throughout the gel with time [Figs. 6(E) and 7(E)]. Although these upper and lower faces of these specimens were not marked before cutting, the top face (exposed to media) of these samples did appear more opaque than the bottom (facing base of cell culture well) when they were removed at day 28.

DISCUSSION

This study was designed to assess the effects of material properties of OPF hydrogels on the osteogenic differentiation of embedded marrow stromal cells (MSCs) cultured *in vitro* with and without dexamethasone. Specifically, two OPF formulations (OPF 10K and 3K) that resulted in hydrogels that were nondegradable over the time course of the study but had different swelling properties[9] were chosen to en-

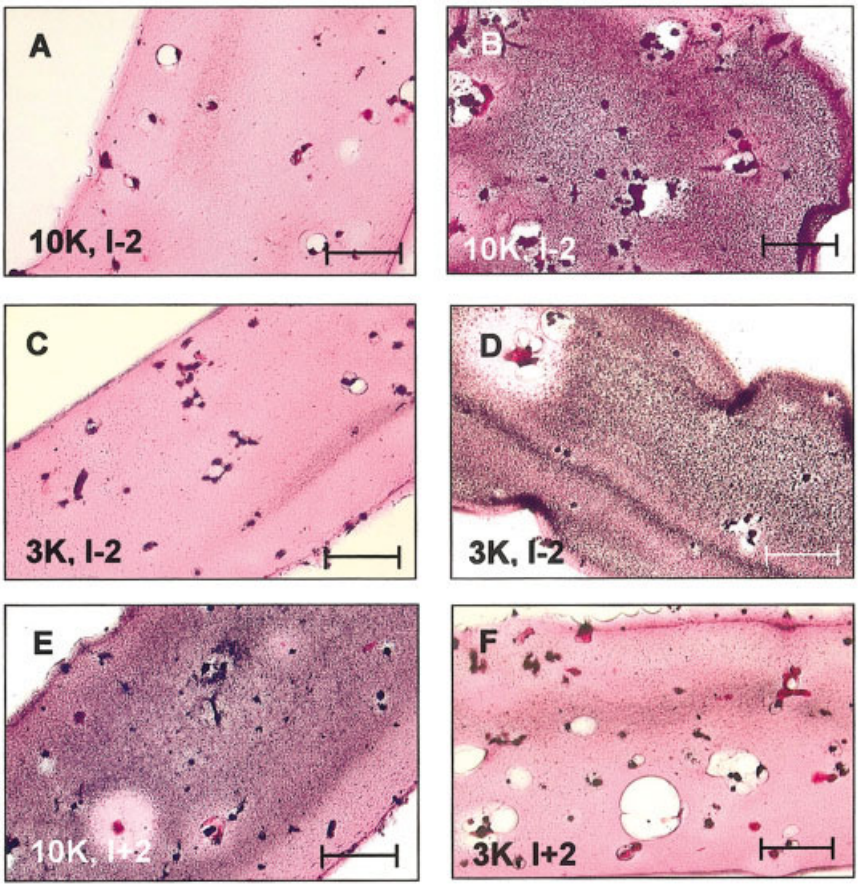

Figure 7. OPF-MSC constructs after 28 days of *in vitro* culture. (A) and (B) represent 10K− hydrogels that showed low and high amounts of calcium deposition, respectively. (C) and (D) represent 3K− hydrogels that showed low and high amounts of calcium deposition, respectively. (E) depicts 10K+ samples (cells for these samples came from I + 2). (F) depicts 3K+ samples (cells for these samples came from I + 2). See Figure 6 for definitions of abbreviations. (See Discussion section for definitions of abbreviations.) Magnification is ×200. Scale bar represents 100 μm. [Fig. 7(E) appeared in modified form in Ref. 9.] [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

capsulate rat MSCs. At each time point, constructs were examined histologically and analyzed for cell number, alkaline phosphatase (ALP) activity, osteopontin (OPN) secretion, and calcium content. The treatments used in this study are referred to as 10K− (OPF 10K hydrogel cultured without dexamethasone), 3K− (OPF 3K hydrogel cultured without dexamethasone), and 10K+ and 3K+ (corresponding hydrogels cultured with dexamethasone).

Biochemical assays for MSC differentiation

In these constructs, although there seemed to be a trend of decreasing cell number over time, this was only significant with the 10K+ samples at day 28 (Fig. 1). Although the APS/TEMED initiator system has been shown in previous work with OPF to be cytocompatible,[9,22] some cell death may occur over the first few days as a result of the encapsulation procedure. At later time points, the increase in mineralized matrix may hinder diffusion of the DNA out of the gel, and thus reduce its availability for the bioassay. Alternatively, the accumulation of matrix may prevent nutrient transport to the cells and cause further cell death with time. A similar apparent decrease in viability over time was seen with rat calvarial osteoblasts embedded in poly(ethylene glycol) diacrylate (PEGDA) hydrogels.[23] In any case, there is no evidence of MSC proliferation after encapsulation in either OPF 10K or 3K hydrogels.

ALP, considered an early marker for osteoblastic differentiation, is a cell membrane associated phosphatase thought to be involved in the onset of extracellular matrix (ECM) mineralization.[24,25] However, in these experiments, there was little difference in ALP content over time, with a significant increase noted only at day 28 for 10K+ and 3K+ samples (Fig. 2). It could be possible that a peak occurred between time points, or that the temporal expression of this marker was altered because of the encapsulation process. Unlike in other systems studying osteogenic differentia-

tion, after encapsulation, there was minimal MSC proliferation, and it has been suggested by experiments with osteoblasts on tissue culture polystyrene (TCPS) that the upregulation of messenger RNA (mRNA) for ALP is connected to the termination of the proliferation stage.[24] However, it is also important to consider that, because this molecule is bound to the cell membrane, there may be problems in extracting it from the gels for analysis, leading to an inability to distinguish trends in expression.

In contrast to ALP, OPN is a secreted marker of osteoblastic differentiation. OPN is a glycoprotein known to possess a cell-binding domain, as well as possible calcium-binding sites.[24,25] Although the exact temporal expression of this protein may vary, OPN levels increase just before or with the onset of mineralization.[24,26,27] In this assay, cell culture media from the different sample types were pooled to represent OPN secretion from days 1–7, 8–14, 15–20, and 21–28. Although this was done to facilitate comparison with the other markers at the same time points, it may decrease the ability to determine exactly when peak OPN secretion occurs in this system. The general shape of the cumulative release curves (Fig. 3) were similar for the two construct types cultured with supplements. Although OPN secretion in the supplemented samples decreased after days 14–21, this was not true for the 10K− samples. The addition of dexamethasone to the culture media immediately after cell isolation could have selected a subpopulation of cells that demonstrated higher OPN production. It should be noted, however, that although cells from the same isolation (I − 2) were used for the 10K− and 3K− samples, this was a different isolation than those used for the 10K+ and 3K+ samples (I + 2).

At all time points, the samples cultured with supplements produced higher levels of OPN than those cultured without, indicative of further differentiation toward the osteoblastic phenotype with the addition of dexamethasone. Although there was no increase in rate of OPN secretion over time in the supplemented samples, there was a decrease after a certain point. Because of the lack of proliferation in this system, the timing of OPN upregulation may have been shifted, or, at later time points, OPN could be increasingly sequestered in the mineralizing ECM and thus not available for assay in the media. In addition, the reports of temporal distribution of osteoblastic markers, in some cases, have been based on mRNA upregulation,[24] which may not correspond to final, active protein amounts. This is particularly true for OPN, where not only production, but subsequent phosphorylation is important for its role in matrix mineralization.[28,29]

In addition, for the constructs cultured in supplemented media, the OPF 10K hydrogels resulted in higher OPN levels than the 3K at all time points. It is not clear from this assay whether this hydrogel formulation encourages production, or just secretion, of OPN, because the OPF 10K hydrogel swells more than the 3K and thus may allow better diffusion of OPN into the media. However, that this trend was found only with 10K+ (rather than all 10K) samples, suggests that the differences in OPN levels cannot be explained solely by increased diffusion.

Calcium, a late marker for osteogenic differentiation,[24,27] was found only in day 21 and 28 samples (Fig. 4). Blank hydrogels showed no significant calcium deposition at any time point, so calcium accumulation in these constructs was not due to passive precipitation from the media. Large variability was found in 10K+ samples on day 21 and 10K− and 3K− on day 28, which can, in part, be explained by tracing the source of the MSCs used for these samples.

In this study, four isolations from three rats each were used, assuming that the pool of donors for each isolation was large enough to mitigate the effects of differences in osteoprogenitor cells between animals. This was found not to be the case. Considering only the OPF 10K samples, at day 21, the 10K− contained two samples with cells from isolation I − 1 and two from isolation I − 2 [see Fig. 5(A)], whereas the 10K+ included samples with cells from isolations I + 1 and I + 2 [Fig. 5(B)]. Although the response from I − 1 and I − 2 cells was similar at this time point, there was a large difference in calcium deposition between I + 1 and I + 2 (I + 1 average: 350 μg/sample; I + 2 average: 23 μg/sample). In contrast, samples from day 28 contained only cells from harvest I − 2 or I + 2 (see Fig. 5). Here, there was little variation between samples grown in supplements, but constructs cultured without supplements demonstrated wide variability in calcium content.

When rat MSCs were cultured on TCPS, a subpopulation of cells that could form mineralized nodules without the presence of dexamethasone was identified, but the addition of dexamethasone increased the amount of these nodules.[15] Because of randomized cell seeding in this study, it could be possible for more of these dexamethasone-independent cells to be placed in one hydrogel than another, accounting for this varied response. The presence of osteogenic supplements, which increased the number of nodules formed from the same amount of progenitors,[15] could mitigate the heterogeneity of the cell population within a single isolation. However, for the 10K+ constructs, each isolation as a whole (I + 1 or I + 2) seemed to differentiate on a different timescale. (There was no difference in calcium content over time for the 3K+ scaffolds with cells from either isolation.) These effects can be reduced in future experiments by using a larger pool of donors, or screening for osteogenic potential before encapsulation.

At both 21 and 28 days, more calcium was produced in the 10K+ than the 3K+ hydrogels. This finding, in

conjunction with OPN results, suggests that the OPF 10K hydrogel promotes MSC differentiation in the presence of osteogenic supplements. This may be due to a higher fold swelling ratio, leading to enhanced diffusion of growth factors, nutrients, and/or mineralized matrix components throughout these hydrogels.

Histology

Images from cross sections of these constructs confirm the variability of calcium deposition for both day 21 and day 28 samples. In all cases, there is evidence of some mineralized (dark) cells by day 21. In the 10K+ (I + 1) constructs, there is mineralized matrix throughout the entire hydrogel at this time point, with only a few nonmineralized cells [Fig. 6(D and G)]. This treatment using cells from I + 2 also shows evidence of mineral deposition, but only partially filling the gel [Fig. 6(E and H)]. The 3K+ samples exhibit only a few areas of localized mineralization at day 21 [Fig. 6(F and I)].

At day 28, the 10K+ (I + 2) specimens show what appears to be darker and more extensive Von Kossa staining than at day 21, indicating that these cells, while not responding as early as the I + 1 cells, continued to produce mineralized matrix over time [Figs. 6(E) and 7(E)]. Some of the samples cultured without dexamethasone had mineralized deposits filling the hydrogel, whereas others stained very lightly, further confirming data from the calcium assay [Fig. 7(A–D)]. With all samples at this time point, there is evidence of mineralized bands not only directly adjacent to cells, but in areas microns away. This level of mineralization is similar to what was reported previously with 10K OPF gels[9] but is more extensive than that seen with rat calvarial cells embedded in PEG-DA hydrogels.[23] As mentioned in a prior study,[9] a possible explanation for the accumulation of mineral throughout the OPF hydrogels is that the polymer network structure may encourage homogeneous and/or heterogeneous nucleation of calcium-phosphate crystals.[25,28,30]

CONCLUSIONS

In this study, the effect of hydrogel material properties on *in vitro* osteogenic differentiation of encapsulated rat MSCs with and without the presence of dexamethasone was investigated. Specifically, two OPF formulations (OPF 10K and 3K) that resulted in hydrogels with different swelling properties were chosen to encapsulate rat MSCs. Evidence of MSC differentiation, including OPN and calcium results, was apparent over the 28-day culture period for samples without dexamethasone, but there was large variability in calcium production between constructs with cells of the same source. Differentiation was also seen in samples cultured with osteogenic supplements, but calcium deposition varied depending on the source pool of MSCs. By day 28, OPN and calcium results suggested that, in the presence of dexamethasone, OPF 10K hydrogels promoted embedded MSC differentiation over OPF 3K hydrogels. In histological sections, mineralized areas were apparent for all sample types many microns away from the cells by the end of the culture period. These experiments explore the use OPF hydrogels as MSC carriers for bone regeneration and indicate that hydrogel material properties can influence differentiation of encapsulated progenitor cells.

The authors thank Dr. L. S. Baggett, Rice University, for assistance with statistical analysis of the bioassay data.

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