Bioactive Glass 13-93 as a Subchondral Substrate for Tissue-engineered Osteochondral Constructs

A Pilot Study

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Abstract

Background Replacement of diseased areas of the joint with tissue-engineered osteochondral grafts has shown potential in the treatment of osteoarthritis. Bioactive glasses are candidates for the osseous analog of these grafts.

Questions/purposes (1) Does Bioactive Glass 13-93 (BG 13-93) as a subchondral substrate improve collagen and glycosaminoglycan production in a tissue-engineered cartilage layer? (2) Does BG 13-93 as a culture medium supplement increase the collagen and glycosaminoglycan production and improve the mechanical properties in a tissue-engineered cartilage layer?

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Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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Methods In Study 1, bioactive glass samples ($n = 4$) were attached to a chondrocyte-seeded agarose layer to form an osteochondral construct, cultured for 6 weeks, and compared to controls. In Study 2, bioactive glass samples ($n = 5$) were cocultured with cell-seeded agarose for 6 weeks. The cell-seeded agarose layer was exposed to BG 13-93 either continuously or for the first or last 2 weeks in culture or had no exposure.

Results Osteochondral constructs with a BG 13-93 base had improved glycosaminoglycan deposition but less collagen II content. Agarose scaffolds that had a temporal exposure to BG 13-93 within the culture medium had improved mechanical and biochemical properties compared to continuous or no exposure.

Conclusions When used as a subchondral substrate, BG 13-93 did not improve biochemical properties compared to controls. However, as a culture medium supplement, BG 13-93 improved the biochemical and mechanical properties of a tissue-engineered cartilage layer.

Clinical Relevance BG 13-93 may not be suitable in osteochondral constructs but could have potential as a medium supplement for neocartilage formation.

Introduction

Osteoarthritis is widely prevalent in the United States, with associated treatment costs of more than \$30 billion a year [24]. Surgical treatments include abrasion arthroplasty [38], autologous chondrocyte implantation, mosaicplasty, osteochondral (OC) autografts and allografts [7], and eventual replacement of the joint with a prosthesis [6]. These methods have limitations related to donor site morbidity, limited tissue supply, immunorejection, and pathogen transmission, as well as prosthesis wear and failure [5].

The tissue engineering of OC grafts requires the combination of two components. Articular cartilage cells are encapsulated in a hydrated matrix that maintains the chondrocyte phenotype. The underlying layer is comprised of a stiffer, porous material to provide mechanical support and integration into the subchondral bone. The optimal osseous analog in a bilayered OC graft should be a mechanically competent porous material that can assimilate with the native bone and support cartilage growth on the chondral interface. We previously reported devitalized allograft bone has deleterious effects on chondral tissue formation in long-term culture, whereas tantalum metal permits the growth of cartilage tissue with properties similar to native articular cartilage [29]. A major disadvantage of using tantalum metal for these purposes is that over time it is not bioresorbable.

Bioactive glasses (BGs) are osteoinductive materials [42]. They can be fabricated into complex geometries with precise control of pore dimensions and overall porosity, including the shape of a proximal human tibia [16]. Depending on the volume fraction of crystals present in the material, BG can either resorb slowly in vivo or undergo localized conversion to a bone-like material after implantation [22, 33, 34]. They undergo a time-dependent conversion to a calcium phosphate material, typically hydroxyapatite, which bonds to bone at body temperatures (eg, 37°C) [17, 18].

Bioglass[®] 45S5 has been widely researched and developed for many biomedical applications, including craniofacial and periodontal repair [12, 13, 19]. The limitations of 45S5 glass and related glass ceramics include unfavorable flow characteristics secondary to their low SiO₂ content and low fracture toughness when compared to human cortical bone [23]. Our work with an alternative silicate-based glass composition, Bioactive Glass 13-93 (BG 13-93), shows this composition can be readily shaped into porous constructs of complex shapes, while retaining the desirable in vitro bioactive characteristics of 45S5 glass and higher mechanical strength [16, 32]. It is known, as BG degrades, it also releases its ion content, which has the ability to stimulate osteoblast activity [3, 4, 40, 42]. However, there is currently a lack of studies investigating the effect of BG 13-93 on chondrocyte behavior and its use in cartilage tissue engineering.

To assess this effect and function, we formulated two studies. In Study 1, we asked whether BG 13-93 as a subchondral substrate would increase collagen and glycosaminoglycan (GAG) production in the tissue-engineered cartilage layer compared to constructs without BG 13-93. In Study 2, we asked whether BG 13-93 as a culture medium supplement would also increase the collagen and GAG production and improve the mechanical properties in the tissue-engineered cartilage layer.

Materials and Methods

Study 1 investigated the effect of BG 13-93 as a subchondral substrate in a bilayered tissue-engineered OC construct (Fig. 1). Study 2 investigated the effect of BG 13-93 when placed in the same culture dish as a chondrocyte-seeded agarose layer at different time points (Fig. 1). Agarose has been used extensively in the tissue-engineering studies of cartilage due to its ability to maintain chondrocytic phenotype in long-term suspension cultures [2, 8, 9, 25]. Additionally, agarose is being used as a copolymer in autologous chondrocyte transplantation (Cartipatch[®]; Banque de tissus, Mions, France) in Europe [39]. Our previous work demonstrates agarose can support the elaboration of functional cartilage in the chondral layer of bilayered tissue-engineered osteochondral constructs [28, 36].

BG with 13-93 composition (wt%) (53SiO₂-6Na₂O-12K₂O-5MgO-20CaO-4P₂O₅) was fabricated into cylinders using a polymer foam replication technique [15], with porosity of 85% ± 2%, dimensions of 4-mm diameter and 4-mm depth, and pore sizes ranging from 100 to 500 µm to match the microstructure of human trabecular bone [15]. The elemental content was confirmed using energy-dispersive xray spectrometry (EDAX) at the Hospital for Special Surgery (New York, NY) (Fig. 2).

To formulate the hydrogel for each study, chondrocytes were harvested from three to four fresh juvenile bovine carpometacarpal joints. The pooled samples were digested in high-glucose Dulbecco's Modified Eagle's Medium (hgDMEM) supplemented with 5% fetal bovine serum and 390 U/mL collagenase Type IV (Sigma-Aldrich, St Louis, MO) for 11 hours at 37°C. The cell suspension produced was filtered through a 70-µm-sized pore mesh and then sedimented in a centrifuge for 10 minutes at a speed of 1000 g. Cell viability was determined using a hemocytometer and trypan blue.

Constructs were cultured in medium consisting of hgDMEM with 1× PSF (penicillin 100 U/mL, streptomycin 100 µg/mL, Fungizone[™] 0.25 µg/mL), 0.1 µmol/L dexamethasone, 50 µg/mL ascorbate 2-phosphate, 40 µg/mL l-proline, 100 µg/mL sodium pyruvate, and 1X ITS + premix (insulin, human transferrin, and selenous acid; Becton Dickinson, Franklin Lakes, NJ). The medium was further supplemented with 10 ng/mL transforming growth factor β3 (TGF-β3; R&D Systems, Minneapolis, MN) for the first 14 days of culture. Medium was changed every 2 to 3 days. Previous work has shown constructs that underwent deformational loading with 2 to 3 weeks' exposure to TGF-β3 had improved mechanical properties compared to those that had continuous exposure [27].

Sixty microliters of molten cell-laden agarose suspension was poured into a custom mold. BG 13-93 cylinders

Fig. 1 A diagram illustrates the study design for the two studies: investigation of BG 13-93 as a subchondral substrate for tissue-engineered osteochondral constructs (Study 1) and investigation of BG 13-93 as a culture medium supplement for a tissue-engineered cartilage layer (Study 2).

Fig. 2A–B (A) A scanning electron microscopic image of the trabecular network and (B) confirmatory EDAX analysis of the BG 13-93 samples used are shown.

(4-mm diameter, 4-mm length; n = 4) were pressed into the gel from above to the desired depth (Fig. 1). For biochemical content, sample wet weight was measured and then lyophilized for 24 hours, followed by measuring of the dry weight. Then samples were digested in proteinase K overnight at 56°C [21]. Aliquots of digest were analyzed for GAG content using the 1,9-dimethylmethylene blue dye-binding assay [14]. The hydroxyproline (HP) content was determined as a measure of total collagen content, using a previously described assay and modified to a

96-well format [37]. A 50-μL aliquot of the gel layer digest solution was mixed with 50 μL 4 N NaOH and the mixture was autoclaved for 20 minutes at 121°C to hydrolyze the sample. The sample was then mixed with the chloramine T reagent and incubated at 25°C for 25 minutes, followed by mixing with Ehrlich aldehyde reagent. The chlorophore was developed at 65°C for 20 minutes. The absorbance was then read at 550 nm and the samples were compared with an HP standard to determine the HP concentration of the sample. Results were standardized to tissue wet weight and

are reported as microgram of hydroxyproline per milligram of gel layer wet weight.

Each biochemical constituent (GAG and collagen) was normalized to the sample wet weight and DNA content (PicoGreen[®] dsDNA Quantitation Assay; Invitrogen, Carlsbad, CA). The media were analyzed for GAG and collagen content and the inflammatory mediators prostaglandin E₂ (PGE₂) and nitric oxide (NO) using the enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) and Greiss assays (Promega, Madison, WI), respectively.

For histologic evaluation, samples were fixed in ethanol, embedded in paraffin, and sectioned (5 μ m) through the transverse plane. These samples were stained with hematoxylin and eosin and Masson's trichrome and evaluated via light microscopy. For immunohistochemical analysis, unstained sections were deparaffinized in xylene and rehydrated in graded ethanol solutions. The samples were permeabilized with a 0.1% trypsin solution at 36°C for 60 minutes and then blocked with 5% bovine serum albumin at room temperature for 60 minutes. Slides were incubated overnight at 4°C in predetermined dilutions of the primary antibodies (in parentheses): proteoglycan (mouse anti-human antibody [Millipore, Billerica, MA], reported to crossreact with members of the aggrecan/versican proteoglycan family in bovine tissue), collagen Type I (rabbit anti-bovine antibody [Millipore]), or collagen Type II (rabbit anti-bovine antibody [Millipore]). The next day, slides were rinsed in Tris-buffered saline before being incubated with the secondary antibody. Proteoglycan was labeled with goat anti-mouse fluorescein isothiocyanate (FITC), and collagen Types I and II were labeled with goat anti-rabbit FITC. Samples were coverslipped and reviewed using fluorescent light microscopy. Negative controls were used as comparison in which the primary (but not secondary antibody) was omitted from the slides to see if any stain was due to fluorescence aside from the target region. Two blinded investigators (PJ, HS) subjectively assessed the immunohistochemical images. The staining was assessed from the center to the periphery of the constructs with attention paid to the degree of staining, the location of staining, and the cellular appearance.

Inductively coupled plasma mass spectroscopy (ICP-MS) technology was used to measure the elemental constituents of 1 mL culture medium (University of Missouri). The pH of the medium was also measured using a commercially available pH meter.

In Study 2, cylinders were placed adjacent to the cell-seeded agarose constructs on Day 28 of culture and assessed for mechanical and biochemical changes at 7 and 14 days postexposure (Fig. 3). Then, using cells from a separate isolation, BG 13-93 samples were added to the culture medium for 2 weeks immediately after cell encapsulation (and coincident with exposure of TGF- β 3,

Fig. 3 A timeline for BG 13-93 exposure in Study 2 is shown. The cell-seeded agarose gel layer had no exposure to BG 13-93 or was exposed to BG 13-93 early in culture (Days 0–14), late in culture (Days 28–42), or continuously (Days 0–42).

Days 0–14). BG 13-93 cylinders were then removed, the medium replaced, and the constructs were cultured for 4 more weeks (Fig. 3). Agarose constructs maintained in culture medium without BG 13-93 and those cultured with BG13-93 continuously for the entire culture period (6 weeks) served as negative and positive controls, respectively.

For mechanical testing, agarose constructs were tested in unconfined compression with loading to 10% strain at a rate of 0.05% per second, after an initial 0.02-N tare load (elastic modulus, or E_Y). Dynamic modulus (G^*) was measured by superimposing 2% peak-to-peak sinusoidal strain at 0.1 Hz. In terms of biochemical content, samples were evaluated for GAG [14], collagen [37], and DNA content (PicoGreen[®] dsDNA Quantitation Assay) normalized to wet weight.

On the harvest day, samples were fixed in acid formalin ethanol and paraffin embedded. Separate 8- μ m sections were stained either with alcian blue or picrosirius red to assess for proteoglycan and collagen content, respectively.

SigmaStat[®] software (Systat Inc, Chicago, IL) was used to perform all statistical tests. In Study 1, to discern the differences in collagen and GAG content and NO and PGE₂ release into the media between BG13-93 constructs and controls, one-way ANOVA tests were performed. The Tukey test was used to compare the release of ions into the medium from BG 13-93 constructs compared to controls. This test was also used to compare the pH of the media. In Study 2, we determined differences in collagen, GAG content, and mechanical properties between groups using one-way ANOVA tests.

Results

In Study 1, at Day 42, BG 13-93-containing constructs had a mean concentration of GAG in the chondrocyte-seeded

Fig. 4A–D Graphs show the biochemical results of Study 1 ($n = 4$) comparing the tissue properties of the OC constructs and also the contents of the culture media: (A) GAG content in the tissue, (B) collagen content in the tissue, (C) GAG content in the media, and (D) PGE₂ content in the media. (A) At Day 42, OC constructs with a BG 13-93 subchondral base had a mean concentration of GAG in the chondrocyte-seeded agarose gel layer of 6.29%ww. (B) The collagen

content of the BG 13-93 constructs was higher ($p = 0.048$) than controls at Day 14, but there were no differences at later time points. (C) GAG and (D) PGE₂ release into the medium from the BG constructs was higher in the middle of the culture period (Days 17–26). There was also a second peak of release for both GAG and PGE₂ late in culture at Day 42 compared to controls. The p values stated are from statistical tests comparing BG 13-93 constructs versus controls.

agarose gel layer of 6.29%ww, which is comparable to native bovine cartilage [30] (Fig. 4A). The collagen content (normalized to the DNA content) of the BG 13-93 constructs was higher ($p = 0.048$) than controls at Day 14, but there were no differences at later time points (Fig. 4B). GAG (Fig. 4C) and PGE₂ (Fig. 4D) release into the medium from the BG constructs was higher in the middle of the culture period (Days 17–26), which corresponds with the withdrawal of TGF- β 3 from the culture medium at Day 14. There was also a second peak of release for both GAG (Fig. 4C) and PGE₂ (Fig. 4D) late in culture at Day 42 compared to controls. There were no differences in NO release among groups (data not shown). Immunohistochemical staining showed BG 13-93 constructs subjectively had a homogenous staining of proteoglycan from the center of the specimens to the periphery (Fig. 5). The intensity of collagen Type II staining for both groups was low. However, BG 13-93 constructs had a collagen Type I rim on the outer surface of the construct, not observed in controls. Subjective assessments of the trichrome-stained histology sections showed constructs with

BG 13-93 produced a less uniform collagen network than controls. ICP-MS data showed higher ($p < 0.001$) silicon content in the media from BG 13-93 constructs but not calcium (Table 1). There was no difference in pH between the media from BG 13-93 constructs and controls (Fig. 6).

In Study 2, the exposure of cell-seeded agarose constructs at Days 28 to 42 to BG 13-93 led to superior mechanical and biochemical properties compared to samples with continuous or no exposure (Fig. 7). These improvements included increased G^* compared to continuous exposure (Fig. 7A), E_Y compared to continuous exposure (Fig. 7B), GAG compared to early transient exposure (Days 0–14) (Fig. 7C), and collagen compared to continuous and early transient exposure (Fig. 7D). The peak construct values achieved in this study were E_Y , 743 kPa; GAG, 7.6%ww, G^* , 2.8 MPa; and collagen, 6.2%ww, which compare favorably with native bovine wrist cartilage [30]. Histology showed a homogeneous extracellular matrix distribution (GAG and collagen) for constructs exposed to BG 13-93 on Days 28 to 42 (Fig. 8).

Fig. 5 Immunohistochemistry and trichrome images for GAG, collagen I (Col 1), and collagen II (Col 2) distribution (original magnification, $\times 20$) from Study 1 are shown. The green color indicates the degree and confluence of staining for the particular matrix constituent. Yellow arrows indicate chondrocytes. Immunohistochemical staining showed BG 13-93 constructs subjectively had a homogenous staining of proteoglycan from the center of the

specimens to the periphery. The intensity of collagen II staining for both groups was low. However, BG 13-93 constructs had a collagen I rim on the outer surface of the construct, not observed in controls. Subjective assessments of the trichrome-stained histology sections showed constructs with BG 13-93 produced a less uniform collagen network than controls.

Table 1. Elemental content of the culture medium from Study 2

Element	Elemental content (ppm)							
	Day 3		Day 14		Day 28		Day 42	
	Control	BG 13-93 construct	Control	BG 13-93 construct	Control	BG 13-93 construct	Control	BG 13-93 construct
Si	ND	32*	ND	35*	ND	39*	ND	30*
Na	3168	3269	3100	2971	3452	3403	3720	3857
K	212	250	210	236	228	233	242	286
Mg	20	30	16	28	22	25	24	33
P	37	36	31	34	41	41	44	53
Ca	64	113	47	110	71	123	76	124

BG = bioactive glass; ND = not detectable; * $p < 0.001$ (comparison between ion content in the media from BG 13-93 constructs versus control constructs).

Discussion

BG ceramics are synthetic materials that enhance bone formation and can integrate with living bone [1, 18]. In our previous *in vivo* work, we found BG 13-93 supports maintenance of grafted cells in a poly(ethylene)glycol hydrogel layer at the articulating surface with integration of bone into the BG layer of tissue-engineered OC grafts [1]. We asked whether BG 13-93 as a subchondral substrate would increase collagen and GAG production in the

tissue-engineered cartilage layer compared to constructs without BG 13-93 and whether BG 13-93 as a culture medium supplement would also increase the collagen and GAG production and improve the mechanical properties in the tissue-engineered cartilage layer.

Our study is subject to a number of limitations. First, at harvest, we did not image the BG 13-93 cylinders themselves primarily because the aim of this study was to investigate the effect of BG 13-93 on a tissue-engineered cartilage layer. Second, when we examined the use of

BG 13-93 as a subchondral substrate in a tissue-engineered OC constructs, we did not investigate the mechanical properties of the cell-seeded agarose gel layer. In Study 2, however, we did examine these properties. Third, although we reported the release of ionic constituents into the medium over time and hypothesized the effect of these constituents on the cell-seeded agarose layer, we were

unable to decipher a direct effect of individual ions in this study. The current results in terms of ion release should therefore be taken to be a possible indicator of an effect needing further clarification.

In Study 1, we investigated the effect of BG 13-93 on a tissue-engineered cartilage layer when it was used as a subchondral substrate. Histologic analysis of the tissue-engineered layer confirmed a homogenous distribution of GAG in constructs from the center to the periphery with BG 13-93 constructs, similar to previous work with Bioglass® 45S5 [41]. BG 13-93 was associated with higher GAG release in the middle of the culture period, which coincided with TGF- β 3 removal from the medium. The release of GAG at this time point could either represent the production of smaller GAG molecules or catabolism of the tissue-engineered samples. If taken together with PGE₂ release into the medium, this more likely represents the latter, which could be of importance for any proposed clinical use of these scaffolds. Further work is ongoing in our laboratory to identify the types of GAG produced by chondrocytes on exposure to this material and the spatial

Fig. 6 A graph shows measurement of the pH of the media from Study 1 at different time points ($n = 4$ at each time point).

Fig. 7A–D Graphs show the mechanical and biochemical results of Study 2 ($n = 5$): (A) G^* (dynamic modulus), (B) E_γ (Young's modulus), (C) normalized GAG, and (D) normalized collagen. In Study 2, the transient exposure of cell-seeded agarose constructs at Days 28 to 42 to BG 13-93 led to superior mechanical and biochemical properties compared to control samples with no exposure. The continuous exposure to BG 13-93 did not cause an increase in these properties. Exposure of gel layers at 28 days to BG 13-93 led to improvement in properties within 2 weeks compared to the control

and continuous exposure groups. These improvements included increased G^* compared to continuous exposure, E_γ compared to continuous exposure, GAG compared to early transient exposure (Days 0–14), and collagen compared to continuous and early transient exposure content. The p values stated are from statistical tests comparing the mechanical and biochemical properties of cell-seeded constructs exposed to BG 13-93 continuously in culture (Days 0–42) and early in culture (Days 0–14) versus those exposed late in culture (Days 28–42).

Fig. 8 Histology images show a homogeneous extracellular matrix distribution (GAG [stained with alcian blue] and collagen [stained with picosirius red]) for constructs exposed to BG 13-93 on Days 28 to 42 (original magnification, $\times 10$).

variation in matrix distribution away from BG 13-93 to further elucidate this issue.

Culture medium examination in our study revealed BG 13-93 OC constructs did not have higher levels of calcium. However, previous studies have shown calcium is known to have an effect on the extracellular matrix deposition of cartilage [35, 43, 44]. The intracellular concentration of calcium channels plays an important role in regulating chondrocyte proliferation and maturation in monolayer [44], and therefore high concentrations of calcium could be of benefit for cartilage tissue engineering. Previous studies with Bioglass[®] 45S5 suggest there is a dose-dependent effect of silicon concentration that is optimal for osteoblast proliferation and differentiation [45, 46]. This study provides evidence that increasing silicon released into the medium via BG 13-93 dissolution could modulate chondrocyte function. BG 13-93 is biodegradable, and when placed in an aqueous environment at 37°C, it converts to a calcium phosphate material analogous to hydroxyapatite [20, 26]. As BG degrades, it also releases its ion content, which has the ability to stimulate osteoblast activity [3, 4, 40, 42]. Previous studies have shown, in an osteoblast cell line, increasing the pH can improve collagen Type I production by increasing cellular metabolism [10, 42]. Due to the limited numbers used in this pilot study, we may not have seen a difference in the pH of the medium from BG 13-93 constructs. A further investigation of this effect would be of interest to decipher whether the ion release in solution could cause an alkaline pH that may have contributed to the collagen I rim on the periphery of the BG 13-93-containing constructs (at the expense of GAG). This hypothesis is supported by Das et al. [11], who reported, at a more alkaline pH, chondrocytes shift from collagen II to collagen I production. However, these findings have not been found with other BGs. Tellisi and Ashammakhi [41] investigated the effect of Bioglass[®] 45S5 on osteoarthritic chondrocytes and found substantial staining of collagen II with maintenance of the chondrocytic phenotype similar to freshly isolated articular chondrocytes. Loty et al. [31] reported a BG containing apatite and wollastonite caused chondrocytes to produce an extracellular matrix with high collagen II and proteoglycan. Our study suggests, when BG 13-93 is

used as a subchondral substrate, it may not adequately maintain the chondrocyte extracellular matrix within the agarose gel layer due to less collagen II content compared to collagen I. This would be a potential disadvantage in terms of articular cartilage tissue-engineering purposes.

In Study 2, we found, when BG 13-93 is cocultured adjacent to chondrocyte-seeded agarose constructs, the positive effects were seen with late transient exposure (last 2 weeks). In these groups, agarose constructs had superior biochemical (GAG and collagen content) and mechanical properties than those exposed earlier in culture or continuously to BG 13-93, suggesting there may be an optimal exposure time associated with these effects. The amount of elaborated matrix in the gel layer (reflecting culture maturity) could be an important modulator of the BG 13-93 response. This is further supported by Study 1 in that there were negative biochemical findings from continuous exposure to BG 13-93 within an OC construct. While the cause for the negative effects on OC constructs remains to be elucidated, it is possible continuous exposure to BG 13-93 is detrimental to chondrocyte metabolism due to the local ion concentrations associated with BG 13-93 dissolution exacerbated by the integration of the BG 13-93 to the gel layer.

Under the conditions of the current investigation, our results suggest, while BG 13-93 may not be a suitable osseous analog for OC constructs, its transient application to culture media (as a supplement) has potential for optimizing the tissue engineering of cartilage.

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