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The Effects of Crosslinking of Scaffolds Engineered from Cartilage ECM on the Chondrogenic Differentiation of MSCs

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Abstract

Scaffolds fabricated from cartilage extracellular matrix provide a chondroinductive environment that stimulates cartilaginous matrix synthesis in a variety of cell types. A limitation of these cartilage-derived matrix (CDM) scaffolds is that they contract during *in vitro* culture, which unpredictably alters their shape. The current study examined the hypothesis that collagen crosslinking techniques could inhibit cell-mediated contraction of CDM scaffolds. We analyzed the effects of dehydrothermal (DHT) treatment, ultraviolet light irradiation (UV), and the chemical crosslinker carbodiimide (CAR) on scaffold contraction and chondrogenic differentiation of adult human bone marrow-derived stem cells (MSCs). Both physical and chemical crosslinking treatments retained the original scaffold dimensions. DHT and UV treatments produced significantly higher glycosaminoglycan and collagen contents than CAR crosslinked and non-crosslinked constructs. Crosslinking treatments influenced the composition of newly synthesized matrix, and DHT treatment best matched the composition of native cartilage. DHT, UV, and non-crosslinked CDM films supported cell attachment, while CAR crosslinking inhibited cell adhesion. These results affirm that collagen crosslinking treatments can prevent cell-mediated contraction of CDM scaffolds. Interestingly, crosslinking treatments influence chondrogenic differentiation. These effects seem to be mediated by modifications to cell-matrix interactions between MSCs and the CDM; however, further work is necessary to elucidate a specific mechanism.

Keywords

articular cartilage; decellularized tissue; tissue engineering; mesenchymal stem cell; cell-mediated contraction; crosslink

Introduction

Tissue engineering combines cells, growth factors, and scaffolds to treat damaged cartilage, and encompasses the repair of focal defects as well as the potential for complete joint

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resurfacing [1-4]. Biomaterial scaffolds play a prominent role in this approach by providing initial mechanical function and geometry to allow for tissue regeneration, as well as environmental cues for regulating cell growth and differentiation [3,5,6]. Scaffold composition and structure govern its biocompatibility, degradation, mechanical function, and bioinductive properties [7]. While synthetic materials can provide biomimetic mechanical properties and predictable, biocompatible degradation, they generally possess a limited capacity to interact with cells without modification or functionalization with defined ligands [8]. In this regard, native extracellular matrices present an attractive material option for scaffold fabrication because they offer a host of tissue-appropriate physical and chemical cues that govern specific cell proliferation, differentiation, and matrix synthesis [9-11].

Previous studies have shown that porous scaffolds fabricated from the extracellular matrix of native cartilage can provide a chondroinductive environment that directly influences chondrogenic differentiation, in contrast to hydrogels, such as alginate [12], that maintain cells in a rounded phenotype but require exogenous growth factors to induce chondrogenesis. These cartilage-derived matrix (CDM) scaffolds have been shown to promote cell proliferation, chondrogenic differentiation, and cartilaginous matrix accumulation by adipose-derived stem cells (ASCs) [13,14], bone marrow derived mesenchymal stem cells (MSCs) [15], and chondrocytes [16]. In the absence of exogenous growth factors, CDM constructs can upregulate cartilage-specific genes and synthesis of cartilaginous proteins in ASCs [13] as well as porcine and human chondrocytes [16]. Studies investigating the chondrogenic capacity of ASCs and MSCs in the presence of various growth factor combinations found that CDM scaffolds enhanced matrix production compared to an alginate bead culture system, and that CDM mitigated the hypertrophic differentiation of MSCs typically observed in the presence of TGF- β 3 [15]. While constructs fabricated from CDM provide many potential benefits for cartilage tissue engineering, they generally possess low compressive moduli (e.g., ~50 kPa at day 0) [13], which make them susceptible to cell-mediated contraction. ASCs and MSCs express α -smooth muscle actin [17,18], and have been shown to contract collagen-glycosaminoglycan (GAG) [19-21] and CDM scaffolds [13,15] during in vitro culture. Cell-mediated contraction of porous constructs can reduce diffusion to the center of constructs as well as the volume available for cellular proliferation and matrix deposition [19]. Scaffold contraction can also alter the overall shape of constructs in an unpredictable manner, complicating the design of anatomically-shaped implants [19] and preventing integration with host tissue [14].

To enhance mechanical properties and prevent cell-mediated contraction, a variety of physical and chemical crosslinking techniques have been applied to biologic scaffolds, including dehydrothermal treatment [22-24], ultraviolet irradiation [22-27], carbodiimide [20,21,28], glutaraldehyde [19,29,30], and genipin [31,32], among other methods [14,19,33,34]. These treatments confer various types and degrees of crosslinking [14,23,29,31,35] and protein denaturation [35-39], which influence mechanical strength [19,23,28,40], scaffold contraction [19-21,30], resistance to enzymatic degradation [24,40,41], and cell-matrix interactions [22]. Previous studies have demonstrated that crosslinking CDM with genipin prevented contraction of ASC-seeded scaffolds and maintained the chondrogenic inductive properties of the CDM [14]. Despite these promising results, genipin can impart certain adverse effects on CDM scaffolds, including loss of GAG during the crosslinking process, decrease in pore size limiting cellular infiltration, and substantial attenuation of collagen and GAG content throughout in vitro culture [14].

The current study investigated the effects of dehydrothermal treatment, ultraviolet irradiation, and carbodiimide crosslinking techniques on cell-mediated contraction of CDM scaffolds and chondrogenic differentiation of MSCs. These crosslinking methods were selected because they preserve the native composition of CDM and unlike genipin do not

incorporate into the constructs; thus minimizing alterations to the natural extracellular matrix. Previous work has demonstrated that CDM stimulated MSCs toward a more mature chondrogenic phenotype than ASCs [15]. Therefore, the current study analyzed the effects of crosslinking treatment on the chondrogenic differentiation of MSCs due to their prominent response toward CDM. In addition to characterizing scaffold contraction and chondrogenic differentiation, we investigated potential mechanisms to explain any differences generated by crosslinking treatment by examining cell attachment to CDM films. We hypothesized that physical crosslinking methods, which impart lower degrees of crosslinking than chemical reagents [19], would be sufficient to inhibit cell-mediated contraction of CDM scaffolds and retain their chondrogenic potency by preserving cell-matrix interactions that become masked under extensive crosslinking [14].

Methods

Preparation of Scaffolds

Articular cartilage was harvested from the knees of skeletally mature (2-3 year old) female mixed-breed pigs, acquired from a local slaughter house immediately *post mortem*. Cartilage was finely minced while still on the bone, and then removed for homogenization. Porous scaffolds were fabricated by homogenizing porcine articular cartilage at a concentration of 0.1 g wet weight/mL distilled water using a post-mounted PRO260 homogenizer (PRO Scientific Inc., Oxford, CT) [13-16]. Cartilage was homogenized for five cycles of 2 minutes homogenization at 30,000 rpm and cooling 2 minutes on ice to prevent overheating. Aliquots of homogenized cartilage (0.75mL) were placed in wells of a 48-well plate, frozen overnight at -80°C, and lyophilizing for 24 h as previously described [13]. Scaffolds were cut using a biopsy punch and scalpel to form constructs 6mm in diameter, approximately 2mm thick, with a dry weight of 4 mg (Fig. 1). This process results in scaffolds with a porosity of 96.5% and an average pore size of 221 μm [13]. After lyophilization, scaffolds were crosslinked by one of the following methods: (a) no treatment (non-crosslinked) control (NON) (b) dehydrothermal treatment by heating scaffolds in a dry environment at 120°C for 24 hrs (DHT), (c) ultraviolet light exposure at an energy concentration of 8 J/cm² from a 254nm source for 80 min (UV), (d) chemical crosslinking with carbodiimide solution (14mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and 5.5mM N-hydroxysuccinimide; Sigma) for 3 hrs at room temperature (CAR). CAR treated scaffolds were washed with a 0.1M Na₂HPO₄ solution for 2 hours to hydrolyze any remaining carboxylic acid groups. Subsequently, CAR treated scaffolds were washed four times with distilled water and lyophilized. After crosslinking, all scaffolds were sterilized using ethylene oxide gas and outgassed for 1 week before use.

Cell Culture

Human MSCs were obtained from the posterior superior iliac crest of healthy donors, with informed consent (University Hospitals of Cleveland Institutional Review Board protocol number 09-90-195) as previously described [42]. MSCs from three female donors (average age 27 years) were combined after initial expansion. Cells were cultured at 5000 cells/cm² through 4 passages in DMEM-low glucose (Gibco, Grand Island, NY) containing 1 ng/mL bFGF (Roche Diagnostics, Florence, SC) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) from a lot selected for its ability to support MSC proliferation and differentiation [42]. MSCs were suspended in culture medium and seeded by pipetting 500,000 cells in 30 μL directly onto 6 mm-diameter CDM constructs, which were placed in 24-well low attachment plates (Corning Life Sciences, Corning, NY). Cells were allowed 1 hr to attach before adding 1mL of culture medium, which was changed every 48 hrs. The culture medium consisted of DMEM-high glucose (Gibco), 1% penicillin-streptomycin (Gibco), 37.5 $\mu\text{g/mL}$ L-ascorbic acid 2-phosphate (Sigma), 40 $\mu\text{g/mL}$ L-proline (Sigma),

and 1% ITS+Premix (Collaborative Biomedical-Becton Dickinson, Bedford, MA). The chondrogenic group (TGF) also contained 10ng/mL human TGF- β 3 (R&D Systems). Constructs were harvested for area determination, biochemical analysis, and immunohistochemistry at 1hr (Day 0), Day 14, and Day 28 time points.

Area Determination

Scaffolds were removed from culture wells and immediately photographed next to a metric ruler. Pictures were analyzed with Image J to quantify circular area.

Biochemical Analysis

Day 0, 14, and 28 biochemical samples (n=6 per group) were digested by incubating in 1 mL of papain buffer [125 μ g/mL papain (Sigma), 100 mM phosphate buffer, 10 mM cysteine, and 10mM EDTA, pH 6.3] for 16 hr at 65°C. DNA content was measured fluorometrically using the PicoGreen fluorescent double-stranded DNA assay (Invitrogen/Molecular Probes, Carlsbad, California) according to the manufacture's protocol (excitation wavelength, 485nm; emission wavelength, 535 nm). GAG content was determined using bovine chondroitin sulfate as a standard and measuring sample content with the dimethylmethylene blue assay [43]. Total collagen content was determined by measuring the hydroxyproline content of the scaffolds after acid hydrolysis and reaction with p-dimethylaminobenzaldehyde and chloramine-T, using 0.134 as the ratio of hydroxyproline to collagen [44].

Cell Viability

Cell survival and apoptosis were assessed using the Live/Dead® Cell Viability/Cytotoxicity Kit, for mammalian cells (Invitrogen/Molecular Probes, Carlsbad, California). Live cells were stained with calcein AM and dead cells were labeled with ethidium homodimer-1 bound to DNA. Stained constructs were imaged using confocal microscopy (LSM 510, Zeiss, Thornwood, NY, USA).

Histology and Immunohistochemistry

For histology and immunohistochemistry, constructs (n=2 per group) were fixed overnight at 4°C in a pH 7.4 solution containing 4% paraformaldehyde and 100 mM sodium cacodylate. Constructs were taken through a series of increasing ethanol solutions and xylene steps to clear the constructs. Samples were then embedded in paraffin and cut into 5 μ m sections. Monoclonal antibodies to type I collagen (ab90395; Abcam, Cambridge, MA), type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), type X collagen (C7974; Sigma-Aldrich). Sections for collagen staining were treated Pepsin (Digest-All; Zymed, San Francisco, CA) to expose the epitopes. The anti-mouse IgG biotinylated secondary antibody (ab97021; Abcam, Cambridge, MA) was linked to horseradish peroxidase and reacted with aminoethyl carbazole (Histostain® Plus Broad Spectrum kit; Invitrogen, Carlsbad, California). General histological staining using 0.1% aqueous Safranin-O, 0.02% fast-green, and hematoxylin was also performed on xylene-cleared sections. Human osteochondral plugs were prepared in the same manner as samples and were used as positive controls for each antibody. Negative controls without primary antibody were also prepared for each slide.

CDM Films

CDM films were fabricated by homogenizing porcine cartilage in distilled water at a concentration of 0.6% weight/weight, and allowing slurry to evaporate for 48 hours in 12 mm silicone wells mounted on glass slides. Films were crosslinked with one of the treatments described above. Films were washed four times with distilled water to remove

non-adherent particles and allowed to dry for 48 hours. On the day of seeding, CDM films were blocked with 3.75% BSA diluted in PBS for 3 hours at 37°C to prevent non-specific binding. After blocking, films were washed with DMEM-low glucose (Gibco). During monolayer expansion, MSCs were cultured in the presence of 4µg/mL 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide (Polybrene, Sigma) and lentiviral vectors containing a green fluorescent protein (GFP) plasmid. Transduction was expedited via centrifugation at 1200G for 30 minutes. After centrifugation, viral supernatant was aspirated and replaced with fresh expansion medium. To verify successful transduction, GFP expression was visualized using 488nm excitation with confocal microscopy (LSM 510, Zeiss, Thornwood, NY, USA). GFP-expressing MSCs were seeded onto films via pipetting at a density of 1,700 cells/mm². Cells were allowed 1 hour to attach. Non-adherent cells were removed by rinsing films twice with DMEM-low glucose (Gibco). Seeded films were fixed for 15 minutes with 4% paraformaldehyde. Films were then mounted with a coverslip and imaged using confocal microscopy. Cells in a 10x field were counted for semi-quantitative analysis. There were six independent samples per treatment group, and three images were averaged for each sample.

Statistical Analysis

Two-factor analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) *post hoc* test ($\alpha = 0.05$) were used to determine significance for cell type and culture condition.

Results

Cell-free scaffolds did not demonstrate any matrix accumulation and remained translucent throughout the 28-day culture period, while cell-seeded scaffolds became increasingly opaque from day 0 to day 28 (Fig. 2). Constructs receiving TGF-β3 supplementation were completely opaque by day 28, and adopted a smooth, shiny texture (Fig. 2).

At day 14, cell-seeded non-crosslinked scaffolds (NON) showed significant contraction, while scaffolds crosslinked in any manner retained their original area throughout the culture period (Fig. 3A). At day 28, TGF-β3 supplemented constructs had areas that were greater than or equal to their respective cell-free scaffolds in all crosslinking treatments (Fig. 4A). Non-crosslinked constructs were significantly smaller than DHT and CAR treated constructs (Fig. 4A). Control constructs were smaller than cell-free scaffolds in all treatments except for CAR (Fig. 4A).

At day 14, there was no difference in DNA content across medium types in the CAR and NON groups (Fig. 3B). The DHT and UV groups demonstrated an upregulation in DNA content in response to TGF-β3 supplementation compared to the respective control medium condition (Fig. 3B). At day 28, all TGF-β3 treated scaffolds had significantly higher DNA content than their respective control media samples (Fig. 4B). Of the constructs that received TGF-β3 supplementation, CAR treatment resulted in the highest DNA content and UV treatment in the lowest (Fig. 4B).

At day 14, there was no significant difference in GAG content among the NON crosslinked scaffolds (Fig. 3C). The UV and DHT treated groups showed no difference between cell-free scaffolds and samples that received control medium (Fig. 3C). However, both physical crosslinking treatments yielded a substantial increase in GAG content in the presence of TGF-β3 (Fig. 3C). The DHT constructs cultured with TGF-β3 had the highest GAG content of any other treatment condition (Fig. 3C). While, the control CAR treated scaffolds had a higher GAG content than their respective cell-free constructs, there was no difference between CAR constructs that received control medium or TGF-β3 (Fig. 3C). The TGF-β3

CAR group had a lower GAG content than either of the physical crosslinking methods in the presence of TGF- β 3 (Fig. 3C). The day 28 GAG data exhibited a significant increase in GAG content across all crosslinking treatments in the presence of TGF- β 3 (Fig. 4C). The DHT crosslinking treatment again yielded the highest GAG content in response to TGF- β 3 (Fig. 4C).

At day 14, no differences were observed in collagen content between the cell-free scaffolds and their respective TGF- β 3 supplemented constructs in any of the crosslinking groups (Fig. 3D). The non-crosslinked scaffolds exhibited a significant decrease in collagen content in the presence of TGF- β 3 compared to their respective cell-free constructs (Fig. 3D). The day 28 collagen data demonstrates that there was no difference in collagen content across any of the cell/medium conditions within each crosslinking treatment (Fig. 4D). Overall, DHT treatment produced scaffolds with the highest collagen content (Fig. 4D).

Histology and immunohistochemistry of cell-free scaffolds revealed empty matrices with large pores (Fig. 5). The cartilage matrix alone did not stain positively for GAGs, type I collagen, or type X collagen; however, it did stain positive for type II collagen (Fig. 5). Non-crosslinked scaffolds that were cultured in control medium for 28 days experienced a reduction in pore volume and an aggregation of cartilage chunks that comprise the CDM matrix (Fig. 6). Collapse of the scaffold pores was not seen in any of the crosslinked scaffolds (Fig. 6). In control medium, the NON, UV, and CAR treated scaffolds demonstrated minimal matrix production, while DHT scaffolds exhibited robust newly synthesized matrix that stained positive for type II collagen, but not for GAGs, type I collagen, or type X collagen (Fig. 6). In all constructs, supplementation with TGF- β 3 greatly enhanced the accumulation of new tissue that stained intensely for GAGs and type II collagen (Fig. 7). Type I collagen was also found in all samples except for DHT treated scaffolds (Fig. 7). Type X collagen was not detected in any sample, and negative controls demonstrated no non-specific or background staining in the absence of primary antibodies (Fig. 7). In the NON and CAR crosslinked constructs, new matrix deposition was non-uniform and was localized to one surface of the scaffolds (Fig. 7). On the other hand, the DHT and UV scaffolds exhibited uniform matrix deposition throughout the full-thickness of the construct (Fig. 7). Interestingly, fluorescent live/dead staining revealed that cells were preferentially distributed on one side of the scaffold in each of the crosslinking methods except for the DHT treatment, which resulted in a high degree of cellularity on both sides of the construct (Fig. 8). The UV and NON crosslinked scaffolds had a higher cell content on the side in which the cells were pipetted, while the CAR constructs had a higher degree of cellularity opposite the cell-seeded side (Fig. 8).

In control experiments, MSCs readily adhered to untreated glass slides (Fig. 9A). This non-specific attachment was eliminated when glass slides were blocked with BSA (Fig. 9A). Semi-quantitative analysis showed cells adhered to CDM films receiving NON, DHT, and UV treatments (Fig. 9B). However, CAR crosslinking prevented cell attachment (Fig. 9B).

Discussion

The findings of this study demonstrated that all of the different crosslinking treatments could prevent cell-mediated contraction of CDM constructs; however, the methods produced distinct effects on the chondrogenic induction of MSCs and cell attachment to films. Non-crosslinked scaffolds experienced substantial contraction. In contrast, all crosslinked samples retained their original shape throughout *in vitro* culture. Area quantification combined with biochemical, histological, and immunohistochemical evaluations showed that chondrogenic differentiation was independent of contraction. DHT and UV constructs retained their original shape and synthesized a greater amount of cartilaginous matrix than

non-crosslinked and CAR groups, which exhibited disparate amounts of contraction. The attenuated chondrogenic response of CAR crosslinked samples corresponded with a concomitant reduction in cell adhesion. These findings suggest that the effects of cross-linking on cell-matrix interactions, rather than construct contraction *per se*, may play a critical role in supporting chondrogenesis of CDM-based scaffolds.

Gross morphology (Fig. 2) and quantitative analysis of scaffold areas (Fig. 3A and 4A) demonstrated that both physical and chemical crosslinking treatments prevented the cell-mediated contraction that has been observed in non-crosslinked CDM scaffolds [13-15]. Previous studies have reported the ability of chemical crosslinking agents such as carbodiimide and genipin to inhibit cell-mediated contraction of collagen-GAG scaffolds [20,21] and CDM constructs [14], respectively. Interestingly, the findings of the current study revealed the ability of DHT and UV treatments to prevent cell-mediated contraction of lyophilized scaffolds, which contrasted with previous results that indicated the minimal crosslinking densities imparted by physical crosslinking methods were not sufficient to overcome contraction [19-21]. The discrepancies between the current results and previous findings could arise from differences in material strength, scaffold density, and concentration of seeded cells. In addition to type II collagen and GAGs, the cartilage extracellular matrix contains numerous fibril-associated collagens [45] and small leucine rich-proteins [46] that play an adhesive role and could enhance the mechanical strength or augment the effects of crosslinking CDM compared to collagen-GAG scaffolds. The CDM scaffolds of the current study also may possess a higher density of proteins and matrix macromolecules than previously reported collagen-GAG constructs [20,21], which has been shown to enhance mechanical integrity [47]. Additionally, variations in the quantity of seeded cells would dramatically alter the contractile forces on the scaffold [18]. Thus scaffolds with a low material content and a high cell-seeding concentration would be expected to have a greater propensity for cell-mediated contraction. Our findings elucidate the ability of physical crosslinking treatments to impart a mechanical integrity sufficient to inhibit cell-mediated contraction given the appropriate porosity and cell-seeding constraints.

CDM constructs alone possessed a limited capacity to induce chondrogenic differentiation of MSCs and required supplementation of exogenous growth factors to promote cartilaginous tissue accumulation. After 28 days of culture in the absence of growth factors, CDM scaffolds elicited minimal matrix synthesis from resident MSCs (Fig. 6), consistent with previous reports [15]. Comparing cell free scaffolds (Fig. 5) to constructs cultured in the absence of growth factors (Fig. 6), dehydrothermal-treated constructs possessed the highest amount of newly synthesized matrix that stained positive for type II collagen. These results indicate that the CDM is able to stimulate MSCs to produce matrix in the absence of growth factors. However, the newly synthesized tissue showed minimal staining for GAG, which is an essential component of native cartilage. Across all crosslinking treatments, exogenous TGF- β 3 supplementation resulted in abundant newly synthesized cartilaginous matrix that stained prominently for GAGs and type II collagen (Fig. 7). While these findings are in contrast to the stronger chondroinductive capabilities of CDM that have been previously reported for ASCs and chondrocytes [13,16,48,49], other studies have shown that CDM can prevent MSCs from entering a hypertrophic phenotype [15] that is typically observed during chondrogenic differentiation [50,51]. Although the current study did not investigate the effects of crosslinking treatment on the gene expression of MSCs, previous studies have demonstrated the ability of non-crosslinked CDM to modulate the gene profiles of ASCs and MSCs in the presence and absence of growth factors [13,15]. These findings suggest an important and potentially synergistic effect between the chondroinductive properties of CDM with those of TGF- β 3.

In the presence of TGF- β 3, physical crosslinking methods (DHT and UV) exhibited greater levels of MSC chondrogenesis than did non-crosslinked or chemically treated CDM constructs, as evidenced by enhanced cellular proliferation and accumulation of cartilaginous matrix. Divergent rates of proliferation, apoptosis, and matrix production (Fig. 3, 4, and 7) amongst the various crosslinking groups could arise from distinct influences of these treatments on CDM properties, such as crosslink density [14,23,29,31,35] and collagen denaturation [35-39]. In turn, many of these characteristics have been shown previously to influence cellular responses to TGF- β 3. For example, cellular responses to soluble growth factors can be modulated by insoluble environmental factors such as cell-matrix interactions [52-54] and substrate stiffness [55,56]. In the present study, Day 0 values (data not shown) demonstrated that CDM scaffolds possessed collagen and GAG compositions equivalent to native cartilage [57]. However, non-crosslinked scaffolds lost large amounts of GAG (Fig. 3C and 4C) and collagen (Fig. 3D and 4D) contents throughout *in vitro* culture. This finding is consistent with previous studies of CDM constructs, and loss of these extracellular matrix components was not prevented by genipin crosslinking [14]. In contrast, the current investigation implemented crosslinking techniques that have been shown to tether GAGs to collagen molecules [58,59], which enhanced GAG retention throughout *in vitro* culture (Fig. 3C and 4C), specifically cell-free DHT scaffolds retained the highest GAG and collagen contents after 28 days of culture (Fig. 4C and 4D). All crosslinking treatments maintained the ability of CDM to prevent hypertrophic differentiation of MSCs [15] as evidenced by the absence of type X collagen staining (Fig. 7). However, a substantial effect on type I collagen production, characteristic of a fibrocartilage phenotype, was observed (Fig. 7). Only DHT treatment inhibited type I collagen deposition, highlighting the ability of specific crosslinking techniques to selectively modulate production of extracellular matrix proteins. A similar effect was documented with ASCs cultured in genipin crosslinked CDM [14].

Chondrogenic induction of MSCs was independent of cell-mediated contraction. The ability of MSCs to undergo chondrogenesis in the absence of scaffold contraction contrasts with previous work in collagen-GAG scaffolds, which found a positive correlation between amount of contraction and matrix production [21]. Interestingly, the current study and previous work [21] both found increased chondrogenesis in physically crosslinked constructs relative to chemically treated scaffolds. While previous work attributed these findings to increased rates of scaffold contraction in physically crosslinked samples [21], the current study did not observe this contraction, and ascribed the enhanced levels of chondrogenesis to the preservation of cell-matrix interactions, which have been shown to become masked at high levels of chemical crosslinking [14]. Blocking cell-matrix interactions between MSCs and CDM potentially eliminates a primary mechanism by which CDM can provide chondroinductive cues to differentiating cells. This mechanism is also consistent with the attenuated cellular proliferation and biosynthesis rates of chemically treated scaffolds (Fig. 3 and 4).

In order to elucidate whether chemical crosslinking treatment obscured cell-matrix interactions between MSCs and CDM, two-dimensional CDM films were used to assess the effects of each crosslinking method on cell attachment. CDM film experiments revealed that bovine serum albumin blocked non-specific cell attachment; non- and physical crosslinking treatments supported MSC adhesion to CDM, while chemical crosslinking completely eliminated cell-matrix interactions that mediated cell attachment to CDM (Fig. 9). The effects of crosslinking treatments on the conformation and structure of collagen molecules and their organization into fibrils has been well characterized [60]. The formation of additional crosslinks [14,23,29,31,35] as well as varying degrees of denaturation [35-39] produced by crosslinking treatments has been shown to influence cellular responses such as migration [22]. Cell attachment results demonstrated that high levels of chemical crosslinking could significantly inhibit the ability of extracellular matrix proteins to interact

with cells (Fig. 9), similar to that observed with genipin crosslinking previously [14]. The ability of crosslinking treatments to alter cell-matrix provides a mechanism to explain the distinct chondrogenic responses produced by the various methods. MSCs have been characterized as anchorage-dependent [61]; therefore, chemical treatments that block cell attachment to CDM are likely to attenuate cellular proliferation and biosynthesis. While MSCs could not adhere directly to chemically crosslinked constructs, cells were still retained in the scaffolds by becoming entrapped in the tortuous pore architecture of the constructs. Indeed, MSCs tended to aggregate at the bottom of the carbodiimide crosslinked scaffolds, as indicated by the increased GAG staining along the bottom edge of the construct (Fig. 7). Once entrapped, MSCs could be stimulated to undergo cellular proliferation and chondrogenic differentiation by growth factor stimulation as well as cell-cell interactions [62,63], even in the absence of potential cell-scaffold interactions. This cellular proliferation enabled MSCs to overgrow the bottom of the scaffold and produce a smooth shiny surface (Fig. 2); however, this architecture was only observed on the bottom of the CAR scaffolds. The resurgence of DNA, GAG, and collagen content in chemically treated scaffolds by day 28 further demonstrates the potency of soluble growth factors in driving differentiation.

The purpose of the current study was to characterize the effects of various crosslinking treatments on cell-mediated contraction and chondrogenic differentiation. However, an important future direction of this work includes a quantitative assessment of the effects of crosslinking on the mechanical properties of CDM constructs. In the present study, emphasis was placed on preserving the chondroinductive properties of the CDM, while eliminating cell-mediated contraction. However, even at the highest degree of crosslinking, it is unlikely that collagen crosslinking alone will be sufficient to confer the mechanical properties necessary to support physiologic loads without other modifications. For example, previous studies have shown that three-dimensional fiber reinforcement can produce CDM scaffolds mechanical properties similar to those of native cartilage [48]. In this regard, crosslinking treatments developed in the present study could be used to immobilize CDM amongst synthetic scaffolds that possess well-characterized, tunable mechanical properties [48].

An important consideration in the interpretation of the current study is that the CDM was not decellularized. Complete removal of cellular debris is crucial for *in vivo* studies, as exogenous cellular components have been implicated as potent inflammatory stimuli [64-67]. However, decellularization approaches are met with several negative consequences including loss of GAGs, denaturation of proteins, and attenuation of mechanical properties [67,68]. Future studies will investigate strategies to remove exogenous cell debris while maintaining the native biochemical and biomechanical properties of the CDM.

Conclusions

Overall, these results highlight the importance of cell-matrix interactions in governing chondrogenic differentiation and suggest that the inhibition of cell adhesion and cell-matrix interactions as one potential cause of attenuated chondrogenesis in chemically crosslinked scaffolds. A reduction in pore size due to cell-mediated contraction [20] does not appear to alter chondrogenic differentiation of MSCs in CDM scaffolds. While the CDM scaffold alone is not sufficient to stimulate MSC chondrogenesis, CDM itself, and the mode of crosslinking, significantly influence MSC responses to TGF- β 3. Taking into account the biochemical, immunohistochemical, and cell-adhesion results together, dehydrothermal treatment produced the most desirable outcomes in terms of increased cellular proliferation, enhanced GAG and collagen production, and inhibition of hypertrophic and fibrocartilage phenotypes. The beneficial effects of DHT crosslinking could potentially arise from preventing scaffold contraction, which offers greater pore volume for cell proliferation and matrix synthesis, as well as preservation of cell-matrix interactions that mediate

chondrogenic differentiation. Future studies are required to elucidate the specific cell-matrix interactions responsible for mediating chondrogenesis in MSCs.

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Figure 1. Low (A) and high (B) magnification scanning electron microscope images demonstrate the high porosity and large pore size of the CDM scaffold. Scale bars: 1mm (A), 200 μ m (B).

Figure 2.

Gross morphology of NON: non-crosslinked; UV: ultraviolet light crosslinked; DHT: dehydrothermally crosslinked; and CAR: carbodiimide crosslinked CDM scaffolds in cell free (CF), control medium (CON), and TGF- β 3 (TGF) culture conditions at 1 hour (Day 0), 14, and 28 days of culture. Constructs cultured in the presence of TGF- β 3 adopted a smooth, shiny surface indicative of cartilage-like matrix synthesis. Crosslinked scaffolds maintained their original size throughout cultivation. Non-crosslinked constructs showed marked contraction at day 14. Scale bars: 5mm.

Figure 3.

(A) Scaffold Areas; (B) DNA content; (C) GAG content; (D) Collagen content of NON: non-crosslinked; UV: ultraviolet light crosslinked; DHT: dehydrothermally crosslinked; and CAR: carbodiimide crosslinked CDM scaffolds after 14 days of culture (n=6). Bars represent means $\pm$ standard error of the means. Groups not sharing same letter have p-values < 0.05. In the presence of TGF- β 3, crosslinking treatments inhibited cell-mediated contraction, while noncrosslinked scaffolds underwent substantial contraction. Physical crosslinking treatments (DHT and UV) produced the highest rates of cellular proliferation, GAG synthesis, and collagen production in MSCs.

Figure 4. (A) Scaffold Areas; (B) DNA content; (C) GAG content; (D) Collagen content of NON: non-crosslinked; UV: ultraviolet light crosslinked; DHT: dehydrothermally crosslinked; and CAR: carbodiimide crosslinked CDM scaffolds after 28 days of culture (n=6). Bars represent means $\pm$ standard error of the means. Groups not sharing same letter have p-values < 0.05. Non-crosslinked constructs possessed smaller areas than crosslinked scaffolds. All constructs demonstrated increased cellular proliferation in the presence of TGF- β 3. DHT treatment exhibited the highest GAG and collagen contents after 28 days of culture.

Figure 5.

Histology (Safranin-O and Fast Green staining) and immunohistochemistry for type II collagen (COL II); type I collagen (COL I); type X collagen (COL X) on cell-free scaffolds after 28 days of culture. CDM lacked native GAG content; only DHT treatment retained minimal amounts of GAG. Cell free scaffolds stained positive for type II collagen and negative for types I and X collagen, which indicated that the collagen composition of the CDM was preserved. Aminoethyl carbazone (AEC) produces red color. Scale bars: 500 μ m.

Figure 6.

Histology (Safranin-O and Fast Green staining) and immunohistochemistry for type II collagen (COL II); type I collagen (COL I); type X collagen (COL X) on control scaffolds after 28 days of culture. In the absence of TGF- β 3, MSCs produced matrix completely devoid of GAGs. However, the newly synthesized tissue stained positive for type II collagen and negative for types I and X collagens. Aminoethyl carbazone (AEC) produces red color. Scale bars: 500 μ m.

Figure 7.

Histology (Safranin-O and Fast Green staining) and immunohistochemistry for type II collagen (COL II); type I collagen (COL I); type X collagen (COL X) on human osteochondral sections (+Control) and CDM scaffolds after 28 days of culture in the presence of TGF- β 3. All images are transverse sections, showing full-thickness of each construct. TGF- β 3 stimulated MSCs to produce mature cartilaginous matrix staining positive for GAGs and type II collagen. Crosslinking treatments altered the composition of newly synthesized matrix demonstrated by variable amounts of type I collagen staining. MSCs did not enter into a hypertrophic phenotype as shown by the absence of type X collagen staining. Aminoethyl carbazone (AEC) produces red color. Scale bars: 500 μ m.

Figure 8.

Live/Dead staining of CDM scaffolds seeded with MSCs after 28 days of culture in the presence of TGF-β3. The NON: non-crosslinked; UV: ultraviolet light crosslinked; and CAR: carbodiimide crosslinked scaffolds exhibited uneven cellular distribution; however, DHT: dehydrothermally crosslinked constructs possessed high cellularity on both sides of the construct. Scale bars: 500μm. Scaffold auto-fluoresces red.

Figure 9.

MSC attachment to CDM films. (A) Green visualizes adherent GFP-expressing MSCs at 1 hour post-seeding. (B) Semi-quantitative assessment of cell attachment by counting adherent cells in a 10x field. BSA treatment blocked non-specific cell attachment. NON: non-crosslinked; UV: ultraviolet light crosslinked; and DHT: dehydrothermally crosslinked films supported MSC adhesion to CDM; while CAR: carbodiimide crosslinking inhibited cell attachment to CDM films. Scale bars: 50 μ m. Groups not sharing same letter are significantly different ($p < 0.05$).