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Effects of Dexamethasone on Mesenchymal Stromal Cell Chondrogenesis and Aggrecanase Activity: Comparison of Agarose and Self-Assembling Peptide Scaffolds

Emily M Florine, B.S.E., Ph.D. [Candidate],

Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

Rachel E Miller, Ph.D. [Postdoctoral Researcher],

Department of Biochemistry and Section of Rheumatology, Rush University Medical Center, Chicago, IL

Ryan M Porter, Ph.D. [Postdoctoral Researcher],

Center for Advanced Orthopedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

Christopher H Evans, Ph.D. [Professor],

Center for Advanced Orthopedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA

Bodo Kurz, Ph.D. [Associate Professor], and

Bond University, Gold Coast, Australia and Anatomical Institute, University of Kiel, Germany

Alan J Grodzinsky, Sc.D. [Professor]

Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, MA

Emily M Florine: eflorine@mit.edu; Rachel E Miller: rachel_miller@rush.edu; Ryan M Porter: rmpor@bidmc.harvard.edu; Christopher H Evans: cevans@bidmc.harvard.edu; Bodo Kurz: bkurz@bond.edu.au; Alan J Grodzinsky: alg@mit.edu

Abstract

Objective—Dexamethasone (Dex) is a synthetic glucocorticoid that has pro-anabolic and anti-catabolic effects in cartilage tissue engineering systems, though the mechanisms by which these effects are mediated are not well understood. We tested the hypothesis that the addition of Dex to chondrogenic medium would affect matrix production and aggrecanase activity of human and bovine bone marrow stromal cells (BMSCs) cultured in self-assembling peptide and agarose hydrogels.

Design—We cultured young bovine and adult human BMSCs in (RADA)₄ self-assembling peptide and agarose hydrogels in medium containing TGF- β 1±Dex and analyzed extracellular matrix composition, aggrecan cleavage products, and the effects of the glucocorticoid receptor antagonist RU-486 on proteoglycan content, synthesis, and catabolic processing.

Mailing address, phone, and fax for corresponding author: Alan J. Grodzinsky, MIT Room NE47-377, 500 Technology Square, Cambridge, MA 02139, Phone: 617-253-4969, Fax: 617-258-5239.

Conflict of Interest Statement

The authors have nothing to disclose.

Informed Consent and Human/Animal Rights

The three patients whose cells were used in this study granted informed consent and surgical procedures were preapproved by the local Institutional Review Board at either Brigham and Women's Hospital or Massachusetts General Hospital (Boston, MA).

Institution where work done: Massachusetts Institute of Technology

Results—Dex improved proteoglycan synthesis and retention in agarose hydrogels seeded with young bovine cells, but decreased proteoglycan accumulation in peptide scaffolds. These effects were mediated by the glucocorticoid receptor. Adult human BMSCs showed minimal matrix accumulation in agarose, but accumulated ~50% as much proteoglycan and collagen as young bovine BMSCs in peptide hydrogels. Dex reduced aggrecanase activity in (RADA)₄ and agarose hydrogels, as measured by anti-NITEGE Western blotting, for both bovine and human BMSC-seeded gels.

Conclusions—The effects of Dex on matrix production are dependent on cell source and hydrogel identity. This is the first report of Dex reducing aggrecanase activity in a tissue engineering culture system.

Keywords

Chondrogenesis; mesenchymal stem cells; degradative enzymes; articular cartilage; extracellular matrix

Introduction

Tissue engineering using bone marrow-derived stromal cells (BMSCs) is an attractive strategy for healing cartilage defects. BMSCs have been shown to differentiate to a cartilage lineage in a variety of scaffolds and create cartilage-like extracellular matrix (ECM).¹⁻⁴ These cells can be extracted without the need for further damage to the joint and expanded for several passages without losing differentiation potential.^{5, 6} Additionally, BMSCs have been found to synthesize ECM with higher dynamic stiffness, longer sulfated glycosaminoglycan (sGAG) chains, and longer aggrecan core proteins than matrix synthesized by chondrocytes, regardless of donor age.⁷ Despite progress in cartilage tissue engineering, further optimization is needed to improve integration with native tissue, mechanical function, and maintenance of the chondrocyte phenotype.^{8, 9}

One strategy for improving our understanding of these complex systems is to investigate the specific effects of chemical factors added to the cellular microenvironment. Dexamethasone (Dex) is a synthetic glucocorticoid frequently added to culture medium for chondrogenesis studies, motivated by research demonstrating pellet cultures of rabbit BMSCs stimulated with TGF- β 1 and Dex (TGF+Dex) grew larger than those cultured with TGF- β 1 alone.² Additional studies found that TGF+Dex increased aggrecan biosynthesis and gene expression in bovine and human BMSCs over TGF- β 1 alone.^{10, 11}

Dex also has anti-catabolic properties. The aggrecanases ADAMTS-4/5 (a disintegrin and metalloproteinase with thrombospondin motifs-4/5) are key destructive enzymes in human osteoarthritis progression and are involved in cytokine-induced aggrecan lysis in cartilage explants.¹²⁻¹⁴ ADAMTS-4/5-generated aggrecan fragments are also found in BMSC-seeded hydrogels.³ Recently, Dex was found to reduce sGAG loss and rescue proteoglycan synthesis in cartilage explants exposed to inflammatory cytokines.¹⁵ The mechanisms through which Dex mediates pro-anabolic and anti-catabolic effects is not well understood, though evidence that the glucocorticoid receptor mediates the increase in aggrecan mRNA levels caused by Dex has been reported.¹⁶

We have used self-assembling peptide hydrogels for cartilage tissue engineering because they support TGF- β 1-induced chondrogenesis of BMSCs in vitro³ and have been successfully used for studies of cartilage repair in animal studies.^{17, 18} Additionally, self-assembling peptides have been used to deliver growth factors,¹⁸⁻²¹ which is important for designing an optimal chondrogenic microenvironment as well as controlling sustained, local delivery of growth factors. While scaffold-free, or pellet, cultures offer important culture

systems for studying chondrogenesis, our objective was to investigate BMSC matrix production and catabolism in hydrogel scaffolds motivated by the long-term translational challenges of growth factor delivery and integration between neocartilage and adjacent native cartilage. We tested the hypothesis that chondrogenesis of human and bovine BMSCs in self-assembling peptide hydrogels, as well as subsequent matrix production and aggrecanase activity, would be responsive to chondrogenic medium supplemented with Dex. We compared the influence of Dex on chondrogenesis of young bovine BMSCs and adult human BMSCs in (RADA)₄ self-assembling peptide hydrogels and agarose hydrogels. By analyzing accumulation of sGAG, DNA, and collagen, the effects of the glucocorticoid receptor antagonist RU-486, and aggrecan cleavage products, we found that cell source and scaffold environment changed the BMSC response to Dex, emphasizing the importance of optimizing these variables for neocartilage generation. Dex reduced ADAMTS-4/5 activity in (RADA)₄ self-assembling peptide and agarose hydrogels for both cell types.

Methods

Bovine BMSC Isolation and Expansion

Bovine BMSCs were extracted from the femora and tibiae of four 1-2 week old calves (Research 87, Marlborough, MA) as described previously.³ Briefly, marrow was isolated aseptically, homogenized in phosphate-buffered saline (PBS), centrifuged, and the cell fraction was plated on tissue culture plastic for 30 minutes to remove rapidly adhering cells. The remaining cell population was plated into flasks at 1×10^6 mononuclear cells/cm². Remaining red blood cells were removed by a medium change two days after plating. Colonies were expanded in low glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc, Manassas, VA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA or ThermoScientific, Logan, UT), penicillin streptomycin amphotericin (PSA) (Sigma-Aldrich, St. Louis, MO), 4-(2-hydroxyethyl)-1-piperzaineethanesulfonic acid (HEPES) (Invitrogen), and 1ng/mL basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN) until reaching 80% confluence as described previously.³ Cells were then removed by 0.05% trypsin/0.53mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and frozen for future use (passage 0, P0). After thawing frozen P0 aliquots, cells were seeded at 6000 cells/cm² and expanded two passages consisting of 3 days each in expansion medium with 5ng/mL bFGF. BMSCs were then seeded into hydrogels for chondrogenesis studies.

Human BMSC Isolation and Expansion

Human BMSCs (hBMSCs) were isolated from intramedullary aspirate generated using a Reamer Irrigator Aspirator device during surgical procedures performed at Brigham and Women's Hospital and Massachusetts General Hospital (Boston, MA).²² The three patients whose cells were used in this study (two males, one female, ages 81, 51, and 37) granted informed consent and surgical procedures were preapproved by the local Institutional Review Board. The bulk aspirate was centrifuged, the red blood cell fraction lysed briefly in a buffer of 155mM NH₄Cl, 10mM KHCO₃, and 0.1mM EDTA (pH 7.2), and BMSCs isolated from the remaining nucleated cell fraction by differential adhesion to tissue culture plastic as above. These donor cell populations have been confirmed to undergo chondrogenic differentiation in pellet cultures in response to TGF- β 1 and Dex.²³ Frozen aliquots of P1 or P2 cells were thawed and seeded at 1000 cells/cm² in expansion medium with 5ng/mL bFGF. Cultures received supplements of bFGF on day 4, passaged on day 6, given more bFGF on day 9 or 10, and were seeded into hydrogels on day 12. One patient's cells required further expansion and were passaged on day 12, given more bFGF on day 14, and cast on day 17.

Hydrogel Encapsulation and Culture

Following BMSC expansion in monolayer, cells were encapsulated in one of two hydrogel materials: (RADA)₄ self-assembling peptide hydrogel (RAD, also known as PuraMatrix, a gift from 3DM, Cambridge, MA) and low melting point agarose (Invitrogen, catalog number 15517-022). BMSCs were encapsulated in 0.5% (w/v) RAD or 2% (w/v) agarose at 10⁷ cells/mL. These hydrogel concentrations were chosen to match previous studies.^{3, 4, 11, 24, 25} The cell/hydrogel mixture was cast as disks (~6mm diameter, 1.5mm thickness) into the center of rings of acellular agarose pre-equilibrated in chondrogenic medium consisting of high glucose DMEM, 1% ITS+1 (Sigma-Aldrich; insulin, transferrin, sodium selenite, bovine serum albumin, and linoleic acid), proline (Sigma-Aldrich), ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), HEPES, PSA, non-essential amino acids (NEAA) (Sigma-Aldrich), and sodium pyruvate (Invitrogen), as described previously.³ The cell-seeded hydrogel disks were cultured in chondrogenic medium with 10ng/mL recombinant human TGF-β1 (R&D Systems) with or without 100nM Dexamethasone (Sigma-Aldrich), labeled TGF+Dex or TGF, respectively. Hydrogels were cultured for up to 21 days with medium changes every 2-3 days. Some hydrogels were immediately cast into buffer containing 50mM tris(hydroxymethyl)aminomethane (Tris) and 1mM CaCl₂ to measure day 0 DNA levels.

Viability Staining

To assess viability of BMSCs for all culture conditions, hydrogels were viewed using a Nikon Eclipse TE-300 fluorescence microscope at ~day 4 with 4μg/mL fluorescein diacetate (live cells) and either 0.875μg/mL ethidium bromide or 35μg/mL propidium iodide (dead cells).

Glucocorticoid Receptor Antagonist Culture

RU-486 (Mifepristone; Sigma-Aldrich), a glucocorticoid receptor antagonist with partial agonist activity,^{26, 27} was added to the culture medium to determine whether observed responses to Dex were mediated by the glucocorticoid receptor. A dose-response study confirmed that 1μM RU-486 would not interfere with BMSC viability or sGAG synthesis. This dose has been used previously.¹⁶ Bovine BMSCs were encapsulated in (RADA)₄ or agarose hydrogels as described above and cultured in medium with 10ng/mL TGF-β1 or 10ng/mL TGF-β1 with 100nM Dex with and without RU-486 for 21 days.

Hydrogel Biochemistry

For the final 24 hours of culture, hydrogels used for biochemical analysis were radiolabeled with 5μCi/mL ³⁵S-sulfate and 10 or 20μCi/mL ³H-thymidine (Perkin Elmer Inc, Waltham, MA) to measure proteoglycan and DNA synthesis, respectively. Unincorporated radiolabel was removed, hydrogels were weighed wet, lyophilized, weighed dry, and digested with Proteinase-K (Roche Applied Science, Indianapolis, IN) as described previously.³ Digested samples were assayed for sGAG content by 1.9-dimethylmethylene blue (DMMB) dye binding,²⁸ DNA content by Hoechst dye binding,²⁹ and radiolabel incorporation by liquid scintillation counting. Hydroxyproline content, as a measure of total collagen, was measured by reaction with p-dimethylaminobenzaldehyde.³⁰ sGAG released to the culture medium was measured by DMMB.

Histology and Immunohistochemistry

Day 21 hydrogels from one experiment were fixed in 4% paraformaldehyde for 2 hours at room temperature and overnight at 4°C. Osteochondral explants were collected from the distal femurs of 1-2 week old bovine calves for collagen staining controls. Explants were fixed in 4% paraformaldehyde, transferred to 70% ethanol, and demineralized. Gels and

explants were then embedded in paraffin, sliced into sections, deparaffinized, and stained as described previously in detail.³ Briefly, for immunohistochemistry, samples were stained for type I or type II collagen; negative controls were incubated without primary antibodies.³¹ Additional sections were stained for sulfated proteoglycans using toluidine blue dye solution.³² For detection of apoptotic cells, sections were stained with haematoxylin. Cells with and without nuclear blebbing were counted using a Zeiss Axiophot microscope with 40x objective (Zeiss, Wetzlar, Germany). For quantification of nuclear blebbing,³³ sections from three different gels per experimental group were evaluated by counting cells in three adjacent, distinct fields of vision in the center of each section. For each individual gel/sample the percentage of apoptotic cells was calculated and the mean value for each experimental group was given (n=3).

Aggrecan Extraction and Western blot Analysis

Additional hydrogels were cultured for 21 days, soaked in PBS with Complete Protease Inhibitors (Roche) for ~2 hours, and frozen at -20°C until extraction. Hydrogels were rotated in 4M guanidine hydrochloride with 100mM sodium acetate plus Protease Inhibitors for two days at 4°C to extract proteoglycans. After centrifugation at 13,000 g for 30 mins, the supernatant was removed and its sGAG content was measured by DMMB. Aggrecan extract was then run through microcentrifuge tubes with a 10,000 MW cut-off (Millipore, Billerica, MA). Retained protein was washed twice with buffer containing 0.05M Tris, 0.05M sodium acetate, and 0.01M EDTA and resuspended in this buffer at a concentration of 1µg sGAG/µL. The aggrecan was then deglycosylated using protease-free chondroitinase ABC (30mU/100µg sGAG), keratanase II (0.5mU/100µg sGAG), and endo-β-galactosidase (0.5mU/100µg sGAG) (Seikagaku Biobusiness Corporation, Tokyo, Japan). sGAG was loaded into a 4-12% Bis-Tris gel (Invitrogen) and run at 200V for 45 mins. Proteins were transferred to a polyvinylidene fluoride membrane and probed with the anti-NITEGE monoclonal antibody AGG-C1³⁴ (a gift from Dr. Carl Flannery, Pfizer) and the anti-G1 antibody G1-2 (a gift from Dr. John Sandy, Rush University).³⁵ Some membranes were stripped following anti-NITEGE imaging and re-probed with anti-G1 antibody. To ensure the removal of the NITEGE antibody following stripping, membranes were exposed to the secondary antibody again and imaged to ensure no signal.

Statistical Analysis

Results are reported as mean±standard error of the mean. A linear mixed model of variance with animal/patient as a random factor and medium condition and timepoint as fixed effects was used to analyze data for experiments testing the effects of Dex on sGAG content, DNA content, proteoglycan synthesis, DNA synthesis, sGAG retention, and hydroxyproline content for bovine and human BMSCs. Data from the agarose and RAD scaffolds were analyzed separately and not compared statistically. Data from experiments with RU-486 were analyzed using the same model with only the medium condition as a fixed effect. Residual plots for all of the above comparisons were investigated and data were transformed as necessary to ensure normality. Apoptotic cell data were analyzed using a general linear model with medium condition as an independent variable. A Kolmogorov-Smirnov test was used to ensure normality and data were transformed as necessary. Tukey post hoc tests with p<0.05 were used to evaluate statistical significance for all pairwise comparisons. Statistical tests were performed using Systat 12 software.

Results

Matrix and Cellular Content of Bovine BMSC-Seeded Hydrogels

sGAG content increased with time in both scaffolds seeded with bovine BMSCs, though there was no statistical difference between days 14 and 21 in agarose hydrogels. By day 21,

sGAG content of RAD hydrogels was nearly 150% of agarose hydrogels. Consistent with previous literature,¹¹ TGF+Dex significantly increased sGAG accumulation over TGF alone in agarose hydrogels (Fig. 1A). In RAD, a decrease in sGAG accumulation was observed with Dex compared to TGF alone on days 14 and 21. DNA content did not change significantly between days 7 and 21 for either scaffold (Fig. 1B). In agarose, the addition of Dex increased DNA content at all timepoints. Whereas little or no proliferation was observed in agarose hydrogels, RAD hydrogels showed a 2.5 fold increase in DNA content over day 0 levels by day 7. DNA content and sGAG normalized to DNA are also reported in Supplementary figure S1.

Proteoglycan synthesis normalized to DNA content increased from day 7 to days 14 and 21 in RAD and remained elevated with TGF+Dex, while synthesis dropped at day 21 with TGF alone (Fig. 1C). Proteoglycan synthesis showed a trend of decreasing over time in agarose. TGF+Dex showed a significant increase compared to TGF alone for agarose at days 14 and 21, consistent with sGAG content. DNA synthesis rates normalized to DNA content were not statistically different over time for the TGF+Dex condition in RAD, while the TGF alone condition showed a significant decrease at day 21 (Fig. 1D). In agarose, DNA synthesis decreased with time in both conditions. Dex supplementation significantly increased DNA synthesis in agarose at day 7 and day 21.

Dex had a greater effect on sGAG retention in agarose than in RAD hydrogels (Fig. 1E). In agarose, sGAG retention decreased from day 14 to day 21 and the addition of Dex significantly increased retention at all timepoints. In contrast, sGAG retention was generally higher in RAD than in agarose at all times with or without Dex. Dex increased sGAG retention over TGF alone in RAD at day 7. Total sGAG produced and total sGAG normalized to DNA are also reported in Supplementary figure S1.

Hydroxyproline content increased over time in both scaffolds (Fig. 1F), similar to the trends in sGAG content. In RAD, addition of Dex decreased hydroxyproline content at all timepoints compared to TGF alone. In agarose, there was no statistical difference between TGF and TGF+Dex at any timepoint.

Histology and Immunohistochemistry

Toluidine blue staining of day 21 hydrogels (Fig. 2A) was consistent with the quantitative sGAG content (Fig. 1A), with TGF+Dex and TGF gels showing similar levels of staining in RAD and TGF+Dex agarose gels showing darker staining than TGF alone. Interestingly, addition of Dex led to more diffuse staining throughout the agarose scaffold compared to the largely pericellular staining for TGF alone.

To investigate the types of collagen present in day 21 hydrogels, immunohistochemistry was performed. RAD hydrogels were positive for collagen type II staining, but agarose gels were not (Fig. 2B). After analyzing multiple sections of RAD gels, a trend of more collagen type II staining in TGF alone gels compared to TGF+Dex gels was seen in RAD, based on the distribution of matrix areas that were stained positively. This was consistent with higher levels of hydroxyproline in RAD gels with TGF alone compared to TGF+Dex (Fig. 1F). Collagen type I staining was not seen in either agarose or RAD hydrogels (data not shown).

Day 21 hydrogels were also analyzed for nuclear blebbing as an indicator of apoptosis by haematoxylin staining.³³ Representative images showed little apoptosis in RAD gels with either medium condition, while agarose gels showed much higher levels of blebbing (Fig. 2C). After reviewing multiple sections, the percentage of apoptotic cells was calculated for each condition (Fig. 2D). Agarose hydrogels showed 20-30% of total cells being apoptotic,

while RAD gels were near 2%. TGF+Dex showed a trend of less apoptosis than TGF alone in agarose (19.0% vs 31.8%, $p=0.186$).

Glucocorticoid Receptor Antagonist Studies

RU-486 was added to the culture medium in ten-fold excess of Dex to determine whether the previous responses were mediated by the glucocorticoid receptor. At day 21, TGF plus RU-486 (TGF+RU) was not significantly different from the TGF alone condition in either scaffold, as expected (Fig. 3A). Importantly, the addition of RU-486 to the TGF+Dex condition significantly reduced sGAG levels such that there was no difference between TGF+Dex+RU and TGF alone in agarose. In RAD, a decrease in sGAG content was observed for TGF+Dex+RU compared to TGF+RU and TGF alone. DNA content was not significantly different among media conditions in either scaffold (data not shown).

Addition of RU-486 to the TGF+Dex condition in agarose reduced proteoglycan synthesis levels to that below TGF alone levels (Fig. 3B), consistent with the results of total sGAG content analysis in Fig. 3A. In RAD, addition of RU-486 lowered the TGF+Dex production of sGAG to that below the TGF alone level.

Bovine Aggrecan Western Blot

Western blots of aggrecan extracted from day 21 hydrogels were performed to analyze G1-NITEGE neoepitope fragments generated by ADAMTS-4/5 cleavage as well as all fragments containing a G1 domain. In agarose, both TGF and TGF+Dex showed NITEGE fragments (Fig. 4A), though the staining was reduced in the TGF+Dex condition. In RAD, NITEGE fragments were found in the TGF alone condition, but were dramatically reduced for TGF+Dex. Addition of the RU-486 GR antagonist resulted in the reappearance of full-intensity NITEGE bands when added to the TGF+Dex condition in both scaffolds. All conditions in both scaffolds showed full-length aggrecan as a dominant species, with NITEGE fragments also staining strongly in the agarose hydrogels (Fig. 4B). The ~140kDa band is consistent with m-calpain activity,^{36,37} although the activity of this enzyme was not investigated specifically in this study. The bands between 40-50kDa are consistent with link protein, which shares close homology to the aggrecan G1 domain.^{38,39}

Matrix Content and Aggrecan Western Blot for Human BMSC-Seeded Hydrogels

To investigate the applicability of these results to a more clinically relevant scenario, the response of human BMSCs (hBMSCs) to Dex when cultured in RAD and agarose scaffolds was also tested. hBMSCs produced minimal sGAG in agarose hydrogels (Fig. 5A), whereas sGAG accumulation in RAD hydrogels seeded with hBMSCs was ~50% of that of young bovine BMSCs in RAD gels. In agarose there was an increase in sGAG with Dex supplementation at both timepoints, while in RAD Dex had no significant effect. In agarose there was no significant increase in sGAG with time, whereas in RAD sGAG content increased significantly from day 14 to 21. DNA content and sGAG normalized to DNA are also reported in Supplementary figure S2.

Neither scaffold showed high levels of proliferation as measured by a fold change in DNA compared to day 0 (Fig. 5B). In RAD, TGF+Dex showed more proliferation than TGF alone at both timepoints. In agarose there was no significant effect of Dex. Neither scaffold showed a significant change in DNA content from day 14 to 21.

Proteoglycan synthesis normalized to DNA content in RAD increased with time and was higher in the TGF alone condition than TGF+Dex at both timepoints (Fig. 5C). Proteoglycan synthesis in agarose increased with time and showed no significant effect of Dex.

Hydroxyproline content in RAD hydrogels increased with TGF+Dex compared to TGF alone at both timepoints (Fig. 5D). Hydroxyproline content increased with time in RAD for both media conditions. Agarose showed identical statistical differences between conditions, though the levels of hydroxyproline content were less than ~33% of those found in RAD.

sGAG retention levels were ~50% in RAD with a significant increase for TGF+Dex compared to TGF alone (Fig. 5E). sGAG retention levels did not change significantly over time in RAD. In agarose, sGAG retention decreased with time and was increased by the addition of Dex. Total sGAG produced and total sGAG normalized to DNA are also reported in Supplementary figure S2.

Consistent with Western blots performed on hydrogels seeded with bovine BMSCs, aggrecan extracted from day 21 hBMSC-seeded RAD hydrogels showed NITEGE fragments with TGF alone, but a dramatic decrease in NITEGE fragments with TGF+Dex (Fig. 5F). Both conditions showed a strong full-length aggrecan band in the G1 blot. Aggrecan from hBMSCs cultured in agarose gels was not analyzed by Western blotting due to the limited quantity of sGAG produced.

Discussion

We hypothesized that Dex would improve matrix production and reduce ADAMTS-4/5 activity in agarose and RAD hydrogels seeded with adult human and young bovine BMSCs. We tested this hypothesis by comparing sGAG, DNA, and hydroxyproline accumulation, apoptosis, and ADAMTS-4/5-generated NITEGE fragments for self-assembling peptide and agarose hydrogels cultured in TGF- β 1±Dex. To our knowledge, this is the first study to report that Dex affects ADAMTS-4/5 activity in a tissue engineering system. We found that Dex reduced ADAMTS-4/5 activity across both hydrogel types and cell sources, but the overall effects of Dex on chondrogenesis depended on the donor species/age and the type of hydrogel. We have demonstrated chondrogenic differentiation through cartilage-like matrix production, including aggrecan and type II collagen. Others have investigated the effects of Dex on TGF- β -induced chondrogenesis at the gene expression level for both bovine and human BMSCs and have found evidence of the pro-anabolic effects of Dex which are consistent with our results.^{3, 11, 16, 40}

For young bovine BMSCs in agarose, Dex caused a two-fold increase in sGAG content, consistent with previous literature.¹¹ This increase was due to the increase in proteoglycan synthesis per cell caused by Dex. Although statistical comparisons were not made between agarose and RAD hydrogel results, as this was the focus of our previous work,³ it is interesting to note one additional finding. Bovine BMSCs proliferated to a greater extent in RAD than agarose, which ultimately resulted in the accumulation of more total sGAG even though sGAG per cell (as measured by sGAG normalized to DNA) was lower in RAD than agarose for the TGF+Dex condition (Fig S1A). sGAG was better retained in bovine BMSC-seeded agarose hydrogels with TGF+Dex compared to TGF alone. A trend of decreasing apoptosis was found in the presence of Dex for young bovine BMSCs in agarose at day 21, while RAD hydrogels with bovine BMSCs showed very little apoptosis in either condition. RAD gels seeded with young bovine BMSCs had reduced sGAG and hydroxyproline content in the presence of Dex compared to TGF- β 1 alone, but the overall levels of these matrix components were still higher than in agarose hydrogels in the presence of Dex at day 21. An increase in proteoglycan synthesis with Dex was observed on day 21; therefore it is possible that longer culture duration with TGF+Dex could have resulted in increased sGAG accumulation. The effects of Dex on sGAG accumulation and synthesis in both scaffolds were mediated by the signaling of Dex through the glucocorticoid receptor, which correlates

well with the ability of RU-486 to reverse Dex-induced aggrecan gene expression and type II collagen production by human mesenchymal progenitor cells derived from bone.¹⁶

Hydrogels seeded with adult human BMSCs showed less matrix production overall compared to young bovine-BMSC-seeded gels. In RAD hydrogels with hBMSCs, the addition of Dex increased DNA and hydroxyproline content, but not sGAG content. We were surprised to find very little evidence of chondrogenic differentiation by hBMSCs in agarose. A previous study of hBMSCs in agarose showed evidence of proteoglycan and type II collagen production at the protein level, but only one cell cluster was shown.²⁵ hBMSCs have been successfully cultured in other scaffold materials, though pellet culture remains one of the most common culture methods for these cells.^{10, 16, 41-44}

ADAMTS-4/5 activity, as measured by anti-NITEGE Western blotting, was decreased by Dex in RAD and agarose hydrogels seeded with young bovine BMSCs and for RAD gels seeded with adult human BMSCs. It is exciting that this finding was consistent across scaffold type and cell species/age. A recent study has shown that Dex does not regulate ADAMTS-4/5 activity at the gene expression level,¹⁵ but suggests the possibility that Dex could be involved in regulating the activation of latent ADAMTS-4/5 enzymes. This is an exciting research area that is the focus of ongoing studies. Use of the glucocorticoid receptor antagonist RU-486 confirmed that the decrease in ADAMTS-4/5-generated NITEGE fragments was mediated by the glucocorticoid receptor. This is an interesting finding that should be investigated further.

There were several limitations to this study. First, apoptosis was only investigated at early timepoints to ensure high viability, and at day 21 to compare to other histology samples. Further study of cell death over the entire culture duration comparing across conditions could aid in optimizing tissue engineering systems. Secondly, proteoglycan and DNA synthesis data were normalized to DNA content (Fig 1, 3, 5), which underestimates the synthesis levels in agarose, since the DNA levels include live and dead cells. Third, hydroxyproline content reflects multiple collagen types and the type present in agarose gels seeded with bovine BMSCs (Fig 1F) was not determined in this study. Since the matrix in agarose was largely pericellular (Fig 2A) and the pericellular matrix (PCM) of primary chondrocytes cultured for 21 days in agarose was found to be rich in type VI collagen,⁴⁵ we believe this is an abundant constituent of the PCM. Finally, our results encompassing young bovine and adult human BMSCs do not allow us to conclude whether differences in outcomes were associated with donor age or species. Both of these factors are important, and given the conflicting evidence about the importance of donor age for BMSC therapies, further studies are warranted.⁴⁶ Recent work by Erickson et al. has shown that BMSCs from young bovine tissue produce cell aggregates with higher sGAG and collagen content than BMSCs from skeletally mature adult donors, bringing forth the hypothesis that the difference in cell donor ages could be a contributing factor in this study.⁴⁷ Despite the limited number of human donors used in this study, we did find reproducible trends, which allowed us to find the statistically significant differences between conditions presented here. The differences we found between the cell types used here highlight the importance of considering the effects of species and age differences when translating in vitro studies into a clinical setting.

Several questions for future work remain, including how cell interactions with the scaffold microenvironment affect differentiation, proliferation, matrix production and remodeling, and cell death. This question is especially interesting given the lack of cartilage-like matrix produced by human BMSCs in agarose. Variables that may affect the response of BMSCs to Dex in different scaffolds include scaffold mechanical properties, scaffold interactions with newly-synthesized matrix, and cell-mediated scaffold compaction. We previously reported

that BMSCs maintain a rounded morphology in agarose throughout the culture duration, whereas BMSCs in RAD spread and elongate to achieve a networked morphology with extensive cell-cell contact early in culture.³ This is likely an important factor since cell-cell contact is an essential aspect of chondrogenesis in limb bud formation.⁴⁸

Dex does affect matrix production by BMSCs in agarose and RAD peptide scaffolds, but the specific results depend on the cell source and the scaffold type. These findings highlight the importance of choosing a scaffold, cell type, and growth factor combination carefully since the interactions between these variables can change the outcome. Dex reduced ADAMTS-4/5 activity in both types of hydrogels and both cell sources, suggesting an exciting new avenue for investigating interactions between Dex and ADAMTS-4/5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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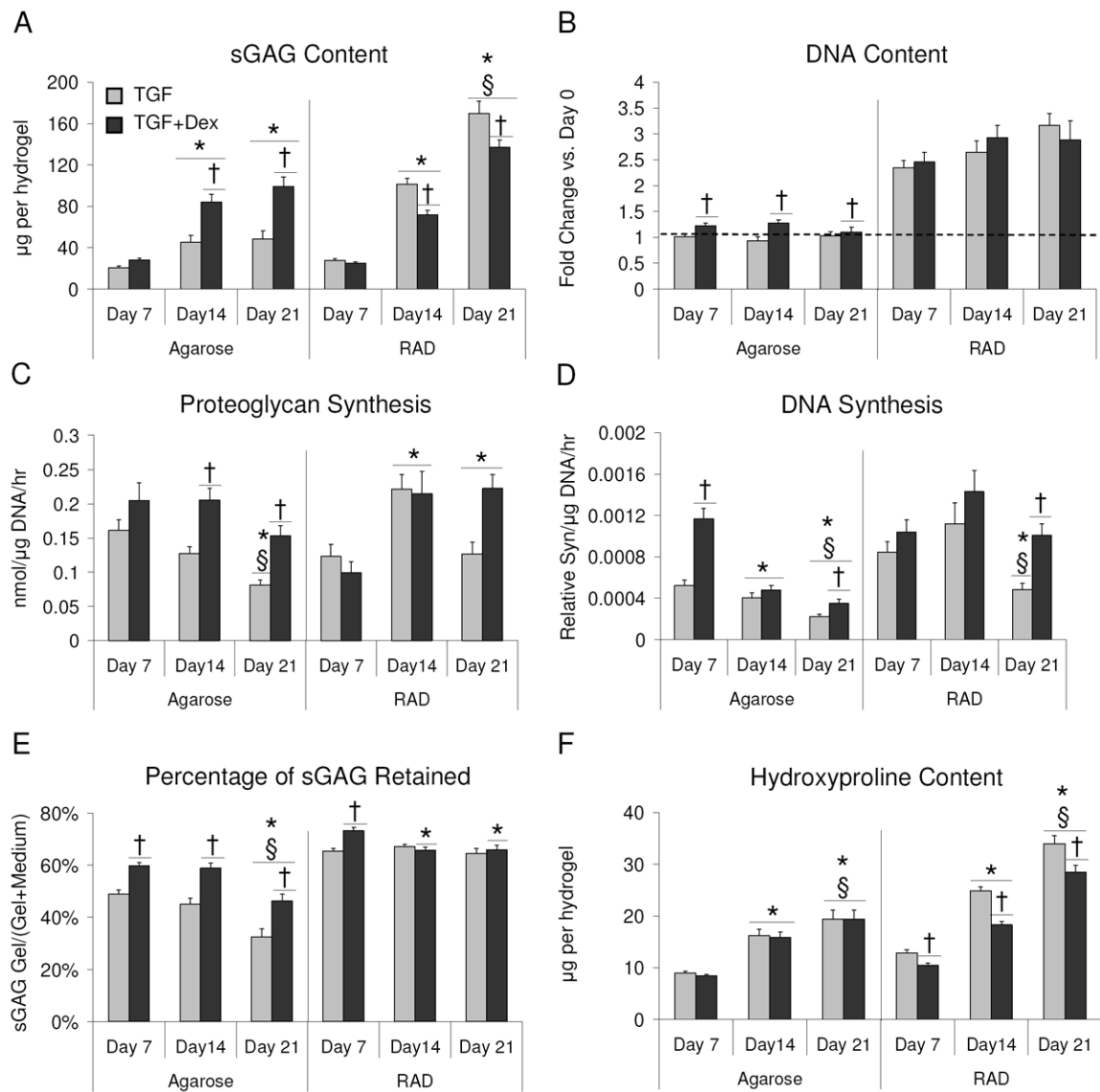


Figure 1. Extracellular matrix and cellular content in agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF or TGF+Dex medium. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content, (C) proteoglycan synthesis, (D) DNA synthesis, (E) percent sGAG retained, (F) hydroxyproline content. Values are mean \pm standard error of the mean. n=13-16 (3-4 hydrogels \times 4 animals). † versus TGF, * versus day 7, § versus day 14, p<0.05

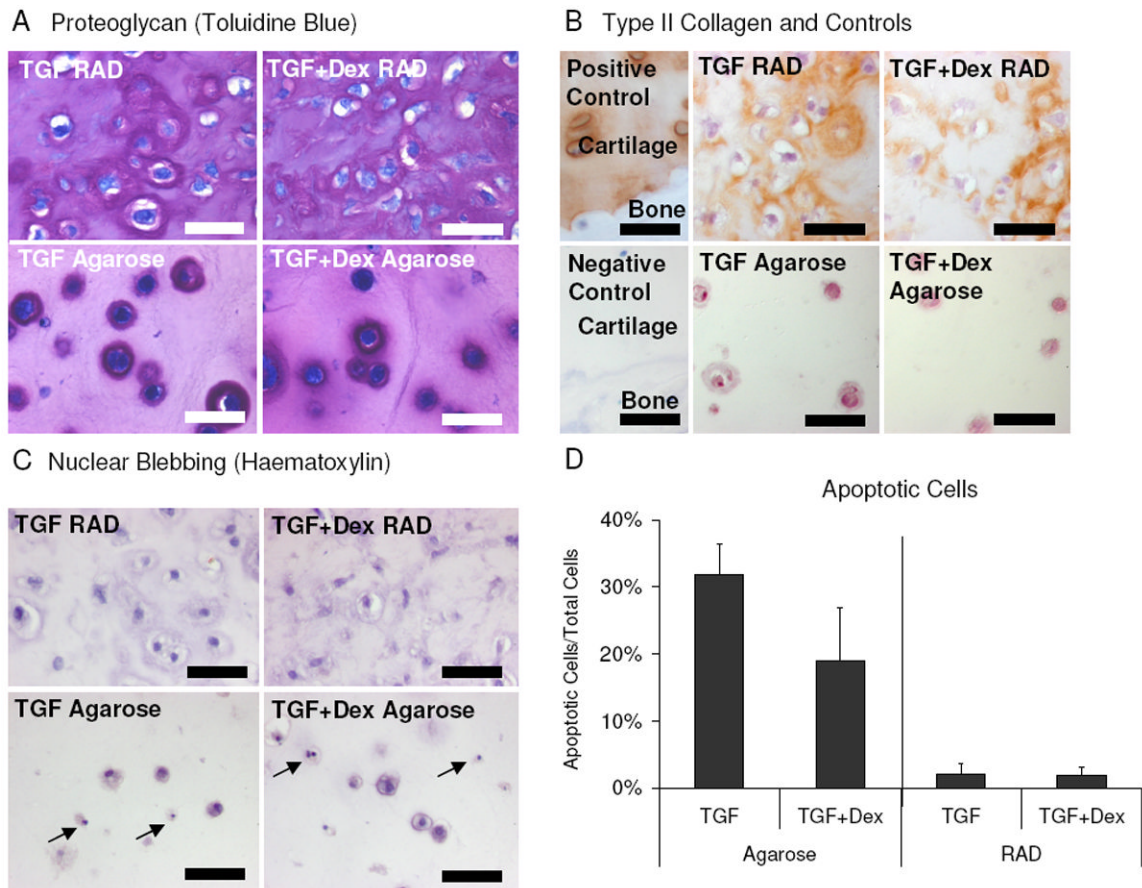


Figure 2. Representative staining of day 21 agarose and RAD hydrogels seeded with bovine BMSCs and cultured with TGF or TGF+Dex medium. (A) Toluidine blue staining for proteoglycans, (B) type II collagen immunohistochemistry, (C) haematoxylin staining for nuclear blebbing (arrows indicate apoptotic cells), and (D) percentage of apoptotic cells, n=3, values are mean \pm standard error of the mean. Controls performed on bovine osteochondral explants. Scale bar is 30 μ m.

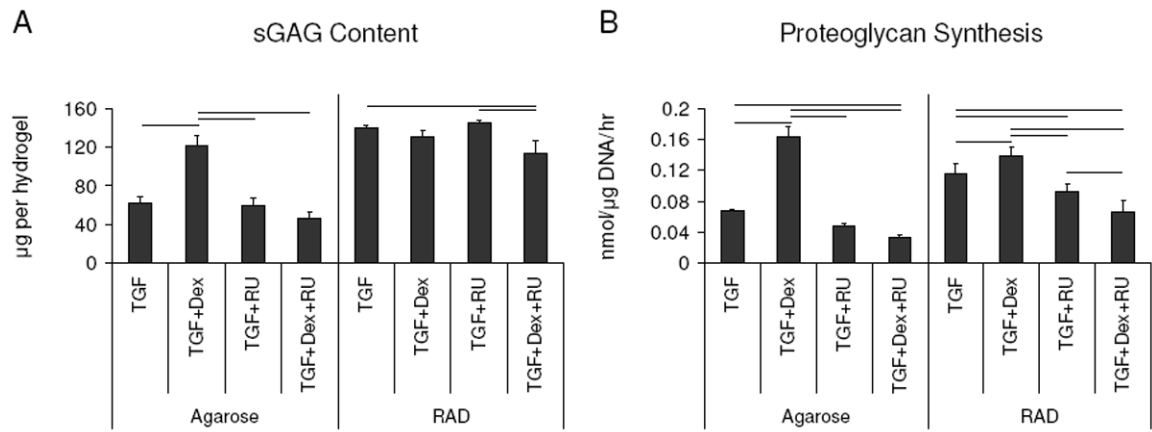


Figure 3.

Effects of RU-486 on matrix production in agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF medium \pm RU-486 and TGF+Dex \pm RU-486 medium for 21 days. (A) sulfated glycosaminoglycan (sGAG) content and (B) proteoglycan synthesis. Values are mean \pm standard error of the mean. n=8 (4 hydrogels \times 2 animals). Line indicates significant difference between two conditions, $p < 0.05$

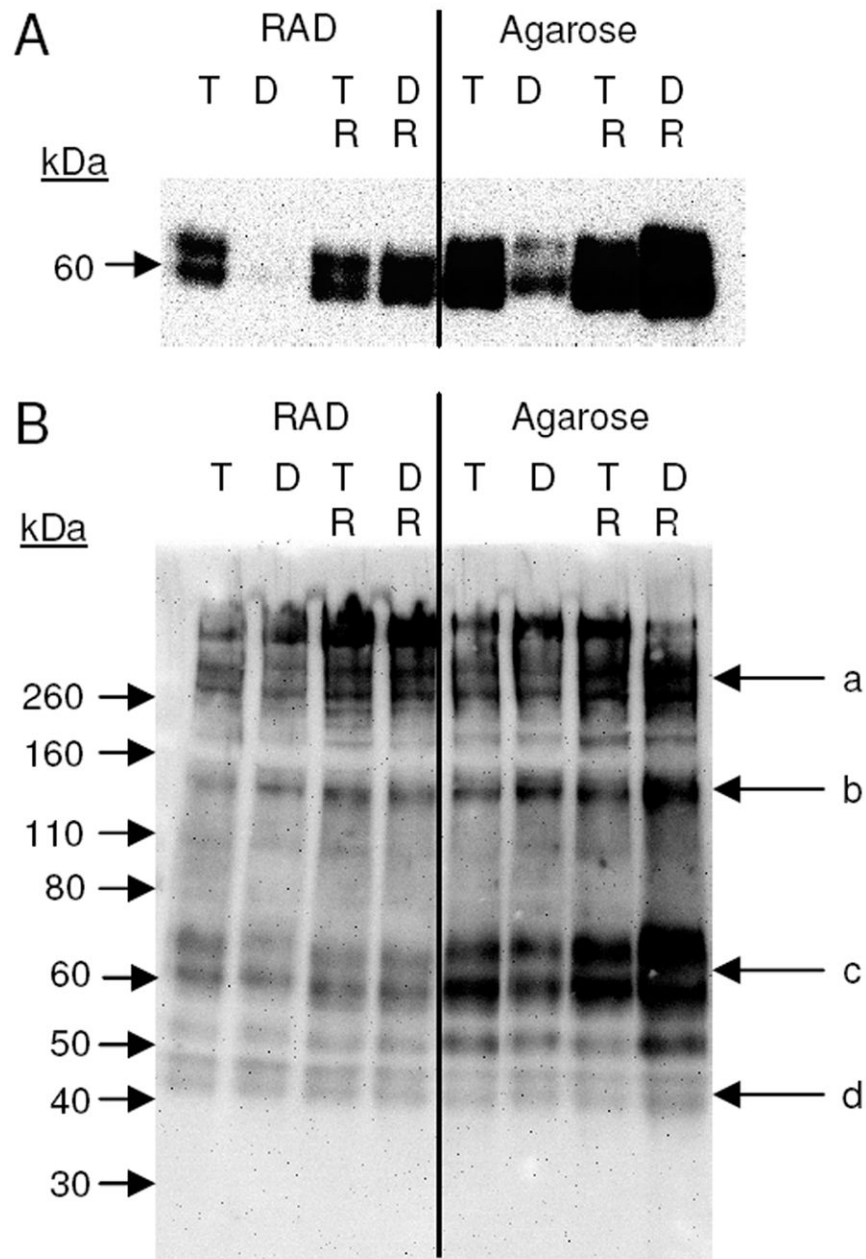


Figure 4. Analysis of aggrecan cleavage products extracted from day 21 agarose and RAD hydrogels seeded with bovine BMSCs and cultured in TGF medium (T) \pm RU-486 (R) and TGF+Dex (D) \pm RU-486 medium. 10 μ g sGAG loaded per lane. (A) anti-NITEGE Western blot and (B) anti-G1 Western blot. Arrows in (B) correspond to (a) full-length aggrecan, (b) potential m-calpain cleavage fragment, (c) G1-NITEGE fragment, and (d) potential link protein. Anti-NITEGE blot was stripped and re-probed with anti-G1 antibody to obtain image 4B. Representative of two repeats for each experiment type.

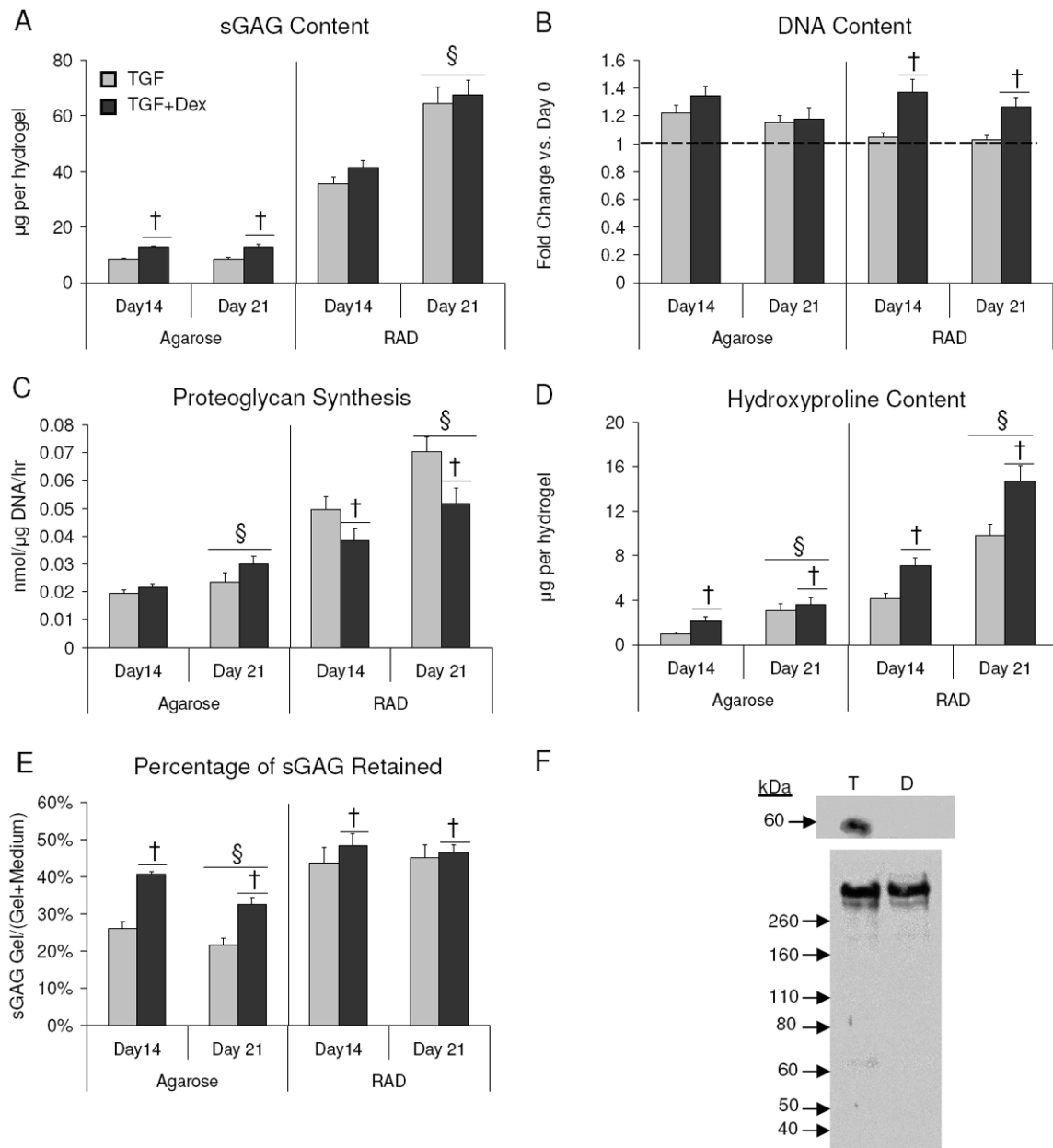


Figure 5. Extracellular matrix and cellular content in agarose and RAD hydrogels seeded with human BMSCs and cultured in TGF or TGF+Dex medium. (A) sulfated glycosaminoglycan (sGAG) content, (B) DNA content, (C) proteoglycan synthesis, (D) hydroxyproline content, (E) percent sGAG retained, (F) Western blot of aggrecan extracted from day 21 RAD gels seeded with human BMSCS and cultured in TGF (T) and TGF+Dex (D) medium. Top blot is anti-NITEGE, lower blot is anti-G1. 20µg sGAG loaded per lane. Values are mean ± standard error of the mean. n=11-17 (3-6 hydrogels × 2-3 patients). † versus TGF, § versus day 14, p<0.05