Effects of TGF-β1 on alternative splicing of Superficial Zone Protein in articular cartilage cultures

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**Objective:** Superficial Zone Protein (SZP) is expressed by the superficial zone chondrocytes and is involved in boundary lubrication of the articular cartilage surface. SZP protein expression is dependent on anatomical location and is regulated by the transforming growth factor-β (TGF-β) pathway. The hypothesis of this study was that between load-bearing, and non-load-bearing locations, of the femoral medial condyle alternative splice isoforms of SZP are different, and regulated by TGF-β1.

**Methods:** Using reverse transcription-polymerase chain reaction (RT-PCR) we identified differentially expressed SZP alternative splicing. Using recombinant proteins of the N-terminal region produced from these isoforms, we identified differences in binding to heparin and the extracellular matrix.

**Results:** We identified a novel splice form of SZP (isoform E), lacking exons 2–5. Differences in alternative splicing were observed between anterior load-bearing locations of the femoral medial condyle (M1) compared to the posterior non-load-bearing location (M4). TGF-β1 increased splicing out of exons 4 and 5 encoding a heparin binding domain. The minimal induction time for changes in splicing by TGF-β1 at the M1 location was 1 h, although this did change total SZP mRNA levels. Inhibition of Smad3 phosphorylation inhibited TGF-β1 induced splicing, and SZP protein expression. Recombinant proteins corresponding to isoforms upregulated by TGF-β1 had reduced binding. The SZP dimerization domain is located within exon 3.

**Conclusions:** In conclusion, alternative splicing of SZP is regulated by TGF-β1 signaling and may regulate SZP interaction with heparin/heparan sulfate or other components in the extracellular matrix of articular cartilage by splicing out of the heparin binding domain.

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**Introduction**

Articular cartilage is an avascular tissue with limited innate potential for repair and regeneration. The tissue provides a low friction surface for movements of the joints. Lubrication of these surfaces is critical to normal function. Products of the friction surface for movements of the joints. Lubrication of these potential for repair and regeneration. The tissue provides a low weight (approximately 345 kD). The full length bovine product is 1195 amino acids in length compared to that of human at 1404 amino acids. This difference is due to fewer mucin repeats within exon 6, while the N- and C-terminal domains are highly homologous. Oligosaccharides attached to the mucin repeats are thought to be responsible for SZP’s lubricating ability as removal of the (1–3) Gal-β1,3GalNAc moieties by endo-N-acetyl-β-galactosaminidase reduced lubricating activity by 77%.

SZP protein expression varies across different anatomical locations of the bovine femoral condyles with locations of higher loading having increased SZP expression. This experimental model is amenable to investigate the influence of chemical and physical forces including mechanotransduction. Osteochondral plugs from the M1 (anterior medial and load bearing) and M4 (posterior medial and...
non-load bearing) regions of the femoral medial condyles were used. These locations have shown the greatest variability of SZP expression and mechanical loading. Furthermore, upregulation of SZP protein by shear was through the transforming growth factor-β (TGF-β) pathway. Exogenous addition of TGF-β1 was sufficient to alter the SZP expression of tissue from non-load-bearing regions (M4) to resemble that of load bearing tissue (M1). Therefore we formulated these hypotheses: (1) the greatest difference in splicing may exist between the load bearing M1 and non-load-bearing M4 locations, (2) control of alternative splicing in articular cartilage between M1 and M4 may be regulated by the TGF-β signaling pathway. We tested if recombinant isoforms had functionally different binding abilities to superficial zone articular cartilage matrix. Because TGF-β splices out a potential heparin binding domain, we also tested and demonstrated binding to heparin.

Materials and methods

Materials

Articular cartilage was harvested from 1- to 3-week old bovine stifle (tibiofemoral) joints obtained from a local abattoir within 6 h of sacrifice. Dulbecco Modified Eagle’s Medium (DMEM)/F12 and antibiotic solution (both from Invitrogen, Grand Island, NY), cell culture components and reagents (from Sigma, St Louis, MO or Fisher Scientific, Tustin, CA), and human recombinant TGF-β1, Activin A, B and AB (R&D Systems Inc., Minneapolis, MN) were used in this study. TGF-β1 Type 1 receptor inhibitor SB431542 and cycloheximide were from Sigma, and TGF-β1 Type 1 receptor inhibitor Type IV (Type IV), and Smad3 phosphorylation inhibitor (SIS3) were from Calbiochem (San Diego, CA).

Tissue acquisition and culture

Bovine calf stifle joints were opened using an aseptic technique, and two side-by-side osteochondral explants were harvested from M1 (anterior and load bearing) and M4 (posterior and non-load bearing) regions of the femoral medial condyles [Fig. 1(A)]. Hereafter, these samples will be referred to as M1 and M4 explants. A 5 mm coring reamer was used to obtain the osteochondral explants while an adjustable custom-made jig was used to trim the explants to lengths of 4 mm removing the subchondral bone. The explants were allowed to equilibrate in serum- and cytokine-free culture medium consisting of DMEM/F12, 0.2% bovine serum albumin (BSA), 1% penicillin/streptomycin, 50 μg/ml ascorbic acid 2-phosphate, and 5% CO₂ at 37°C for 24 h. The medium was replaced after the equilibration period. Subsequently, the explants were incubated in a medium supplemented with 10 ng/ml TGF-β1 or 5 mM HCl with 0.1% BSA (vehicle control) for 1 and 2 days at 37°C and 5% CO₂. Inhibitors (SB431542, Type IV, SIS3) or cycloheximide treated samples were treated 1 h prior to addition of TGF-β1. All other wells were treated with the vehicle control [1 ml/ml dimethyl sulfoxide (DMSO) for SB431542, ethanol for Type IV, phosphate buffered saline (PBS) for SIS3 and cycloheximide]. Following treatment the top 200 μm was removed with a custom cutting jig and frozen in liquid nitrogen for RNA extraction.

Monolayer culture

Bovine calf stifle joints were obtained from a local abattoir and dissected under aseptic conditions. A Silvers miniature skin graft knife (Integra, Plainsboro, NJ) was used to obtain superficial zone samples from locations M1 and M4 of approximately 200 μm.

Fig. 1. SZP alternative splicing varies by anatomical location. (A) Tissue was harvested from the anterior location M1 (load-bearing) and posterior M4 (non-load bearing) of the femoral medial condyle. (B) Alternative splicing of SZP in M1 and M4 (assembled from a single larger gel). (C) Five SZP isoforms (A–E) were identified in the bovine superficial zone of articular cartilage.
Cartilage slices were digested with 0.2% collagenase P (Roche, Indianapolis, IN) in culture medium containing 3% fetal bovine serum (Gibco BRL, Grand Island, NY) for 3 h. Isolated chondrocytes were plated as monolayers at a density of $2.5 \times 10^5$ cells/well in 12-well culture plates (Corning, Corning, NY). Chondrocytes were allowed to equilibrate and attach for 16 h in 10% serum containing culture medium consisting of DMEM/F12, 0.2% BSA, 1% penicillin/streptomycin, 50 μg/ml ascorbic acid 2-phosphate, and 5% CO₂ at 37°C. The medium was replaced after the equilibration period with serum free DMEM/F12 and treated as described for explants11,13.

RNA extraction and RT-PCR

Total RNA was harvested from superficial zone cartilage by pulverizing samples using a metal impact chamber submerged in liquid nitrogen. RNA extraction was performed using the Qiazol Lipid RNeasy Kit (Qiagen, Valencia, CA). This utilizes a modified phenol–guanidine–chloroform extraction with on-membrane DNase I digestion to avoid genomic DNA contamination. cDNA was made from 5 μg of total RNA using Superscript First-Strand Synthesis System with random hexamer primers (Invitrogen, Carlsbad, CA). Primers were designed using Primer Express 1.9 (Applied Biosystems) following the recommended protocols using Primer Express 1.9 (Applied Biosystems). Forward and reverse primers are: 5’-AACAGCCCATCAGTAGGAAATACT-3’ located in exon 1 and 5’-AGTCCACTTCCATCTCTTACAGT-3’ located in exon 6, and straddle the spliced exons. The PCR reaction was optimized to ensure all isoforms produced remained within the linear range during amplification. The following amplification cycles were employed: 5 min at 95°C followed by 30 cycles of 95°C for 15 sec, 60°C for 3 min, 72°C for 3 min, and extension at 72°C for 7 min. PCR products were electrophoresed on 2% agarose gels for imaging and the bands were excised and sequenced to identify the specific isoforms [Fig. 1(C)].

Real-time quantitative PCR was done in triplicate on the cDNA with an ABI 7700 Sequence Detector (Applied Biosystems) following the recommended protocols using the last amino acids of exon 1 (QQASS) through the chondroitin sulfate attachment site at the beginning of exon 6 from each of the splice forms identified. In brief, these were cloned into a C-terminal egfp-c2 plasmid (Invitrogen) and a V5 tag was attached to the N-terminal end. A sixth clone representing empty green fluorescence protein (GFP) vector was also used as a negative control. Plasmids were transfected using Lipofectamine 2000 (Invitrogen) into human embryonic kidney (HEK) 293A cells. After 48 h cell lysate was harvested.

Recombinant binding ELISA

An enzyme-linked immunosorbent assay (ELISA) was performed to determine if different isoforms bind differently to the superficial zone extracellular matrix. Superficial zone articular cartilage was removed from load-bearing regions of the lateral condyle of bovine stifle joints, frozen in liquid nitrogen and then pulverized while frozen. Extracellular matrix was extracted with 4 M guanidine–HCl, 0.05 M sodium carbonate pH 7.4 solution containing phenyl methyl sulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma) overnight at 4°C. After extraction the solution was spun down to remove insoluble components. Extracted superficial zone extracellular matrix (10.0 μg/ml) or sodium heparin (2.0 μg/ml) was diluted in 0.05 M sodium carbonate and incubated overnight at 4°C in Maxisorp black well plates (Thermo Fisher Scientific). Extracellular matrix adsorbed plates were blocked for 1 h at room temperature with 1% BSA and washed. Recombinant truncated SZP isoforms were serially diluted in PBS and applied to ELISA plates for 1 h. Isoform binding was visualized using 1:5000 mouse anti-V5 primary antibody (Invitrogen), and 1:3000 goat anti-mouse HRP-linked secondary antibody (Bio-Rad). Signal was visualized using SuperSignal West Femto Chemiluminescence Substrate (Thermo Scientific). ELISA signal was normalized to equivalent amounts of protein run on immunoblot and analyzed for densitometry in ImageJ.

Statistical analysis

Differences in SZP mRNA expression following cytokine treatment were evaluated using a paired t-test (EXCEL Microsoft, Redmond, WA). A significance level of $P < 0.05$ was used to determine differences between groups, based on values from the gel densitometry.

Results

SZP alternative splicing varies by anatomical location

We mapped the alternative splice forms by anatomical location based on the previously identified mapping scheme [Fig. 1(A)] between the anterior load bearing M1 and posterior non-load-bearing region M410. SZP isoform ratios differed between the M1 and M4 locations in freshly isolated tissue [Fig. 1(B)]. There are five splice forms all in-frame for exons 2 through 5. The SZP isoforms identified in the superficial zone of articular cartilage (A–E) are listed in Fig. 1C. Isoform E lacking exons 2–5 was novel. In the M1...
location isoform C (splicing out of exons 4 and 5) was increased compared to isoform A (containing exons 1–5). This was designated as “full length” as this band is derived from an mRNA containing all of the exons in the alternatively spliced region and therefore all of the coding exons 1–12. This was reversed in M4 (n = 7).

**SZP alternative splicing is altered by TGF-β1**

To investigate whether TGF-β1 regulated alternative splicing of SZP we treated explant cultures for 24 and 48 h with 10 ng/ml TGF-β1. TGF-β1 was able to increase isoform C and decrease the full length isoform A in M1 and M4 explant tissues [Fig. 2(A)] with 24 h of treatment (n = 7). Temporal differences in total SZP were also observed between the M1 and M4 locations by qRT-PCR. Explants from M1 upregulated total SZP mRNA faster than M4 explants [Fig. 2(B)]. A significant increase in total SZP mRNA was seen by 24 h (5.07 ± 1.3 fold n = 7) and 48 h (9.5 ± 1.8 fold n = 3) in M1 explants (P = 0.007, 0.011) in M1 explants but not until (P = 0.044) 48 h (c) (3.9 ± 1.4 fold) in M4 explants (error bars represent 95% confidence interval, n = 6, 9, 7, and 3 for 1 h, 4 h, 24 h and 48 h timepoints respectively).

**SZP splicing is mediated through the TGF-β Type 1 receptor kinase and Smad3**

An inhibitor of TGF-β1 Type 1 receptor kinase (SB431542) was used to determine if this splicing signal was transduced through the TGF-β Type 1 receptor. Although 10 μM SB431542 was able to inhibit a decrease in isoform A by TGF-β1 after 1 h (n = 4) in M1 explants [Fig. 4(A)], the increase in isoform C by TGF-β1 only was weak, likely due to the use of DMSO as a vehicle control. Therefore this was repeated in 48 h monolayer culture, where addition of 10 μM SB431542 was able to inhibit the increase in isoform C and decrease in isoform A by TGF-β1 (P = 0.009, n = 3) [Fig. 4(B)]. This finding was then also confirmed in 48 h (P = 0.019, n = 3) monolayer cultures with 1 μM of Type IV inhibitor (Calbiochem), a different TGF-β receptor Type 1 kinase inhibitor, with similar results [Fig. 4(C)].

As Smad3 is one of the downstream targets of the TGF-β1 Type 1 receptor, we inquired whether Smad3 phosphorylation was required for splicing. Therefore 10 μM of SIS3 (Calbiochem), a specific inhibitor of Smad3 phosphorylation, was used. Addition of 10 μM SIS3 was sufficient to inhibit upregulation of isoform C in 1 h (n = 3) treatment of explants by TGF-β1 [Fig. 4(D)] (P = 0.006), and 48 h (n = 3) monolayer [Fig. 4(E) right] (P = 0.005). Furthermore, SIS3 was able to downregulate total SZP protein secreted into the media (n = 3) [Fig. 4(F)]. Addition of SIS3 downregulated both endogenous levels of SZP protein and that stimulated by TGF-β1. Differences in total SZP secreted into the media were also observed between M1 and M4 monolayer cultures although both upregulated SZP protein in response to TGF-β1 treatment.

**Exons 4 and 5 increase superficial zone articular cartilage extracellular matrix binding**

To identify differences in matrix binding between isoforms, truncated recombinant isoforms of SZP were produced [Fig. 5(A)]. Recombinant truncated isoforms of SZP bound strongest to extracellular matrix components present in guanidine extracts of superficial zone articular cartilage when exons 4 and 5 were present. Binding of isoform A (exons 2–5) and isoform B (exons 3–5) was...
stronger than that of isoforms with exons 4 and 5 spliced out (C–E) which had weaker binding observed [Fig. 5(B)]. Levels of binding of isoforms C and D were similar to that of isoform E, which contained only a partial exon 6 and the C-terminus. The level of signal at the no isoform control is similar to isoforms C–E at all concentrations. Therefore this represents background signal and indicates a lack of binding for isoforms C–E. As exon 4 encodes a heparin binding domain, we also tested if this was competent to bind to sodium heparin. Binding of isoforms A, B with exons 4 and 5 present was strongest, with weaker binding of forms lacking exons 4 and 5 (C–E) [Fig. 5(B)].

Exon 3 is a potential dimerization domain

SZP has been reported to exist in both monomeric and dimeric forms through a disulfide bond within the N-terminal domains.
To determine the dimerization domain of SZP, the recombinant truncated SZP isoforms were run on SDS-PAGE under reducing and non-reducing conditions. Bands of the correct size for dimers were observed for all forms except E (corresponding to isoform E), which lacks exons 2 through 5 [Fig. 5(C) left]. No dimers were observed on the reducing gel [Fig. 5(C) right]. Isoform D which only contains exon 3 (but not 2, 4, or 5) had a band of the correct size for a dimer. This supports exon 3, one of two repeated Somatomedin B domains, being sufficient for dimerization of SZP.

### Discussion

Alternative splicing of RNA permits the production of distinct protein isoforms from a single gene. Several splice forms of SZP have been identified in normal cartilage involving exons 2, 4, and 5. SZP alternative splicing in tendon has been shown to vary based on anatomical location corresponding to load. Shear loading induced upregulation of SZP protein through the TGF-β Type 1 receptor kinase. Previous work has demonstrated SZP protein expression increases with dynamic loading, and that this alters the ratio of the protein product sizes found when compared to unloaded controls.

It is noteworthy that alternative splicing of RNA in cartilage explant cultures is different in the various anatomical sites such as load bearing M1 compared to the non-load-bearing M4 site. In the M1 site splicing out of exons 4 and 5 is upregulated. Treatment of the non-load-bearing site M4 explant with TGF-β1 alters the alternative splicing to resemble load bearing M1.

The rapidity of the splicing response (1 h treatment) to TGF-β1 treatment in the absence of protein synthesis in cartilage explant cultures is remarkable. Inhibition of Smad3 phosphorylation demonstrates that phosphorylation of Smad2 is not sufficient to regulate splicing out of exons 4 and 5 in 1 h M1 explants or 48 h monolayer culture. However, this action could be indirect due to blocking of TGF-β induced upregulation of TGF-β signaling.

Disulfide bonded dimerization of SZP has been identified to occur within the N-terminal region of the molecule, localized to a region of exons 2 through 5. Although not definitive, the presence of dimers
of the apparent correct molecular size lends support to the Somatomedin B domain encoded within exon 3 as being the likely dimerization domain, it remains possible that exon 2 which contains a similar Somatomedin B domain is also capable of dimerizing. This remains unlikely to exist in nature though, as exon 3 is present in all alternative splice forms identified except E, and no isoforms were identified containing exon 2 but not 3. Loss of exon 3 appears to be relatively rare, as isoform E appeared to be of low abundance.

The functional differences between the alternatively spliced RNA forms are critical. The SZP C-terminal domain is critical for binding to extracellular matrix and recently a model supporting N-terminal domain binding abilities has been described. Although the binding conditions are quite different, this is puzzling in the light of, previous recombinant work that showed minimal binding of the N-terminal domain in recombinant proteins to intact cartilage superficial zone. Recent work has proposed that SZP may exist as a horseshoe configuration with both N and C terminals bound to the extracellular matrix, leaving the highly glycosylated central mucin domain exposed for interactions during boundary lubrication. Here we investigated the role of the N-terminal domains to bind matrix components, as potentially modulated by splicing. Compared to the isoforms containing exons 4 and 5 (A and B), those isoforms lacking exons 4 and 5 resulted in reduced matrix or heparin binding. However, as these matrix components were guanidine extracted, this may not represent native tissue conditions. This does suggest that SZP lacking exons 4 and 5, as upregulated by TGF-β, may have different binding capabilities.

Splicing may serve as a mechanism to control the ratio of SZP bound to the articular cartilage compared to SZP free floating in the synovial fluid, which can have consequences on lubrication. Therefore, in vivo conditions (changes in biochemical homeostasis or mechanical loading) might change binding ability of SZP to matrix, and subsequently lubrication of the joint. Furthermore, mechanistically this suggests that Smad3 may regulate both SZP matrix, and subsequently lubrication of the joint. Furthermore, mechanical loading might change binding ability of SZP to extracellular matrix and subsequently lubrication of the joint. Consequently, this may also represent native tissue conditions. This does suggest that SZP lacking exons 4 and 5, as upregulated by TGF-β, may have different binding capabilities.

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Study design and conception: GDD, AHR.
Data acquisition: GDD, SMTC.
Data analysis and interpretation: GDD.
Article drafting and revision: GDD, SMTC, AHR.
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Conflict of interest

The authors have no conflicts of interest to disclose.

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