Acta Scientific Orthopaedics (ISSN: 2581-8635)

Volume 3 Issue 8 August 2020

Case Report

Is TSG-6 the Most Important Biomolecule in the Setting of Orthopedic Surgical Immunobiologics? Scientific Concepts and Case Report with Cartigram® Imaging

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DOI: 10.31080/ASOR.2020.03.0195

Received: June 25, 2020

Published: July 18, 2020

Abstract

Bone marrow concentrates for knee arthritis have received much attention over the last decade as a viable treatment for osteoarthritic knee pain and disability [1]. In spite of the availability of these bone marrow concentrates, few authors have suggested a gold standard treatment protocol [2]. We first introduced our signaling cell procedure to orthopedic surgery in 2006 [3]. The procedure has undergone multiple iterations since that time. In this article, we introduce our seventh-generation technique for signaling cell treatment in the setting of osteoarthritis of the knee and discuss the role of tumor necrosis factor stimulated gene six protein in our signaling cell product.

We identified TSG-6 as an important chondroprotective biomolecule that is critical for the assembly and maintenance of the cartilage extracellular matrix. TSG-6 is an inflammation-induced protein that is produced at pathological sites, like synovial joints during arthritic degeneration. TSG-6 protects against joint damage through anti-plasmin activity and de-activation of serine protease during inflammation [4]. TSG6 also limits neutrophil migration and has an anabolic, immunomodulatory effect in synovial joints [5].

Modern signaling cell techniques are unable to capture or concentrate TSG-6 due to its small molecular size, that is coincident with the undesirable pro-inflammatory molecules. These molecules are on the order of 30 kD in comparison with anti-inflammatory molecules that typically are greater than 600 kD in size. Hyaluronic acid typically has a molecular weight of 3000 - 4000 kD and is bound by one of the domains of TSG-6, causing biological activation of the complex [6].

We combine our cell concentration product and a commercially prepared hyaluronic acid to formulate a growth factor concentrate that is activated as a scaffold for signaling cells we harvest from autologous bone marrow aspirate taken at the anterior gluteal pillar during immunobiologic procedures. We believe that by concentrating TSG-6 and including it as a component of our signaling cell transplant procedures, a biologically superior injectate is able to be assembled that may favor cellular and tissue anabolism. In addition, we harvest autologous clotting proteins (Thrombin/factor 2) to lock the product in the subchondral bone after micro core of the stiff, subchondral bone using a commercially available device.

Keywords: TSG-6; Cartigram®; Bone Marrow

Introduction

Tumor necrosis factor (TNF) stimulated gene 6, or TSG6, the only biochemical to carry a "chondroprotective" designation, is often overlooked and is almost universally lost in translation from bench science to clinical application of orthopedic immunobiologics, even with 5th and 6th generation techniques for signaling cell product concentration. Here we describe the mechanism of action of TSG6 and propose how that translates to the treatment of joint arthritis and subchondral remodeling with our algorithm for Nanoplasty™ and mechanical axis deviation (NAMAD®) previously described by us as a technique to manage unicompartmental gonarthrosis with MicroCore™.

Hyaluronan is the natural bioactivator of TSG-6. We explain the role of activated TSG-6 and the mechanism of action of hyaluronic acid in the setting of arthritis. Because the canonical and non-canonical pathways of both inflammatory (Rheumatoid type arthritis

or RA) and load dependent arthritis (osteoarthritis or OA) have a common signaling pathway tree, the biomolecule is critical in the setting of both processes to protect the articular surface regardless of the clinical arthritic condition. We have developed a simple technique for capturing and concentrating TSG6 in the setting of orthopedic immunobiologics for arthritis and arthritic joint pain using an FDA approved, indicated drug (HA) while maintaining adherence to strict standards of 'minimal manipulation' of harvested and concentrated bone marrow put forth by the FDA concerning the regulatory considerations for human cells, tissues, and cellular and tissue-based products: Minimal Manipulation and Homologous Use. The technique strictly adheres to Title 21 of the Code of Federal Regulations (CFR) Part 1271, specifically the 21 CFR 1271.10(a)(1) criterion of minimal manipulation and the 21 CFR 1271.10(a)(2) criterion of homologous use [7].

The process is FDA cleared for safety. Application of cell processors in the setting of signaling cell transplantation for arthritis constitutes off-label use and is experimental, although many clinical studies have defined the emerging role in the management of arthritis symptoms.[1] This fact is made perfectly clear to any patient who is considering, or who is offered our treatment algorithm, which has strict inclusion criteria. While the science is significant, verification of clinical translation to improved patient care with TSG-6 concentration has not been scientifically investigated by anyone other than us, to our knowledge. We are currently compiling our data to determine whether our unique approach to arthritis actually halts the progression of and reverses organic joint disease or simply relieves the symptoms through previously described nanomolecular, immunomodulatory, anti-inflammatory mechanisms described with simple intra-articular, autologous signaling cell concentrates. Intra-articular procedures performed without addressing the genesis of osteoarthritis at the subchondral bone level may be based in futility without attention to deviation in the mechanical axis [8]. However, with mechanical axis deviation, cartilage aggregates of repair have been demonstrated conclusively [9].

TSG6 is a 35000 Da, or 35 kDa protein composed of contiguous Link and Complement 1r/s, Sea Urchin epidermal growth factor (Uegf) and Bone Morphogeneic Protein (CUB) modules [10,11]. CUB modules are ubiquitous, multifunctional extracellular domains found in 16 functionally diverse proteins. Most CUB modules are involved in developmental pathways. Link modules are found in many proteins that interact with the ubiquitious glycosaminoglycan (GAG) Hyaluronic acid (HA), a vital component of the extracellular cartilage matrix that chondrocytes elaborate, reside in and migrate through [12]. The HA binding site of Link-TSG6 has been mapped by nuclear medicine spectroscopy and site-directed mutagenesis, identifying five key residues contributing to HA binding [13-16]. It is now clear that BMP-1 domains act in dorsal-ventral patterning via activation of transforming growth factor beta (TGFbeta)-like proteins BMP2, BMP4 and play a major role in patterning of extracellular and intracellular matrix formation [17]. In vertebrates, the BMP-1/TLD-like metalloproteinases play key roles in regulating formation of the extracellular matrix (ECM) via biosynthetic processing of various precursor proteins into mature functional enzymes, structural proteins, and proteins involved in initiating mineralization of the ECM of hard tissues [18].

Extracellular matrix (ECM) remodeling is the key feature of TSG6 production that draws our attention in the clinical setting of orthopedic immunobiologics. TSG6 interacts with ECM GAG and HA as well as two other ECM components, Chondroitan-4-sulfate and aggrecan [19]. TSG6 also binds the serine protease inhibitor, inter-alpha-inhibitor (IaI), which leads to crosslinking of hyaluronan chains and increased anti-plasmin activity, limiting matrix destruction. TSG-6 is a novel target that may offer structural matrix stability and remodeling when combined with axial load management strategies that reverse gravity and musculoskeletal vector-induced arthritic remodeling due to chronic inflammatory signaling. The clinical efficacy of hyaluronic acid is likely attributable to the presence and concentration of TSG-6 characterizing arthritis.

TSG-6 is presents in bone marrow aspirate and concentrates in the platelet poor plasma fraction of the isopycnic product. Even more advanced signaling cell transplant procedures that eliminate pro-inflammatory cytokines and preserve growth factors and healing response molecules through nanofiltration fail to conserve TSG-6 due to its molecular size exclusion. Commercial nanofilters have pore sizes that are approximately 50-60 kD and were designed to capture Alpha-2 macroglobulin, once described as the master molecule of inflammation [20]. We believe TSG-6 could be a more important biomolecule that should be preserved and concentrated in every case of signaling cell transplant for arthritis, particularly due to the ease of capture/concentration with an FDA approved drug ligand.

Tumor necrosis factor stimulated gene 6, or TSG6 is the only biochemical to carry a "chondroprotective" designation and may be the most important molecule in cartilage and joint metabolism when considered at the quantum, or nanomolecular, level. The immunomodulatory mechanisms of TSG-6 serve to protect articular hyaline cartilage from destruction and amplify anabolic cell signaling that favorably affect sapien symptoms arising from arthritic joint disease. As the name implies, TSG6 is indeed stimulated by the presence of tumor necrosis factor and interleukin-1 in joint fluid that is secreted by type A synoviocytes after mechanochemical stimulatory co-activation [21].

Arthritis is a naturally progressive process that comes from the body's biological cellular response to gravitational overload that occurs because of attempts to stabilize the physical machine against the pull of gravity. This constant 9.8 m/s^2 instantaneous force is countered by musculotendinous force vectors experienced with bipedal human locomotion that contribute to tissue overload. These gross force vectors that follow Newtonian physics are translated biomechanically to biochemical signals through immunomodulatory mechanotransduction mechanisms that dictate physical roles at the nanomolecular level, where particle physical laws predict physical outcome. Thanks to technology like single cell proteomics, flow cytometry and ELISA, we have been able to dissect the immunomodulatory signaling pathways that underlie arthritic diseases in the human under both primary and secondary inflammatory conditions [22]. Understanding these signaling pathway and their overlap with the basic metabolic requirements of other cells and tissues has expanded therapeutic opportunity in biologics in every field in medicine.

We designed and developed a specific signaling cell harvesting and processing protocol to capture and concentrate TSG-6 that was proven using enzyme linked immunosorbent assay (ELISA) techniques previously described [23]. We describe the application of our protocol to human orthopedic science and translation to a clinically viable algorithm for signaling cell transplantation in the setting of symptomatic arthritis of the knee.

Mechanotransduction and the genesis of TSG-6 secretion

In the setting of matrix destruction, like that seen in arthritic conditions, the secretion of tumor necrosis factor (TNF-a) and interleukin-1 (IL-1B) by type A synoviocytes (monocytes) prompts TSG production by chondrocytes, macrophage/monocytes, type B synoviocytes (fibroblasts) and vascular smooth muscle cells. TSG6 is rapidly upregulated by the presence of pro-inflammatory cytokines like IL-6 and IL-8 and serves as a spatiotemporal buffer to matrix metalloproteinase destruction during the first phase of inflammation. Overexpression of TSG6 causes an increase in progenitor cell proliferation, which confirms its potential role in the clinical management of arthritis where conversion to anabolic metabolism is favorable [24]. TSG6 maps to human chromosome 2q23.3 [25,26]. Synthesis is tightly regulated in a wide variety of cell types. Perhaps more important in the context of joint disease, growth factors like TGFB, EGF, FGF upregulate TSG6 in some cell types like chondrocytes [27]. Interestingly, TSG6 has not been shown to have osteogenic properties.

TSG6 is not constitutively secreted. Like endocannabinoids, it is secreted on demand by many of the cells involved in the immunomodulatory, anti-inflammatory cascade, that naturally follows the pro-inflammatory cascade and constitutes sapien healing. TSG6 appears in vascular smooth muscle cells following musculosketal injury and mechanical strain [28]. For our purposes, we are interested in the active secretion of TSG-6 by both synovial inflammatory cells (monocytes) and mesenchymal stem cells (MSCs). TSG6 is produced in the context of inflammatory mediators and is not found in healthy adult tissues. Interestingly, TSG6 expression is induced by PGE2, the only prostaglandin with an anabolic designation [29]. TSG6 mediates the function of MSCs to improve the structure and the attachment strength of the healing tendon-bone interface. TSG6 can be found in the synovial joint fluid, interstitial tissue fluid and even in the sera of patients with arthritis, pointing to a potential point-of-care marker for arthritis [30].

Since the anti-inflammatory cascade is part of the inflammatory cascade, it cannot be manipulated *in vivo* for therapy without first concentrating higher molecular weight anti-inflammatory proteins *in vitro* before returning them as an immunobiologic product as described for our treatment. Without TSG-6, the full anti-inflammatory complement is absent and may compromise the repair/regeneration stage of inflammation, which could affect the long-term remodeling third phase that concludes the process where extracellular matrix elaboration is critical. Modulation of the repair/regeneration and remodeling stages may lead to less fibroblastic scarring with more desirable tissue and better biomechanical properties.

The problem of TSG-6 exclusion in this setting is obvious. Our efforts to design and develop a nanofilter that would retain TSG-6 and was met without success initially. Once we understood the importance of the biomolecule, our attention was focused on how to capture and concentrate it. Our simple remedy has been to mix the marrow plasma with hyaluronic acid before passaging the marrow plasma across the filter. The HA binds to and activates TSG-6, which restricts serine protease destruction and has global anabolic tissue effects as described above.

TSG-6 is upregulated in many cell types in response to pro-inflammatory mediators and growth factors [31]. The protein has specific application in joint disease due to its chondroprotective effects in models of arthritis where extracellular matrix remodeling appears to be its primary responsibility. TSG-6 is part of a negative feedback loop that is capable of both matrix stabilization and immunomodulatory downregulation. TSG-6 is secreted by synovial type A cells in response to elevated levels of TNF-alpha and interleukin 1-Beta concentration ratios. Elevated levels of TNF-a and IL-1B secretion come at the expense of physical matrix degradation and recently enhanced proteome sequencing has revealed an interesting phasic response based on load application in both weight bearing and non-weight bearing joints that are susceptible to in-

flammatory conditions as the result of a non-concentric articular loading condition and mis-directed musculoskeletal force vectors.

Case Report

The patient is a 60-year-old female with symptomatic, unicompartmental varus gonarthrosis of the right knee. The patient initially presented in May 2018 with complaints of medial-sided knee pain severely limiting recreational and vocational pursuits and was felt appropriate for the procedure approximately eight weeks later. The patient was seen and evaluated by two other orthopedic surgeons who recommended total knee arthroplasty as the only treatment option. The patient presented to our clinic for alternative treatment strategies including orthopedic surgical immunobiologics and also for an explanation of pathology. She had also visited two pain medicine clinics proclaiming to offer regenerative medicine treatments who offered her wharton's jelly or umbilical cord cells respectively with a list price just under $8500 for one injection in each instance, representing more out of pocket cost than the autologous concentrated marrow Nanoplasty™.

The patient's history was significant for prior partial medial meniscectomy and use of meloxicam (Mobic®) daily for years. The patient had failed multimodality conservative measures including physical therapy, topical and systemic non-steroidal anti-inflammatory medications (NSAIDs) and corticosteroid (CS) and hyaluronic acid (HA) injections over a more than two-year period of time without adequate relief. The patient had a past medical history including controlled hypertension and gastroesophageal reflux disease optimized medically.

The patient reported no allergies. Upon physical examination, the patient was initially noted to lack full terminal range of motion equal to the contralateral, unaffected varus left knee. Pre-habilitation including manual stretching and strengthening of core and lower extremity was initiated. Achieving full, active extension without demonstrable contracture after stretching out the posteromedial capsuloligamentous structures of the knee is mandatory in all patients. No difference in side-to-side strength was detectable once the patient had completed the pre-habilitation phase, which is not required in some patients who demonstrate clinical appropriateness for therapy. Full, terminal range of motion is critical to achieving an adequate clinical result with this technique. Core, flexor and extensor strength should be optimized and balanced prior to proceeding with the algorithm.

The patient complained of pain in the anteromedial aspect of the ipsilateral right knee and a small effusion was noted. The left knee examination was normal. Lumbosacral and hip evaluation were normal including hip version. The patient had two finger-breadths of varus in the pre-procedural setting without a varus thrust on the right side. Some static correction of the varus deformity of the right knee was able to be clinically demonstrated, indicating ligamentous compliance and suggesting tolerance for unloader brace wear. Motor evaluation was normal and there were no gross neurovascular deficits distally. No asymmetry was detected. Foot and ankle examination were normal. Clinical images are depicted in figure 1.

Figure 1: Clinical presentation.

Plain films of the lumbosacral spine including flexion and extension views, hip and pelvis were normal. No phleboliths indicating prior VTED or primary lesions were noted. There was normal mineralization, no sacroiliac joint arthrosis or lumbosacral spondylosis or facet arthropathy. Radiographs of the knee, including long-standing films for alignment revealed medial compartment joint disease with joint space narrowing, tibial and femoral osteophytes and subchondral sclerosis of the concave and the convex sides of the medial joint. There was preservation of the lateral tibiofemoral and patellofemoral joints. Normal patellofemoral articulation. Radiographs are shown in figure 2.

Pre-procedural magnetic resonance imaging is standard in all of our patients who are considered potential candidates for regenerative cartilage procedures. A T2 wetmap Cartigram® (General Electric Health®, Chicago, IL) was obtained and findings on imaging sequences including the Terracon® and Cartigram® views are shown in figure 3. Preoperatively, the T2 images were used to approximate trajectory and to further visualize the area of dense microstructure (Figure 4).

Figure 2: Radiographs.

Figure 3

Figure 4: Pre-operative planning on T2 MRI of right knee.

The patient was deemed appropriate for the intervention based on her ability to consent to the experimental procedure with a full understanding of all risks, benefits and alternatives to orthopedic surgical immunobiologic treatment, including no treatment. The patient's youth, remaining articular cartilage, absence of exclusionary alignment issues with demonstrable ligamentous compliance, ability and desire to comply with all treatment recommendations, excellent fitness and reasonable expectations made her a candidate for the procedure. It was felt that the patient could adequately benefit from the clinical NAMAD® program as follows.

The patient received pre-operative assurance from her primary physician that she was appropriate to tolerate the office procedure. The patient was admitted to the clinic. One hour prior to arrival, the patient self-dosed an oral sedative-hypnotic and a dose of antibiotic. The patient performed Chlorhexidine 3% scrub at home the evening before and the AM of the procedure at the harvest and target sites. Procedure briefing was accomplished before proceeding.

We positively identified the anterior ipsilateral gluteal pillar as the harvest site and the right knee joint as the primary target surgical site. Indelible marker was used to mark the targets with the MDs initials. The patient was fit for an Ossur Rebound unloader prior to the intervention. The patient has the unloader today for application post procedure. The patient's vitals were taken and found to be stable and within normal range, consistent with the prior clinical visit metrics. Once the patient was deemed appropriate to proceed for preparation, they were transported to the procedural suite via wheelchair and helped onto the procedure table into a seated position.

A 'time-out' assessment was called and the op site was confirmed by the attendant RN and MD visually by indelible marker. Patient confirmed NKDA. The patient was then carefully placed into the supine position for the marrow aspiration procedure. Full sterile isolation barriers were used during the procedure following the prep. In each setting, DuraPrep® sponges were used to paint the intended harvest and target sites followed by application of sterile sticky drapes and maintenance of sterile precautions. The gluteal pillar of the left hemi-pelvis was marked four fingerbreadths back (7 cm) from the ASIS at the thickest part of the iliac crest. Carefully, 10-15 mL of local anesthetic containing Lidocaine 1% plain mixed 1:1 with Marcaine 0.25% with epinephrine was used to infiltrate the skin, subcutaneous tissue and periosteum about the right anterior iliac crest at the level of the gluteal pillar extraction. We let the local anesthetic take full effect so that it could be obviously demonstrated before proceeding. Patient stated VAS pain 3-4 for aspiration, coincident with mean of 3 typically in our experience.

The appropriate entry site was identified and marked, coinciding with the thickest portion of the anterior crest at the gluteal pillar. The Jamshidi was aimed towards the femoral neck. Using a 260 gram surgical mallet, the Jamshidi was gently and carefully advanced approximately 2.5 cm into the core of the pillar. The obturator was removed revealing the expected slow, but immediate egress of bone marrow.

12 mL of marrow plasma was extracted first for Arthrocare® thrombinator processing to allow for extraction of autologous thrombin for scaffold activation upon interosseous cell transplantation.

Next, six 10 mL syringes were used to aspirate bone marrow in sequential fashion with care taken to reposition the needle tip with rotation and/or advancement after each 9-10 mL aspiration to ensure the most efficient mesenchymal cell capture from the marrow.

Following completion of the bone marrow aspiration the Jamshidi was removed uneventfully. Pressure was held for 3 minutes followed by application of a sterile pressure dressing with 2% Bactroban ointment over a flexible fabric band-aid. The wound was checked prior to discharge of patient from the clinic and noted to be dry. The syringes were sequentially handed off as drawn to the RN assistant who immediately injected them through the clot/particulate filter into the sterile Celling Biosciences® ART® II Plus processor in preparation for centrifugation. Once all of the marrow had been transferred to the processing unit, it was counterweighted to 258g. The centrifuge was then loaded and set at a revolution speed of 3200 rpm for 14 minutes duration. Upon completion of centrifugation there were three distinct layers noted in the processor window including a robust buffy coat of nucleated cells. A commercially available, FDA approved and indicated hyaluronic acid (HA) compound was added to the concentrate and platelet poor fractions followed by an additional one minute spin at 3800 rpm. ILLUSTRATION 1 The plasma fraction was actively passaged with the HA through the processor's built-in A2M nanofilter to capture the TSG-6 heavy growth factor concentrate into a total of 5.0 mL and concentrate TSG6. This was mixed with the autologous thrombin and $CaCl_3$ in order to establish pliable ECM cellular scaffold for injection that would gel interosseously at the time of cell transplantation. The nucleated cellular injectate from the bone marrow fraction was aspirated from the ART II plus processor into a volume of 5.0 mL. Total volume for injection was 5.0 mL BMC and 5.0 mL GFC. The product was split into injection syringes in preparation for the Nanoplasty™.

Next, the mini C-arm was draped sterile in preparation for the subchondral nanoplasty as the patient was prepped and draped in standard fashion. Initially, attention was turned towards the subchondral component of the nanoplasty. The bony anatomy was palpated and marked using an indelible marker. The insertion of the pes footprint serving as the starting point with trajectory directed towards the central depression of the medial compartment and confirmed in orthogonal planes to cross the physeal scar. The angle was set at approximately 40-45º relative to a vertical line based off of the medial malleolus to approach the most affected area of the native load cylinder. We identified the ideal starting point and retrograde trajectory to converge on the most sclerotic subchondral zone which was manually fine-tuned in surgical fashion. Care was taken to avoid joint penetration at the zone of calcified cartilage and to cross the physeal scar during the approach (Figure 5).

Figure 5

Avoidance of the joint cavity was confirmed during the intra-articular component of the procedure where only clear, yellow synovial fluid was observed without particulate as the Jamshidi remained intraosseously, impregnating the subchondral bone.

A 3 mm stab incision was made and a 260 gram surgical mallet was used to advanced a fresh Jamshidi® so that the distal tip was parked in the subchondral bone beneath the subchondral plate on image intensification. Once the position of the Jamshidi was confirmed in multiple planes, 15 mL bone marrow aspirate was aspirated and passed off the table. Following dilution and nucleocount, the cell tally was below the detection limit of the Nucleocounter 250 device, <5k/mL TNC.

Next the 1.0 BMC/1.0 GFC/1.0 thrombin injectate was very slowly injected interosseously with rotation of the Jamshidi to achieve a "rain-bird" effect, which the patient tolerated well. We have found this to be consistently the most uncomfortable part of the procedure for the patient with VAS pain 4 - 5 even with adductor blockade, which we have abandoned in favor of local anesthetic due to equal clinical efficacy in our experience. Once the injection was accomplished, the Jamshidi obturator was replaced and the injectate allowed to sit 2 - 3 minutes while the thrombin took full effect. Once the interosseous injection had been accomplished and with the Jamshidi indwelling, aspiration of the knee was accomplished. Aspiration of clear synovial fluid from the joint during the next step is confirmatory of extra-articular and appropriate Jamshidi introduction outside of the synovial folds of the knee joint.

14 mL of clear, straw colored synovial fluid without particulate was aspirated from the knee using an 18 ga needle. There was no evidence of hemarthrosis. Injection of the remaining 8.0 mL cellular/scaffold concentrate was then easily accomplished through the same right lateral para-patellar approach without difficulty using the indwelling 18 ga needle, which was removed promptly after scaffold injection. The injection site was cleaned with chlorhexidine 3%, dried, followed by application of Bactroban 2% ointment and a sterile flexible strip, which is standard for all of our puncture sites. A sterile compressive wrap was applied. Following the knee joint injection, the joint was placed through a full ROM several times including patellofemoral translation. Motion was excellent and the injection was successful. The Ossur® Rebound® unloader was then applied and adjusted. The patient tolerated the procedure extremely well and there were no complications.

A final set of vitals were taken, found to stable and within normal limits. No ice or any anti-inflammatory measures are to be used to preserve natural healing. Follow up in fourteen days for evaluation and release to physical therapy. ADLs with ambulation using Ossur Unloader Brace only with daily LHW orthotic use. The patient will call me immediately with any questions or concerns:

- SCi Nanoplasty-1.0 BMC/1.0 GFC/1.0 autologous thrombin
- Articular Injection-4.0 BMC/4.0 GFC

In the setting of severe posteromedial structure contracture, we consider pie crusting versus Tenex-Health TX-2 cicatrix release® to free up tight medial structures including deep and superficial MCL where necessary.

Unloader Brace and bilateral custom lateral heel wedge orthotics. No impact activity for six weeks. The patient will call me immediately with any questions or concerns. Pt has my mobile number, home number and email contacts and was personally contacted on the night of the procedure to ensure an excellent outcome.

Low intensity pulsed ultrasound (LIPUS)

We now provide a handheld personal low intensity pulsed ultrasound (LIPUS) device to all patients with instructions on appropriate use in the post-procedural setting. All living organisms are subject to external physical forces in their environment. The conversion of these physical forces into biochemical signals and integration of these signals into a functional response is termed mechanotransduction. On a cellular level, a mechanical stimulus generates a biochemical signal, which in turn can bring about a number of intracellular processes. These include activation of complex, spatiotemporally bound signaling pathways, upregulation or downregulation of gene expression and alteration of protein synthesis, resulting in adjustment of the intracellular and extracellular environment in response to the initial mechanical stimulus. This mechanosensitive feedback modulates cellular functions as diverse as migration, proliferation, differentiation, and apoptosis, and is crucial for organ development and homeostasis [32].

Low-intensity pulsed ultrasound (LIPUS) has been commercially available since its 1994 Food and Drug Administration (FDA) approval as an adjuvant therapy in the healing of primary fractures [33]. It exerts a micromechanical stress over its target site and has been shown *in vitro* to increase the incorporation of calcium ions in cultures of cartilage and bone cells while stimulating gene expression implicated in the healing process.

In vivo work by Naruse., *et al.* implicates COX-2 as the central protagonist for mediating LIPUS-induced osteogenesis [34]. Application of LIPUS on bone marrow stromal cells elevated levels of IGF-I, OCN, and bone sialoprotein mRNA, which were in turn eliminated by application of a COX-2 inhibitor. These findings have been substantiated by Tang., *et al*. who demonstrated increased expression of COX-2 in osteoblasts via activation of integrins and subsequently kinase pathways following application of ultrasound [35].

The technology is now widely used in clinical practice, despite the exact biological mechanism of function remaining unknown. Low-intensity pulsed ultrasound was prescribed by 21% of Cana-

dian trauma surgeons in the management of acute tibial fractures in 2008, and LIPUS technologies are available to be prescribed on the NHS in the UK. LIPUS promises a relatively low-cost, non-invasive technology to assist in fracture healing and orthopedic surgical immunobiologic regenerative medicine options. Unfortunately, despite the presence of many trials in the field, a definitive answer in support of its use remains elusive.

It is evident from review of the literature that the application of molecular-level mechanical forces has a clear osteogenic effect on both *in vitro* and *in vivo* cells. Macroscopically, the clinical evidence is less convincing. Current mechanotransductive technologies have failed to prove their utility despite tantalizing *in vitro* evidence and plausible biochemical mechanisms, leaving patients with the deleterious effects of fracture nonunion. A global consensus regarding the optimal way to treat nonunion remains undecided, but stimulating de novo bone formation is likely to be pivotal in the development of an effective therapy and may play a unique role in the stimulation of subchondral bone remodeling in the setting of cartilage sparing procedures.

Given how far the understanding of the physiological mechanisms underpinning mechanotransduction has progressed, together with the osteogenic properties that numerous *in vitro* and *in vivo* mechanotransduction studies have demonstrated, it is hopeful that future research will identify effective novel targets for de novo bone formation utilizing mechanotransduction.

Total nucleated cell count

The patient's total nucleated cell count was obtained immediately following the concentrated cell matrix procedure. Samples were preserved in heparin for enzyme linked immunosorbent assay assessment including bone marrow aspirate (BMA), bone marrow concentrate (BMC), platelet poor plasma (PPP) and growth factor concentrate (GFC) (Figure 6). Total nucleated cell count was 3.22 x 10^8 with 98% viability. We do not routinely plate for CFU-fs. Samples of bone marrow aspirate (BMA), platelet poor plasma (PPP), growth factor concentrate/TSG-6 (GFC6) and bone marrow concentrate were preserved for ELISA.

Post procedure

Post procedure the patient was allowed normal WBAT for two weeks while she continued leg extension exercises and core fitness. Unloader brace wear was at all times while gravity dependent, avoiding stairs. Gait and balance were the primary focus for the first two weeks. The patient was seen at two weeks and pain relief was approximately 50%. The patient was allowed to progress on our physical therapy algorithm specific to this procedure which demands achievement of full range of motion before progressing to strengthening. At six weeks the patient was seen progressing well and was allowed unloader brace wear with activity only and limited impact. At twelve weeks, the patient remained symptom free and able to enjoy recreational and vocational pursuits unhindered.

Figure 6: Laboratory analysis including total nucleated cell count and viability is done immediately following the immunobiologic procedure.

The patient is approaching three years follow up with no return of clinical symptoms and has returned to playing doubles tennis and pickleball without limitations. The patient is able to enjoy all of their desired recreational and vocational pursuits and indicated they would have the procedure again and that they felt the cost justified.

We perform serial MR Cartigram® Imaging at one-year intervals with the results shown in figure 7A and 7B. At one year, there has been interval healing of the subchondral bone on the convex side of the joint and healing on the concave side of the joint where arthritis was originally generated.

Figures 7A and 7B: T2 wetmap MRI demonstrating changes at one-year post procedure. The subchondral bone is healing and the arthritis is no longer progressing. New tissue is evident. (GE Cartigram®, GE Health®, Chicago, IL).

Conclusion

TSG-6 is a member of the Link module superfamily and binds hyaluronan, and other glycosaminoglycans a vital part of the extracellular matrix by the link module. TSG-6 forms covalent and non-covalent complexes with inter-alpha-inhibitor, which is a serine protease inhibitor (SERPIN), potentiating its alpha-2 antiplasmin activity. Plasmin is an important enzyme that participates in fibrinolysis and protein catabolism [36]. TSG-6 is significantly associated with inflammatory mediators including TIMP1, ATM, MMP3, VEGF, VCAM1, ICAM1 and IL-6 and stimulated by pro-inflammatory cytokines, growth factors and hormones [37].

TSG-6 is perhaps the most important molecule in cartilage repair metabolism and deserves its designation as "chondroprotective". TSG-6 shares a similar molecular weight to all of the pro-inflammatory proteins in the 25-30 kD range. TSG-6 is not found in articular fluid until arthritis has been initiated by an inflammatory process, as determined by the presence of TNF-a and IL-1. Our lab has performed quantitative assays for TSG-6 in bone marrow aspirate, platelet poor plasma, growth factor concentrate and bone marrow concentrate.

These assays have demonstrated the presence of the protein in bone marrow and platelet poor plasma as well as the growth factor concentrate. We have demonstrated the ability to concentrate TSG-6 using a very simple, FDA-approved, third party payer reimbursed procedure. We believe this next generation modification should be performed with every cell transplant procedure that is currently performed. Our early clinical data is encouraging and the technique may represent part of a best clinical practice model for an evolving gold standard in cell based therapy (CBT) management. Prior to using the next generation techniques we now employ, patients reported improvement of symptoms within three weeks. With next-gen TSG-6 capture, patients have reported relief the day following the procedure. We believe there is a major role for an unmet need where arthritis is concerned and that many if not most knee arthroplasty procedures can be at least delayed through judicious use of these treatment techniques. Autologous biologics are based in science and on immunologic signaling pathways. We describe a method that is safe, cost effective, scientifically based and clinically proven, at a cost far less than clinically available biosimilars produced by big pharma demand.

We have developed a novel technique to capture TSG-6 in spite the fact that TSG-6, unlike other anti-inflammatory cytokines, matches the molecular weight of the pro-inflammatory molecules. TSG-6 does not appear in normal synovial fluid. As its name implies it is not released until tumor necrosis factor alpha and interleukin-one (IL-1) activate fibroblast-like synovial lining cells (FLS). Es-

tablished programs should have written, consistent protocols including the use of a few therapists who understand the treatment concepts and techniques behind orthopedic surgical immunobiologics.

Current commercially available bone marrow processors intended to capture the nucleated signaling cell population does not retain proteins with MWCO of less than 65000, or 6500 kDa, even when equipped with protein nanofilters. The Celling Biosciences® processor ART II BMC kit comes standard with a polyethersulfone nanofilter. Emcyte Corporation® has separate filters available for disposable use in the setting of cell concentrating procedures. With the more specific filtration we describe that captures and concentrates TSG6, we believe a better biologics product for patient delivery can be constructed in the point-of-care setting.

Specifically, TSG6 and transforming growth factor Beta, both conclusively demonstrated to be critical components of cartilage metabolism are involved in the mechanotransduction pathways. Progenitor cells will not specialize into chondrocytes in the absence of TGF-B.

We believe that custom engineering an autologous scaffold from the harvest is essential in delivering the best possible orthobiologics product. Fibrin forms a basis for cells to anchor via integrins and cadherins. Conglomeration of cells sets up a relatively hypoxic environment that encourages cell adhesion and activation upon injection. The injectate may be more effective if activated by an appropriate CaCl (or CaGluconate) and thrombin concentration that allows the product to 'set up' permanently in the intraosseous compartment of the subchondral bone.

Mechanotransduction comes from adherence of cells to each other and to the matrix molecules they elaborate. Protein cadherins and integrins account for cell-to-cell and cell-to-matrix interactions that are load responsive. Activation of mechanoreceptors leads to intracellular signaling and spatiotemporally based immunomodulation of living tissues. Coupled with subchondral mechanotransduction is the osteoclastic resorption front that leads to osteoblast migration and osteocyte and matrix formation in the challenged subchondral bone. Additional supportive bone is laid down in a support column corresponding to the mechanical axis and bordered by the physeal scar, where mechnoreceptors are concentrated on loaded bone and cartilage cells. This feature is easily seen on terracon MR image sequences. Otherwise the concave, elastic subchondral bone is remodeled and takes on the material characteristics of cortical bone, which is much more stiff and unable to absorb the impact shock from the convexity. As additional bone is remodeled, intraosseous pressures become elevated and oppose new vascular flow and ingrowth, compounding the problem as subchondral bone becomes relatively avascular.

TS6 is a small biomolecule with a molecular weight of approximately 35 kD. It is the smallest of the anti-inflammatory biochemicals of interest, and perhaps one of the most important. Alpha-2-macroglobulin, often called the 'master regulator' has a molecular weight of 800 kDa and is typically snagged and concentrated using commercially available nanopore filters with a pore size of 65 kDa when advanced cell concentrating techniques are applied clinically. With a molecular weight of 30 kDa, the native TSG-6 is lost during nanofiltration due to an inability to be captured based on size exclusion principles. TSG-6 is the only biochemical that has a 'chondroprotective' designation. We have proven using ELISA that the additional steps does concentrate TSG-6. These additional steps have the potential to allow clinicians to elaborate a more aggressive signaling cell product with novel targets, producing better clinical outcomes.

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