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Dynamic Mechanical Compression of Devitalized Articular Cartilage Does Not Activate Latent TGF- β

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Abstract

A growing body of research has highlighted the role that mechanical forces play in the activation of the latent TGF- β in biological tissues. In synovial joints, it has recently been demonstrated that the mechanical shearing of synovial fluid, induced during joint motion, rapidly activates a large fraction of its soluble latent TGF- β content. Based on this observation, the primary hypothesis of the current study is that the mechanical deformation of articular cartilage, induced by dynamic joint motion, can similarly activate the large stores of latent TGF- β bound to the tissue extracellular matrix (ECM).

Here, devitalized deep zone articular cartilage cylindrical explants (n=84) were subjected to continuous dynamic mechanical loading (low strain: $\pm 2\%$ or high strain: $\pm 7.5\%$ at 0.5 Hz) for up to 15 h or maintained unloaded. TGF- β activation was measured in these samples over time while accounting for the active TGF- β that remains bound to the cartilage ECM. Results indicate that TGF- β 1 is present in cartilage at high levels (68.5 ± 20.6 ng/mL) and resides predominantly in the latent form ($>98\%$ of total). Under dynamic loading, active TGF- β 1 levels did not statistically increase from the initial value nor the corresponding unloaded control values for any test, indicating that physiologic dynamic compression of cartilage is unable to directly activate ECM-bound latent TGF- β purely mechanical pathways and leading us to reject the hypothesis of this study.

These results suggest that deep zone articular chondrocytes must alternatively obtain access to active TGF- β through chemical-mediated activation and further suggest that mechanical deformation is unlikely to directly activate the ECM-bound latent TGF- β of various other tissues, such as muscle, ligament, and tendon.

Keywords

TGF- β activation; articular cartilage; dynamic loading; growth factors; extracellular matrix

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Conflict of Interest Statement

We do not have any conflicts of interest with regard to this study and the materials contained herein.

Introduction

The maintenance of articular cartilage is dependent on the well-coordinated metabolic regulation of chondrocytes through mechanical and biochemical factors. Cartilage requires mechanical stimulation, in the form of physiologic joint activity (Jurvelin et al., 1986; Sood, 1971), as well as access to a variety of signaling molecules, including growth factors and other cytokines (Malemud, 1993). Transforming growth factor beta (TGF- β) is one of the most extensively investigated regulators of cartilage metabolic activity (Blaney Davidson et al., 2007; Trippel, 1995) due to its high levels in synovial joints (Morales et al., 1991) and well-characterized ability to modulate the biosynthesis of a large number of cartilage extracellular matrix (ECM) products (Chandrasekhar and Harvey, 1988; Hiraki et al., 1988; Morales and Roberts, 1988; Niikura and Reddi, 2007; O'Keefe et al., 1988; Recklies et al., 1998; Redini et al., 1988; Schmidt et al., 2008). However, the extracellular regulation of TGF- β activity and the physiologic mechanisms through which chondrocytes access the growth factor continue to remain unclear.

In cartilage, as in other tissues, TGF- β is initially synthesized in an inactive latent complex (complexed with the latency-associated peptide (LAP) and latent TGF- β binding protein (LTBP)) that is unable to bind to membrane receptors, and thus unable to induce a biological response (Annes et al., 2003; Hyytiainen et al., 2004). Large stores of latent TGF- β are bound to the cartilage ECM, reported at ~300 ng/mL (of which ~80 ng/mL is the TGF- β 1 isoform) (Morales et al., 1991). However, the mature peptide must first undergo activation (release from LAP), before it can modulate the metabolic activity of chondrocytes.

In general, TGF- β activation mechanisms are believed to be tissue specific and, for a given tissue, several mechanisms may be involved (Hyytiainen et al., 2004). It has traditionally been understood that latent TGF- β primarily undergoes physiologic activation through a variety of chemical mediators (Barcellos-Hoff and Dix, 1996; Lawrence et al., 1985; Lyons et al., 1990; Murphy-Ullrich and Poczaek, 2000; Yu and Stamenkovic, 2000). However, a growing body of research has highlighted the important role that mechanical forces may play in the activation of latent TGF- β (Tenney and Discher, 2009). Originally, this concept was advanced through the demonstration that cells can activate TGF- β in their surrounding ECM through integrin-mediated contractile forces (Annes et al., 2004; Wipff and Hinz, 2008; Wipff et al., 2007). Subsequently, it has been demonstrated that in the absence of cells, mechanical fluid shearing can activate latent TGF- β in platelet releasate (Ahamed et al., 2008; Ahamed et al., 2009). Since the TGF- β peptide is loosely (noncovalently) bound to the LAP, it has been suggested that the molecular conformational alterations of latent TGF- β , induced by fluid shearing, are sufficient to promote the release of the TGF- β peptide (Tenney and Discher, 2009).

In a recent experimental investigation, we have further demonstrated that mechanical forces play a critical role in TGF- β activation in synovial joints (Albro et al., 2012). During joint motion, opposing articular surfaces slide relative to one another, producing high levels of mechanical fluid shear ($\sim 10^4 \text{ s}^{-1}$) in the synovial fluid (Mow and Ateshian, 1997). Our investigation demonstrated that the *in vitro* application of these physiologic shearing levels rapidly activates a large fraction of the soluble latent TGF- β present in synovial fluid. In principle, newly activated TGF- β is available to influence the metabolic activity of surrounding articular cartilage. In a subsequent experimental investigation, we have demonstrated that due to the presence of an overwhelming supply of non-specific binding sites in the cartilage ECM, active TGF- β from an external bathing solution predominantly binds to, and accumulates in the superficial zone (0–250 μm deep) and is unable to penetrate deeper into articular cartilage (Albro et al., 2013). Theoretical predictions from reversible binding kinetics analyses confirm that it would take over a year for active TGF- β to reach

equilibrium at a depth of 1 mm below the articular cartilage; under physiologic conditions, it is highly likely that activated TGF- β will undergo protease-mediated degradation or cellular internalization well before reaching this state. Overall, this characterization demonstrates that TGF- β activated in synovial fluid can reach high concentrations in superficial articular cartilage, but it is unable to transport into the middle and deep zones of the tissue. Thus, it remains unclear how chondrocytes in these deeper regions obtain access to active TGF- β , which is believed to be required for their metabolic function (Chen et al., 2012).

In addition to synovial fluid shearing, physiologic joint motion directly imparts mechanical deformation to the cartilage tissue. Although the shear rates of pressure-driven fluid flow through the interstitium of the tissue are far lower than those experienced in synovial fluid (due to the low permeability of the tissue (Ateshian et al., 2007)), joint motion also imparts elastic deformations to the tissue ECM in the form of tensile, compressive, and shearing strains (Canal et al., 2008). These deformations could impart conformational changes to the ECM-bound latent TGF- β that may be sufficient to induce activation. Therefore, the primary hypothesis of this study is that physiologic levels of dynamic (cyclic) mechanical compression of cartilage induce the activation of the large stores of ECM-bound latent TGF- β in the tissue.

Several inherent challenges exist in measuring activation of TGF- β in biological tissues (Jurukovski et al., 2005). Upon activation, TGF- β may remain in the tissue, where it may bind to the ECM and cell receptors, possibly getting internalized, or desorb into its surrounding bathing solution. Therefore, the measurement of TGF- β activation inside cartilage, and the testing of the current hypothesis, requires the ability to accurately measure active TGF- β levels in both of these pools. To preclude cellular internalization, devitalized cartilage explants are used in this study. In this system, upon activation, due to the natural presence of an extremely high concentration of high affinity nonspecific ECM binding sites, active TGF- β substantially binds to and remains in the tissue (Albro et al., 2013). Furthermore, active TGF- β is known to bind to various types of plastic-ware (Reisenbichler and Jirtle, 1994), potentiating its loss from the surrounding bathing solution during testing. As a result of these challenges, solely measuring the active TGF- β levels in a tissue sample's conditioned media may substantially underestimate the activation response. In light of these issues, this study proposes an experimental system to overcome these challenges in measuring TGF- β activation in devitalized cartilage tissues.

Methods

Materials

Articular cartilage was harvested from 4–6 week old bovine calves. Deep zone cylindrical explants of varying thicknesses were prepared from the femoral condyles after excising the top 1 mm of tissue. Explants were devitalized through freezing and stored at -30°C until testing. For all experiments, tissue and fluid samples were collected in siliconized microcentrifuge tubes (Fisherbrand low retention). Media used in all experiments was prepared from high glucose DMEM containing 1% ITS+ Premix (insulin-transferrin-selenium), 100 nM dexamethasone, 100 $\mu\text{g}/\text{mL}$ sodium pyruvate, 50 $\mu\text{g}/\text{mL}$ L-proline, 1% PSAM. Prior tests show that this formulation substantially reduces the binding of active TGF- β to plastic-ware, predominantly due to the action of the high levels of bovine serum albumin (BSA) from the ITS+ premix (1.25 mg/mL final concentration); BSA acts as a TGF- β stabilizing carrier protein (Reisenbichler and Jirtle, 1994). Exogenous active TGF- β 1 was acquired from R&D Systems (human recombinant; 240B). Tris/CHAPS buffer was prepared with 50mM Tris-HCl, 0.5% CHAPS, 150 mM NaCl at pH 7.2. Guanidine hydrochloride at 4M was prepared in phosphate buffered saline (PBS). The NaCl concentration in PBS has been shown to optimize TGF- β 1 extraction (see supplemental

materials). Hypertonic salt solutions were prepared by supplementing media with additional NaCl (media+150 mM, media+750 mM, and media+2000 mM). All solutions in this study were supplemented with protease inhibitors (Complete tablets, Roche Applied Science).

Active TGF- β measurement techniques

The experimental aim of this study was to assess whether the physiologic levels of dynamic loading of devitalized cartilage explants activates their endogenous latent TGF- β content. The measurement of TGF- β activation requires the ability to distinguish between active and latent TGF- β in the tissue. For all experiments, a TGF- β 1 enzyme-linked immunosorbent assay kit (ELISA; DY240, R&D) specific for the active form of the TGF- β 1 isoform (human and bovine) was used. The use of this assay in measuring active TGF- β levels in a tissue requires a technique to exclusively extract its active TGF- β content without further activating its latent TGF- β content. Treatment of cartilage with chaotropic agents, such as 4M-guanidine hydrochloride, has been shown to activate all latent TGF- β in the tissue, and indiscriminately extract its total TGF- β content (active+latent). Alternatively, it has been reported that active TGF- β can be extracted from biological tissues through the use of a Tris buffer supplemented with detergent (Morales et al., 1991; Recouvreux et al., 2011).

Four validation tests were conducted to determine the suitability of these techniques in extracting and measuring the total and active TGF- β 1 content from devitalized cartilage explants. First, the required duration of guanidine hydrochloride exposure to extract the total TGF- β 1 content from explants was determined. Secondly, the ability of the Tris/CHAPS buffer to exclusively extract the explant entire active TGF- β 1 content was assessed. Here, the extraction ability of hypertonic salt solutions was further characterized to assess the affinity of active TGF- β in the cartilage ECM. Thirdly, the stability of active TGF- β in devitalized cartilage explants was assessed. Fourthly, TGF- β “safe” materials, to which active TGF- β 1 does not bind, were identified for use in the dynamic loading experiments.

Total TGF- β extraction validation—Devitalized explants ($\varnothing$ 5.2.5 mm) were finely minced and individually exposed to 4M guanidine hydrochloride in a microcentrifuge tube at 4°C. After 0.15, 1, 2, 6, or 20 h of exposure (n=5 explants per time point), the extracted TGF- β 1 content was measured. All guanidine hydrochloride extract solutions were subjected to the optimal 1:20 dilution in media prior to assay (see supplemental materials). Due to the outcome of this test (see results), all subsequent guanidine hydrochloride extractions were conducted under these conditions for 6 h.

Active TGF- β extraction validation—For this test, a measurable quantity of exogenous active TGF- β 1 was introduced into explants to examine the suitability of using Tris/CHAPS and hypertonic salt solutions to extract active TGF- β 1 from the tissue. Thin devitalized cartilage explants ($\varnothing$ 4.0.25 mm) were incubated in media for 24 h at 22°C as untreated, or treated with 10 ng/mL of exogenous active TGF- β 1, yielding samples with ECM-bound active TGF- β 1 in addition to the stores of endogenous ECM-bound latent TGF- β 1. After this incubation period, all explants were individually weighed, finely minced, and collected in microcentrifuge tubes. Samples (TGF- β 1 treated and untreated, n=5 explants each) were exposed to guanidine hydrochloride solution for 6 h at 4°C, in order to measure their total (active+latent) TGF- β 1 content. The difference in the total TGF- β 1 content between treated and untreated samples represents the amount of exogenous active TGF- β 1 introduced and bound to the explant ECM during the treatment procedure. In an effort to exclusively extract the active TGF- β content, an additional set of treated samples were exposed to Tris/CHAPS buffer for 0.5, 4, or 24 h, or to a hypertonic salt solution (isotonic media, media+150 mM, media+750 mM, or media+2000 mM for 24 h, n=5 samples per group). To verify that these

solutions did not activate latent TGF- β 1, additional sets of untreated samples were exposed to these solutions for 24 h (n=5 explants per group).

For TGF- β 1 measurements, the Tris/CHAPS and hypertonic solutions were optimally diluted 1:5 in media (see supplemental materials). The 750 mM and 2000 mM extracts were initially dialyzed in PBS.

Active TGF- β stability in cartilage—In order to assess the stability of active TGF- β in devitalized cartilage, exogenous active TGF- β 1 was introduced to thin devitalized explants (n=18), as performed in the prior test, but with 60 pg/mL TGF- β 1 in media. Subsequently, explants were individually maintained in a TGF- β -free media solution (150 μ L per explant). After 0, 6, and 20 h, individual explants and their corresponding solutions were collected and frozen (n=6 per time point). Active TGF- β was extracted from explants with Tris/CHAPS buffer. Finally, active TGF- β 1 levels in extracts and corresponding media solutions were determined through ELISA.

TGF- β “safe” materials—Our conventional setup for the dynamic mechanical loading of explants requires the use of a sample and bathing solution containment dish, an actuating loading platen, and a rigid annulus to laterally surround the explants and maintain them under the loading platen. A preliminary test was conducted to identify TGF- β “safe” materials to which the active growth factor will not bind, for use in this setup. Here, a solution of 0.5 ng/mL active TGF- β 1 in media was exposed to various materials (see below) for 24 h at 22°C. Aliquots of solution were removed before and after exposure and assayed for their active TGF- β 1 content. In order to assess the amount of TGF- β 1 lost from solution due to material surface binding, a TGF- β 1 solution retention ratio was calculated based on the ratio between the final to initial concentration. The tested materials (n=3 for each) included silanized glass (treated with Sigmacote, Sigma), prefabricated plasticware: tissue culture treated and nontreated polystyrene (Falcon Petri dishes), polypropylene (Ted Pella Petri dishes), siliconized polypropylene (Fisherbrand low retention microcentrifuge tubes); and a series of stock materials (McMaster-Carr) which were machined into sample dishes: polypropylene (chemical-resistant grade), stainless steel (alloy 316L), and acrylic plastic. Furthermore, thin rigid annuli (50 mm OD, 18 mm ID, 1.5 mm thick) were prepared by modifying the lids of untreated polystyrene Petri dishes with a laser cutter (Versa). The solution retention ratio of these annuli was measured with and without prior Sigmacote treatment.

Dynamic mechanical loading

Devitalized cartilage explants ($\varnothing$ 4.3 mm, n=84 from 3 joints) were subjected to a continuous dynamic (cyclic) loading regimen (DL) with a custom-made mechanical actuator as described previously (Albro et al., 2011). Explants were subjected to a low physiologic strain regimen ($\pm$ 2%) or high regimen ($\pm$ 7.5%), both superposed over a 10% static strain offset at 0.5 Hz. These loading regimens were selected based on physiologic compressive strains previously measured in human and bovine articular cartilage (Canal et al., 2008; Guterl et al., 2009). For testing, explants (n=6 per time point) were placed in a 60 mm polystyrene Petri dish (Falcon, untreated) and maintained centered with a polystyrene annulus (Sigmacote treated) while immersed in 6 mL of media. Samples were dynamically compressed with the underside of a 35 mm polystyrene petri dish, reinforced with an aluminum shaft from above (Fig. 1). This configuration ensured that samples and the surrounding bathing solution were only in contact with prefabricated Falcon polystyrene materials during testing, thus preventing the potential loss of activated TGF- β 1 during these experiments (see results for testing materials). Sample loading was performed for 1, 5, or 15 h for low strain and 5 or 15 h for high strain testing at 22°C. As a control group, an identical

set of explants (n=6 per time point) were maintained unloaded under free swelling (FS) conditions, similarly in 6 mL of media while on an orbital shaker.

For all groups, upon completion of testing, each sample was diametrically cut in half; one half was processed for active and the other for its total TGF- β 1 content. The surrounding bath solution was also processed for its active and total TGF- β 1 content. Based on the results of the validation experiments, active TGF- β 1 was extracted with the Tris/CHAPS solution over a 24 h period.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed ($\alpha=0.05$, $p<0.05$) to determine the effect of time on active TGF- β 1 extractability (Fig. 3A) and stability (Fig. 4) in explants. A two-way ANOVA was used to determine the effect of loading condition (DL versus FS) and duration on active and total TGF- β 1 levels in explants. A one-tailed test using a *T*-statistic was performed to determine whether TGF- β 1 solution retention ratios were less than unity (Table 1).

Results

Total TGF- β extraction validation

Large amounts of endogenous TGF- β 1 were extracted from explants with guanidine hydrochloride. Extracted concentrations increased at an exponentially decreasing rate over time, leveling off at 65.8 ± 18.8 ng/mL after 2 h (Fig. 2).

Active TGF- β extraction validation

The average amount of exogenous active TGF- β 1 introduced to treated explants was 203.3 ± 62.9 ng/mL. Tris/CHAPS buffer was able to extract this active TGF- β 1 over time, reaching 188.4 ± 14.2 after 4 h (Fig. 3A). The effectiveness of this extraction is solely due to the action of the CHAPS detergent (see supplemental data). In contrast, in the absence of CHAPS, hypertonic salt solutions were only able to extract a fraction of this active TGF- β 1 content, even after 24 h of exposure (Fig. 3B).

Exposure of non-treated explants to Tris/CHAPS buffer and hypertonic salt solutions extracted only small amounts of TGF- β 1 (0.53 ± 0.14 ng/mL Tris/CHAPS buffer; 0.90 ± 0.22 ng/mL isotonic media; 1.47 ± 0.30 ng/mL media+150mM; 2.25 ± 0.29 ng/mL media+750mM; 2.95 ± 0.25 ng/mL media+2000mM), indicating that, as opposed to guanidine hydrochloride, these solutions were not capable of effectively activating and extracting endogenous latent TGF- β 1.

Active TGF- β stability in cartilage

For this experiment, the average amount of exogenous active TGF- β 1 introduced to treated explants was 7.3 ± 1.0 ng/mL (Fig. 4). Subsequently, in TGF- β -free solution, the explant concentration of active TGF- β 1 exhibited no statistical decrease with time ($p=0.24$) for up to 20 h, suggesting that active TGF- β 1 is stable in the devitalized tissue explants utilized in this study. During this period only a small fraction of active TGF- β 1 was released into the surrounding solution (0.5 ± 0.1 ng/mL after 6 h, 1.2 ± 0.2 ng/mL after 20 h; on tissue volume basis), consistent with the results in isotonic media from the prior test.

TGF- β “safe” materials

The materials tested in this study exhibited disparate TGF- β binding properties. For untreated polystyrene and polypropylene, active TGF- β 1 exhibited little or no binding

(solution retention ratios > 0.96, Table 1). In contrast, for machined materials (stainless steel, polypropylene, and acrylic), a great degree of binding was observed (solution retention ratios < 0.29). The annular laser-cutting of polystyrene increased TGF- β 1 binding (solution retention ratio = 0.68 ± 0.15). However, its binding properties could be mostly restored with Sigmacote treatment (retention ratio = 0.89 ± 0.03). These results guided our selection of materials for dynamic loading experiments.

Dynamic mechanical loading

Prior to dynamic mechanical loading, high levels of total TGF- β 1 were measured in explants (79.4 ± 21.2 ng/mL low strain; 57.6 ± 14.1 ng/mL high strain batch at 0h, Fig. 5A & 6A). At the same time, active levels were extremely low (0.7 ± 0.3 ng/mL low strain; 0.7 ± 0.2 ng/mL high strain at 0h, Fig. 5B & 6B), indicating that *in situ*, endogenous stores of TGF- β 1 are predominantly in the latent form (>98% of total). Active TGF- β 1 concentration levels under dynamic loading did not vary from this initial value ($p=0.57$ low strain; $p=0.65$ high strain) nor from the corresponding control value at any time point ($p=0.15$ low strain; $p=0.65$ high strain), indicating that dynamic loading did not significantly activate ECM-bound latent TGF- β 1. In all experiments only low levels of active and total (active+latent) TGF- β 1 were detected in the external bathing solution (average of 0.19 ng/mL active; 1.21 ng/mL total, calculated on explant volume basis, Table 2). Raw measures of active TGF- β 1 in the bath were below the assay detection limit (< 31.2 pg/mL).

Discussion

The results of this study indicate that physiologic dynamic compression of devitalized cartilage is unable to directly activate ECM-bound latent TGF- β 1 purely mechanical pathways, leading us to reject the hypothesis of this study. These results contrast the behavior of latent TGF- β 1 in synovial fluid, which undergoes rapid activation in the presence of physiologic levels of mechanical fluid shearing (Albro et al., 2012). In consideration of the conformational change (stretching) believed to be responsible for activating soluble latent TGF- β 1 during fluid shearing (Tenney and Discher, 2009), this result suggests that mechanical deformations of the cartilage ECM and interstitial fluid shearing are insufficient to induce similar conformational changes to the molecule. The results of this study further suggest that mechanical deformation is unlikely to directly activate the ECM-bound latent TGF- β 1 of various other tissues, such as muscle, ligament, and tendon.

These results have important implications for understanding the metabolic role of TGF- β 1 in articular cartilage. Although physiologic synovial fluid shearing induces the activation of large amounts of TGF- β 1, due to the extremely high concentration of nonspecific binding sites in the cartilage ECM, this active TGF- β 1 accumulates in the superficial zone and is unable to penetrate deeper into the tissue (Albro et al., 2013). In spite of this limitation, it has been clearly demonstrated that TGF- β 1 is required for maintaining the biochemical integrity of the middle and deep zones of the tissue (Chen et al., 2012). Therefore, these deeper tissue zones are alternatively required to access active TGF- β 1 from the large endogenous stores of ECM-bound TGF- β 1. In agreement with a prior report (Morales et al., 1991), these stores appear to be entirely present in the latent form, suggesting that, as with other tissues (Annes et al., 2003; Rifkin, 2005), activation is the rate limiting step for TGF- β 1 activity.

The demonstration that mechanical loading is unable to activate these latent stores indicates that they must alternatively undergo activation through the action of chemical mediators or integrins. Although chemical activation mediators have been characterized for a variety of tissues, studies have rarely addressed these mechanisms in articular cartilage. It has previously been proposed that transglutaminase may play a role in the activation of latent

TGF- β in aging articular cartilage (Rosenthal et al., 2000). Several chemical mediators, such as lysophospholipids (Gay et al., 2004), matrix metalloproteinase-3 (MMP-3) (Maeda et al., 2001), and MMP-13 (D'Angelo et al., 2001) have been implicated in the activation of the latent TGF- β in growth plate cartilage. The relative contribution of each of these mechanisms in healthy articular cartilage remains unclear and will have to be addressed in future studies. It should also be noted that although dynamic mechanical compression is unable to directly activate ECM-bound latent TGF- β , mechanical loading in live tissue may alternatively modulate the secretion of various chemical activation mediators, such as MMPs and other proteases, and thus indirectly induce TGF- β activation. In consideration of the high levels of MMPs secreted in response to traumatic joint injury (Stevens et al., 2009; Vasara et al., 2009), one may speculate that the large stores of latent TGF- β in the ECM may serve as an anabolic reservoir involved in tissue protection and repair. Furthermore, perturbations to activation regulation may occur during the onset of osteoarthritis or rheumatoid arthritis where elevated protease levels are sustained (Kleesiek et al., 1986; Walakovits et al., 1992).

To the best of our knowledge, this study represents the first explicit validation of a technique to measure active TGF- β cartilage explants. The results of this study confirm the anticipated challenges in performing such measurements and highlight the importance of extracting active TGF- β from the cartilage ECM and avoiding its loss through binding to testing materials. From the validation testing, it is clear that active TGF- β binds with high affinity to the nonspecific binding sites in the cartilage ECM; hypertonic salt solutions only extract a small fraction of the active TGF- β content from explants (Fig. 3B). As previously suggested (Morales et al., 1991), in contrast to these solutions, Tris/CHAPS buffer can rapidly extract the entire active TGF- β content from the cartilage ECM (Fig. 3A) while maintaining the latent content intact. The demonstration that isotonic media can only extract a small fraction of exogenous active TGF- β content from explants (Fig. 3B) strongly suggests that upon activation, endogenous TGF- β also remains bound to the cartilage ECM.

Furthermore, validation testing confirms that active TGF- β can exhibit a high degree of binding to materials such as metals and plastic-ware. Although this issue does not appear to influence measurements of TGF- β activation in cartilage explants (due to long term retention of active TGF- β in the ECM), material selection may be an important consideration in other TGF- β activation investigations. For example, in studies that feature tissues with a sparse ECM or short diffusional distances, such as engineered tissue constructs or cellular monolayers, active TGF- β may significantly desorb from the tissue and subsequently bind to the surrounding containment materials. Our results indicate that while conventional prefabricated plastic-ware (polystyrene and polypropylene) appear to be TGF- β “safe,” alternative materials with surfaces that have been subjected to machining or tissue culture treatment (for cell monolayer adherence), promote substantial loss of active TGF- β , and thus are unsuitable for use in measurements of active TGF- β . In any event, the current data strongly highlight the importance of material validation for any studies which involve TGF- β supplementation or activation measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cross-sectional schematic diagram of apparatus with TGF- “safe” materials used for dynamic compressive loading of devitalized cartilage explants.

Figure 2. Extraction of total (active + latent) TGF-β1 content over time from cartilage explants with 4 M guanidine hydrochloride solution (mean ± standard deviation). Solid curve represents exponential fit through data.

Figure 3.

Extraction of exogenous active TGF- β 1 from cartilage explant slices ($\varnothing$ 5 mm $\times$ 0.25 mm). (A) Extracted concentration over time with Tris/CHAPS buffer (mean $\pm$ standard deviation) with exponential fit (solid curve). Dashed horizontal line represents mean concentration of exogenous active TGF- β 1 originally introduced and bound to ECM of explants. * $p < 0.001$ indicates level significantly below the introduced exogenous active TGF- β 1 concentration. (B) Extracted concentration after 24 h of exposure with Tris/CHAPS buffer, isotonic media, and media supplemented with additional NaCl (hypertonic salt solutions).

Figure 4. Stability of active TGF- β 1 in devitalized cartilage explants. Concentration of exogenous active TGF- β 1 in cartilage explant slices (mean $\pm$ standard deviation) during 20 h recovery period in TGF- β -free media.

Figure 5. Concentrations of (A) total (active + latent) and (B) active TGF-β1 in cartilage explants after continuous low strain dynamic loading (DL) or free swelling (FS) for up to 15 h (mean ± standard deviation). *p < 0.05 indicates significant increase above initial value.

Figure 6. Concentrations of (A) total (active + latent) and (B) active TGF-β1 in cartilage explants after continuous high strain dynamic loading (DL) or free swelling (FS) for up to 15 h (mean $\pm$ standard deviation). *p<0.05 indicates significant increase above initial value.

Table 1

Characterization of active TGF- β 1 loss from solution due to binding to various materials. Solution retention ratio represents ratio of final to initial active TGF- β 1 concentration in solution after 24 h of material exposure (mean $\pm$ standard deviation).

Material type	TGF- β 1 solution retention ratio
Silanized microcentrifuge tube	1.08 $\pm$ 0.05
Polypropylene (nontreated)	0.94 $\pm$ 0.08
Polystyrene (nontreated)	0.96 $\pm$ 0.09
Polystyrene (culture treated)	0.37 $\pm$ 0.02 *
Silanized glass	0.68 $\pm$ 0.06 *
Polypropylene (machined)	0.14 $\pm$ 0.04 *
Stainless steel (machined)	0.22 $\pm$ 0.18 *
Acrylic (machined)	0.07 $\pm$ 0.04 *

*
p<0.05 indicates ratio less than unity.

Levels of active and total (active+latent) TGF- β 1 released from dynamically loaded (DL) and free swelling (FS) cartilage explants into bathing solution during testing. Concentrations are calculated on the basis of the volume of cartilage explants in the test. Each measurement represents the bathing solution of the six explants collectively maintained during a single experiment. Raw levels for all active TGF- β 1 measurements in the bath were below the detection limit of the assay.

Table 2

Time [h]	High strain loading				Low strain loading			
	Active TGF- β		Total TGF- β		Active TGF- β		Total TGF- β	
	DL	FS	DL	FS	DL	FS	DL	FS
1	-	-	-	-	0.39	0.01	0.80	0.66
5	0.22	0.10	1.11	0.68	0.14	0.14	0.84	0.82
15	0.27	0.15	1.38	1.67	0.46	0.35	1.93	2.72