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Transport and Binding of Tumor Necrosis Factor-α in Articular Cartilage Depend on its Quaternary Structure

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Abstract

The effect of tumor necrosis factor-α (TNFα) on cartilage matrix degradation is mediated by its transport and binding within the extracellular matrix (ECM) of the tissue, which mediates availability to cell receptors. Since the bioactive form of TNFα is a homotrimer of monomeric subunits, conversion between trimeric and monomeric forms during intratissue transport may affect binding to ECM and, thereby, bioactivity within cartilage. We studied the transport and binding of TNFα in cartilage, considering the quaternary structure of this cytokine. Competitive binding assays showed significant binding of TNFα in cartilage tissue, leading to an enhanced uptake. However, studies in which TNFα was cross-linked to remain in the trimeric form revealed that the binding of trimeric TNFα was negligible. Thus, binding of TNFα to ECM was associated with the monomeric form. Binding of TNFα was not disrupted by pre-treating cartilage tissue with trypsin, which removes proteoglycans and glycoproteins but leaves the collagen network intact. Therefore, proteoglycan loss during osteoarthritis should only alter the passive diffusion of TNFα but not its binding interaction with the remaining matrix. Our results suggest that matrix binding and trimer-monomer conversion of TNFα both play crucial roles in regulating the accessibility of bioactive TNFα within cartilage.

Keywords

cartilage; TNFα; cytokine transport; binding; osteoarthritis; post traumatic osteoarthritis

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1. Introduction

Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine involved in cartilage matrix degradation and associated joint damage in osteoarthritis (OA) [1]. In addition to other cytokines including as IL-1 and IL-6, TNFα is known as a major factor that can cause cartilage destruction by suppressing proteoglycan synthesis [2, 3] and inducing matrix proteolysis caused by upregulation of aggrecanase [1, 4] and matrix metalloproteinase [5] activities. Following traumatic joint injury, the concentration of TNFα in the synovial fluid is significantly higher than that observed in normal joints [6-8], indicating that TNFα may play an important role in the progression to post-traumatic osteoarthritis (PTOA). Due to the critically important role of cytokines such as TNFα in OA pathogenesis, limiting the catabolic effects of TNFα by delivering anti-TNFα antibodies has been explored as a potential therapeutic for OA patients [9].

Since adult articular cartilage is avascular and alymphatic, the transport of TNFα into and through cartilage is mediated by the dense extracellular matrix (ECM) [10]. Interestingly, the bioactive form of TNFα is a 51 kDa homotrimer of 17 kDa monomeric subunits [11, 12], suggesting that steric hindrance of TNFα diffusion by the ECM would depend on the quaternary structure of TNFα due to its different sizes [13]. The isoelectric pH of TNFα is ~ 5.3, which suggests that TNFα is slightly negatively charged under physiological conditions [14]. The reversible conversion between trimeric and monomeric forms of TNFα in solution, and hence the proportion of those two TNFα species, is determined by the concentration of TNFα. The trimer is relatively stable at nanomolar concentrations and higher but slowly dissociates into monomers at sub-nanomolar concentrations [11, 15]. Dissociated monomers can rapidly reassociate to form trimers in solution when additional TNFα is added [11]. The concentration of TNFα in the synovial fluid is in the sub-nanomolar range even at elevated states after the joint injury (i.e., ~ 40 pg/ml = 2.4 pM, calculated on the basis of monomeric TNFα), suggesting that TNFα would preferentially exist in the monomeric form in the synovial fluid [6-8]. Monomeric TNFα would thereby diffuse faster than trimeric TNFα into and within cartilage tissue. However, the trimer, not the monomer, is the bioactive form that binds to cell (chondrocyte) receptors to trigger biological responses [11, 12].

In addition, the binding of TNFα to macromolecular sites within the ECM could significantly alter the transport of TNFα into cartilage. Diffusion-reaction transport kinetics affected by binding can result in the effective diffusivity of a protein being orders of magnitude lower than its diffusivity in the absence of binding [16, 17], thereby slowing transport. Previous studies have shown that TNFα binds weakly to collagen type I [18], type II [18], and type IV [19], as well as to heparin [20], fibronectin [18], laminin [21], decorin [22], biglycan [22], and dermatan sulfate glycosaminoglycans (GAGs) [22]. Interestingly, TNFα does not bind to chondroitin sulfate [22], which is the major GAG chain of aggrecan proteoglycans in cartilage ECM. To examine the transport of TNFα in cartilage, given the issue of TNFα trimer-monomer conversion, we need to examine binding of trimers as well as monomers within the tissue. Therefore, a study of TNFα transport and binding to cartilage ECM constituents should account for the role of the quaternary structure of TNFα on binding interactions.

To better understand the role of molecular structure and binding of TNFα on its transport into native cartilage tissue, our objectives were to characterize the equilibrium binding and transient transport kinetics of TNFα in cartilage, accounting for trimer-monomer conversion. A competitive binding assay revealed that TNFα binds to sites within cartilage, thereby enhancing and sustaining the uptake of TNFα. By cross-linking TNFα to preserve the trimeric form, we found that only monomeric TNFα exhibited significant binding to cartilage ECM. Binding of TNFα was not disrupted by bovine trypsin pre-treatment of cartilage, which removes intratissue proteoglycans and glycoproteins but essentially leaves the biomechanically functional collagen network intact [23].

2. Materials and Methods

Bovine tissue harvest

Bovine cartilage explants were harvested from the femoropatellar grooves of 1-2 weeks old calves (Research 87, Marlborough, MA) [24]. A total of 8 joints from 5 different animals were used. Briefly, 9-mm diameter cartilage-bone cylinders were cored and mounted on a microtome. The top superficial layer was removed to obtain 0.5 mm-thick middle zone slices. Four or five disks (3 mm-diameter, 0.5 mm-thick) were cored from each slice using a dermal punch. For studies with live organ culture explants, cartilage specimens were equilibrated in serum-free medium (low-glucose Dulbecco's modified Eagle's medium [DMEM; 1 g/L]) supplemented with 1% insulin-transferrin-selenium (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium, Sigma, St. Louis, MO), 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/mL ascorbic acid, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B in a 37°C, 5% CO_2 incubator. For studies of transport and binding of TNFα in explants in which cells were first devitalized but the extracellular matrix was normal and not chemically fixed, explants were maintained in 1× phosphate buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA), 0.01% sodium azide (NaN_3) and protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN) at 4°C prior to experiments at 4°C or 37°C.

Postmortem adult human tissue

While most experiments were performed using bovine cartilage, a cross-species comparison for TNFα uptake into cartilage was performed using normal human knee cartilage obtained from a human subject (44-year-old, male) 36 hr postmortem from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL). All procedures were approved by the Office of Research Affairs at Rush–Presbyterian–St. Luke's Medical Center and the Committee on Use of Humans as Experimental Subjects at Massachusetts Institute of Technology. All joint surfaces of the knee joint were scored as grade 0-1 according to modified Collins scale [25]. Only the joint surfaces scored as grade 0 (i.e., normal) and from unfibrillated areas to visual inspection were harvested. After coring 3-mm diameter cartilage cylinders from femoropatellar groove and femoral condyles using a dermal punch, 0.8-mm thick slices were cut from the top surface to include the intact superficial zone. The culture medium for these live human cartilage explant disks (3-mm diameter, 0.8-mm thick) was the same as that for the bovine tissue but supplemented with high-glucose DMEM (4.5 g/L) and 1 mM sodium pyruvate.

Solute preparation

Unlabeled TNFα was purchased from R&D Systems (Minneapolis, MN) and from PeproTech (Rocky Hill, NJ); the PeproTech material was used for experiments involving cross-linking of TNFα to maintain its trimeric form. Iodinated TNFα was purchased from PerkinElmer (Waltham, MA). Before all experiments using ^{125}I-TNFα, Sephadex G25 chromatography was used to separate and remove any small ^{125}I-species that may have resulted from degradation of ^{125}I-TNFα, as previously described [16] (0.7 × 50 cm gravity-fed columns using an elution buffer of 1×PBS plus 0.1% BSA with or without 0.01% NaN_3, and the void volume collected for the desired ^{125}I-TNFα).

Concentration dependent quaternary structure of TNFα

The quaternary structure of ^{125}I-TNFα was analyzed by Sephadex G75 chromatography after incubating ^{125}I-TNFα with a graded amount of unlabeled TNFα (2.94 nM = 50 ng/ml, 20 nM = 340 ng/ml) for 48 hr at 4°C (Fig. 1). The G75 columns were calibrated using molecular weight markers (GE Healthcare, Piscataway, NJ), including albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13.7 kDa), dextran blue (2 MDa), and phenol red (354 Da). The amount of protein markers in the elution volume from the column was quantified using a Nanodrop 1000 Spectrophotometer (Agilent Technologies, Santa Clara, CA). The amounts of dextran blue and phenol red were determined using a microplate reader (VMax Kinetic ELISA Microplate Reader, Molecular Devices, Sunnyvale, CA). All concentrations of TNFα were converted to molar concentration assuming the molecular weight of is the monomeric form (17 kDa), regardless of its quaternary structure.

Measurement of uptake ratio of ^{125}I-TNFα

To measure the partitioning of TNFα into cartilage, and to determine whether TNFα may bind to sites in the tissue, the uptake ratio of ^{125}I-TNFα was measured in both bovine and human cartilage (Fig. 2). Cylindrical disk specimens were incubated in a bath at 37 °C for 48 hr containing 0.15 nM (= 2.55 ng/ml) ^{125}I-TNFα along with graded amounts of unlabeled TNFα. For each bath concentration, the *uptake ratio* was measured as the concentration of ^{125}I-TNFα in the cartilage disks (free and bound, per intratissue water weight) normalized to the concentration of ^{125}I-TNFα in the equilibration bath [16, 26]. We assumed that unlabeled TNFα partitions into cartilage with the same uptake ratio as labeled ^{125}I-TNFα. The equilibration bath consisted of 1×PBS, 0.1% BSA, 0.01% NaN_3 and protease inhibitors (Figs. 2B, 5, 7). To test the effect of cell viability on the equilibrium uptake of ^{125}I-TNFα, live bovine cartilage explants were incubated in DMEM supplemented with 1% ITS, and 0.1% BSA in the absence or presence of 0.01% NaN_3 and protease inhibitors (Fig. 2A). Multiwell plates containing cartilage explants and equilibration baths were placed on a rocker during the incubation to maintain well-mixed conditions. At the end of experiments, disks were collected from the bath and briefly rinsed in fresh 1×PBS; the surface of each disk was quickly blotted with Kimwipes and the wet weight was measured. The ^{125}I-radioactivity of each cartilage disk and aliquots of the corresponding equilibration baths were quantified individually using a gamma counter (model B5002, Packard Instrument Company, Meriden, CT). After lyophilizing, the dry weight of each disk was measured, and the water weight of each disk was calculated as the difference of the wet and dry weights. The sulfated glycosaminoglycan (sGAG) content of each individual disk was measured using the dimethylmethylene blue (DMMB) dye binding assay after the disks were digested with proteinase-K (Roche Applied Science, Indianapolis, IN) [24]. sGAG release to the medium during the incubation was quantified by measuring sGAG content of aliquots of the equilibration bath using the DMMB dye binding assay. The uptake ratio of ^{125}I-TNFα was corrected to take into account the presence of any small labeled species that may have accumulated from degradation of ^{125}I-TNFα during the incubation, using methods described previously [27]. Aliquots from the equilibration baths were analyzed by Sephadex G75 chromatography to determine the amount of small labeled species, assuming the small species to be ^{125}I. The uptake ratio of ^{125}I alone was measured in a separate experiment [26].

Transient uptake ratio of ^{125}I-TNFα

To determine the transport kinetics of TNFα into cartilage tissue, the uptake ratio of ^{125}I-TNFα into bovine calf cartilage was measured with or without unlabeled TNFα over a 48 hr period (Fig. 3). The bath consisted of DMEM supplemented with 1% ITS, and 0.1% BSA.

At selected time points, disks were collected from the equilibration bath and the uptake ratio of each disk was measured as described above. Aliquots of the bathes were also collected and analyzed with G75 chromatography to confirm the state of the quaternary structure of the TNFα.

Cross-linking TNFα

Both ^{125}I-TNFα and 1 unlabeled TNFα were cross-linked with the bifunctional reagent *Bis*[2-(succinimidyloxycarbonyloxy)ethyl]sulfone (BSOCOES, Pierce Chemical Co., Rockford, IL) [12]. Briefly, ^{125}I-TNFα in 1×PBS with 0.1% BSA or unlabeled TNFα in 1×PBS was reacted with 1 mM BSOCOES for 10 min at 4°C. To quench the reaction, 1M glycine in 0.1 M sodium phosphate buffer, pH 7.5, was added to a final concentration of 100 mM glycine and incubated for at least 15 min at 4°C. To remove uncross-linked species, reacted ^{125}I-TNFα or unlabeled TNFα were passed through 30 kDa-cutoff centrifugal filter (Millipore, Billerica, MA) and retentates were collected, which contained molecules larger than 30 kDa. Untreated ^{125}I- or unlabeled TNFα were also filtered using the same method for comparisons (Fig. 4). For chromatographic analysis, retentates of cross-linked ^{125}I- or unlabeled TNFα were diluted to sub-nanomolar final concentrations. The concentrations of ^{125}I-TNFα and unlabeled TNFα were determined by liquid scintillation counter (1450 MicroBeta TriLux, PerkinElmer, Waltham, MA) and the BCA protein assay (Thermo Scientific, Rockford, IL), respectively. Unlabeled TNFα was run through SDS-PAGE (10% Bis-Tris gel, Invitrogen, Grand Island, NY) under non-reducing conditions and detected by silver staining (Invitrogen, Grand Island, NY) to reveal its molecular weight distribution.

Uptake ratio of cross-linked TNFα

To determine the effect of cross-linking of TNFα on its binding to sites in the cartilage tissue, the uptake ratio of cross-linked ^{125}I-TNFα into bovine calf cartilage was measured with addition of graded amounts of cross-linked unlabeled TNFα (Fig. 5). Cartilage disks were incubated in 1×PBS with 0.1% BSA, protease inhibitors, and 0.01% NaN_3 for 24 hr at 37°C. For comparison, the uptake ratio of non-cross-linked ^{125}I-TNFα was measured with or without adding non-cross-linked unlabeled TNFα.

Catabolic effects of native and cross-linked TNF

Live bovine cartilage disks were cultured for 6 days in culture medium supplemented with either native or cross-linked TNFα (25 ng/ml and 100 ng/ml). Culture medium was replenished every two days. Medium collected during culture was analyzed for sGAG content using the DMMB dye binding assay. Cumulative sGAG release to the medium was calculated as the total sGAG release normalized to the total sGAG content of each disk (Fig. 6).

Effects of removal of cartilage proteoglycans on TNFα uptake ratio

To aid in the identification of potential ECM binding sites within intact cartilage, TNFα uptake was measured after trypsin treatment of cartilage to remove aggrecan and other proteoglycans and glycoproteins (Fig. 7). Prior to uptake measurement, cartilage disks were either untreated or incubated with 0.1 mg/ml trypsin (bovine pancreatic, Sigma, St. Louis, MO) in 1×PBS with 0.1% BSA, protease inhibitors (except during trypsin treatment), and 0.01% NaN_3 for 48 hr at 37°C. Uptake of ^{125}I-TNFα was then measured with or without unlabeled TNFα at 37°C for 24 hr. The bath consisted of 1×PBS with 0.1% BSA, protease inhibitors, and 0.01% NaN_3.

Statistical Analysis

One-way ANOVA was used to test the effect of adding cross-linked unlabeled TNFα on the uptake ratio of cross-linked ^{125}I-TNFα. One-way ANOVA with post-hoc Dunnett's test was used to test the catabolic effect of native and cross-linked TNFα on bovine cartilage, with culture in the absence of TNFα as the reference for Dunnett's test. Two-way ANOVA was used to test the effect of trypsinizing cartilage disks and adding unlabeled TNFα on the uptake ratio of ^{125}I-TNFα. For all statistical tests, a *p*-value less than or equal to 0.05 was considered significant. Systat 12 software (Richmond, CA) was used to perform all analyses.

3. Results

Quaternary structure of TNFα is a function 1 of TNFα bath concentration

TNFα can spontaneously dissociate to monomers or associate to trimers depending on the total concentration of TNFα in solution [11, 15]. In order to study the role of the quaternary structure on the transport of TNFα, we first confirmed that our methods were able to detect the effects of TNFα concentration on monomer to trimer conversion. The size of ^{125}I-TNFα species was analyzed using Sephadex G75 gel filtration chromatography after incubating with or without unlabeled TNFα for 48 h at 4°C in 1×PBS supplemented with 0.1% BSA (Fig. 1). In the presence of ~ 3 nM unlabeled TNFα, ^{125}I-TNFα was predominantly found in the trimeric form (51 kDa); with 20 nM unlabeled TNFα, a larger and sharper trimeric peak was detected between the two molecular weight standards, albumin (67 kDa) and ovalbumin (43 kDa). Without unlabeled TNFα, ^{125}I-TNFα formed the peak between ribonuclease A (13.7 kDa) and chymotrypsinogen (25 kDa), consistent with the monomeric form (17 kDa), clearly showing that the quaternary structure of TNFα in solution depended on the total TNFα concentration, as previously reported [11, 15]. Similar results were observed with incubation at 37°C (rather than 4°C) except that a peak at the void volume (~ 6 ml) was detected, which was generated by non-specific binding of BSA and ^{125}I-species (Fig. 3B). Shortening incubation to 24 h did not significantly change the distribution of ^{125}I-TNFα, suggesting that the trimer-monomer conversion of TNFα in these experiments reached equilibrium by 24 h (Fig. S1).

Equilibrium and transient uptake ratio of ^{125}I-TNFα into cartilage depends on bath TNFα concentration

In order to understand the transport of TNFα within intact cartilage, we first measured the equilibrium and transient uptake ratio of ^{125}I-TNFα in tissue explants. Equilibrium uptake was measured by incubating cartilage disks in a bath containing ~ 50-100 pM ^{125}I-TNFα with graded amounts of unlabeled TNFα (Fig. 2). If TNFα was bound to sites within the tissue, unlabeled TNFα would compete with ^{125}I-TNFα for the same binding sites, which would decrease the uptake ratio of ^{125}I-TNFα with increasing amounts of unlabeled TNFα [17].

The equilibrium uptake ratio of ^{125}I-TNFα in immature bovine (Fig. 2A) and adult human (Fig. 2B) cartilage after 48 h incubation at 37°C was measured as ~ 6-7 with no added unlabeled TNFα and decreased dramatically as unlabeled TNFα was added from 0 to 100 nM (tests, below, showed that uptake reached equilibrium by 48 h). It is important to note that these trends in uptake ratio were observed with cartilage samples representing a wide difference in age (immature versus adult) as well as from different species. Considering the molecular weight of TNFα (17-51 kDa) and its net negative charge in physiological pH (isoelectric point = 5.3), the uptake ratio would be expected to be less than 1 without binding [13, 16]. Therefore, these results suggest that certain binding sites for TNFα exist within the tissue. The addition of sodium azide and protease inhibitors did not substantially alter the uptake ratio (Fig. 2A, viable cells vs. non-viable cells), indicating that cellular activity was not the major determinant of the equilibrium uptake of TNFα. Also, since TNFα can induce cell-mediated GAG depletion, we quantified the GAG loss (GAG released to media normalized by total GAG content in the explant) from individual cartilage explant after 48 h incubation. For viable cell conditions, GAG loss per plug was 5.8-11% and for non-viable conditions 3.0-5.8%, depending on the bath concentration of TNFα. Although viable conditions induced slightly more GAG loss from each plug over 48 h, the uptake ratio was not significantly affected, suggesting that GAG molecules might not be major binding sites for TNFα (see Results below). The uptake ratio measured at 4°C in bovine cartilage showed a similar decrease with addition of unlabeled TNFα (Fig. S2), suggesting that the effect of temperature on the equilibrium uptake ratio was not significant.

Transient uptake of ^{125}I-TNFα into bovine cartilage at 37°C was also measured at various time points up to 48 hr (Fig. 3A) in order to determine whether the transport kinetics was affected by the presence of unlabeled TNFα. Chromatograms showing ^{125}I-TNFα collected at the end of the 48 hr experiments confirmed that its quaternary structure varied with the concentration of TNFα (Fig. 3B). The transient uptake ratio measured without unlabeled TNFα was consistently higher than that measured with unlabeled TNFα (Fig. 2). The characteristic time constant (τ) of TNFα transport into cartilage explant was calculated by fitting data to a model of first order (exponential) kinetics in which the uptake ratio is represented as $A(1 - e^{-t/\tau})$, where A is the final (asymptotic) uptake ratio at infinite time. In the absence of unlabeled TNFα, the time constant was $\tau = 7.7$ hr and in the presence of unlabeled TNFα, it was $\tau = 12$ hr, indicating that the transport of TNFα into cartilage was affected by the bath concentration of TNFα. In addition, since diffusive transport equilibrium is generally reached in 3-5 diffusion time constants, τ [28], the final TNFα concentration inside cartilage samples in the uptake experiments of Fig. 2 (and Figs. 5, and 7, below) should be at or near equilibrium by 24-48 h of incubation.

Equilibrium uptake ratio of cross-linked ^{125}I-TNFα was significantly lower than that of untreated ^{125}I-TNFα

The time constant τ governing TNFα transport kinetics (Fig. 3A) is determined by the diffusivity of TNFα in cartilage, the TNFα binding parameters within the cartilage tissue (i.e., the binding site density and the binding dissociation constant) as well as the partition coefficient (i.e., the ratio of TNFα concentration just inside the tissue to the concentration in the surrounding bath) [28]. Since addition of unlabeled TNFα would convert monomeric TNFα to the trimeric form, the effective decrease in diffusivity of the larger trimer would slow the transport. At the same time, unlabeled TNFα would compete for the same binding sites as ^{125}I-TNFα, thereby altering transport of ^{125}I-TNFα by another mechanism. Therefore, the effects of the presence of unlabeled TNFα on the trends of the uptake of ^{125}I-TNFα seen in Figs 2 and 3A could be due to either altered size and/or the binding properties of ^{125}I-TNFα. To distinguish between these effects, we cross-linked ^{125}I-TNFα to prevent dissociation to the monomeric form, thereby keeping solute size constant.

Using BSOCOES as a cross-linking agent, both ^{125}I-TNFα and unlabeled TNFα were successfully cross-linked to their trimeric forms (Fig. 4). Cross-linked ^{125}I-TNFα remained in the trimeric form at a sub-nanomolar concentration, but at the same concentration, untreated ^{125}I-TNFα spontaneously dissociated to the monomer (Fig. 4A). Non-cross-linked unlabeled TNFα, which was analyzed by SDS-PAGE after removing smaller species (< 30 kDa) via a centrifugal filter, appeared in three distinctive bands, representing the trimer, dimer, and monomer (Fig. 4B). This result indicated that some of the untreated unlabeled TNFα had dissociated to the monomeric form during the analysis, as has been reported previously with SDS-PAGE analysis [29, 30]. However, cross-linked unlabeled TNFα showed a single band at the trimer location (Fig. 4B). The position of trimeric band of cross-linked TNFα was slightly different from untreated trimeric TNFα, suggesting that cross-linking altered the mobility of TNFα in SDS-PAGE [31]. The equilibrium uptake of cross-linked ^{125}I-TNFα was measured in bovine calf cartilage with graded amounts of cross-linked, unlabeled TNFα for 48 hr at 37°C (Fig. 5). The uptake ratio of cross-linked ^{125}I-TNFα remained between ~ 0.85 and ~ 1.2 (the average value of 5 different concentrations was ~ 0.98) and not affected by the addition of cross-linked unlabeled TNFα (1-way ANOVA, effect of cross-linked unlabeled TNFα, $p = 0.23$). However, the uptake of untreated (non-cross-linked) ^{125}I-TNFα was significantly decreased from ~ 5 to ~ 1.5 by adding untreated unlabeled TNFα, similar to the trends observed in Figs. 2 and 3. These results suggest that trimeric TNFα exhibits less binding to cartilage matrix sites than the monomeric form. Therefore, the decrease in uptake ratio with increasing amounts of TNFα (Fig. 2) appears driven more by the conversion of monomeric to the non-binding trimeric form at higher TNFα concentrations, and less associated with competitive binding between ^{125}I-TNFα and unlabeled TNFα.

Binding of TNFα to cell receptors and extracellular matrix

To further explore the binding properties of TNFα in cartilage, the ability of cross-linked TNFα to bind cell receptors was compared to that of non-cross-linked TNFα by testing the expected catabolic response of cartilage tissue to TNFα. Treatment of cartilage with TNFα is known to upregulate aggrecanase activity, resulting in cleavage and loss of aggrecan fragments and concomitant loss of GAG. The release of GAG to the medium was measured over 6 days after adding untreated or cross-linked TNFα to the incubation baths. Although cross-linked TNFα would diffuse more slowly into cartilage than monomeric TNFα, a 6-day incubation would be long enough to observe biological activity of the cross-linked TNFα after reaching equilibrium by 24-48 h (Fig. 3A). GAG release increased upon addition of untreated TNFα at 1.47 nM and 5.88 nM concentration (Fig. 6, Untreated, 1-way ANOVA, $p < 0.05$ for post-hoc Dunnett's test with control as a reference). GAG loss induced by cross-linked TNFα was only significant at 5.88 nM (Fig. 6, X-link), suggesting that cross-linking did not completely disrupt TNFα-cell receptor binding but matrix proteolysis induced by cross-linked TNFα was somewhat less effective compared to untreated TNFα.

To identify possible binding sites for TNFα within the extracellular matrix of cartilage, we first removed proteoglycans and glycoproteins using trypsin, and the uptake ratio was measured. Interestingly, trypsin treatment did not significantly alter the uptake ratio of ^{125}I-TNFα regardless of the addition of unlabeled TNFα, demonstrating that the binding sites remained unaltered by trypsin treatment (Fig. 7, 2-way ANOVA, p & 0.0001 for the effect of TNFα, p = 0.164 for the effect of trypsin). In addition, treatment with chondroitinase ABC following trypsin treatment, to quantitatively remove any residual chondroitin sulfate, did not change the uptake ratio (Fig. S3). These results further confirmed that chondroitin sulfate GAGs were not the major binding sites of TNFα. Since trypsin cannot remove collagenous proteins, those proteins or other molecules not removed by trypsin treatment would be potential binding sites for TNFα.

4. Discussion

The results of this study suggest that TNFα binds to sites within the cartilage matrix, which can lead to enhanced uptake of TNFα within the tissue compared to its concentration in the synovial fluid. However, significant binding interactions were observed only with the monomeric form of TNFα. As a result, trimer-monomer conversion of TNFα appears to play a key role in the transport and intra-tissue concentration of this inflammatory cytokine in cartilage, which can thereby affect its local bioactivity. Trypsin treatment of explants did not significantly alter the extent of intra-tissue binding of TNFα, suggesting that the trypsin-cleavable families of proteoglycans and glycoproteins within cartilage ECM are not among the potential binding partners. The equilibrium uptake ratio of ^{125}I-TNFα decreased markedly with addition of graded amounts of unlabeled TNFα, suggesting that there was significant binding of TNFα to the sites in tissue (Fig. 2). However, TNFα that was cross-linked to retain the trimeric form exhibited significantly less binding than the monomeric form (Fig. 5). This observation suggests that the marked decrease in uptake following addition of unlabeled TNFα (e.g., in Fig. 2) was mainly due to monomer-to-trimer conversion of ^{125}I-TNFα, though competitive unbinding of ^{125}I-TNFα by added unlabeled TNFα might partially contribute to this result.

It is important to note that the local TNFα concentration near chondrocytes is not the only factor that governs the local bioavailability of TNFα. Trimer-monomer conversion as well as binding affinity to matrix sites (which also depends on TNFα quaternary structure) can play a major role in regulating local bioavailability. In the synovial fluid, the concentration of TNFα is in the picomolar range [6-8], suggesting that it is primarily in its monomeric form. As monomeric TNFα enters cartilage, substantial binding to cartilage matrix can occur, which slows the penetration of TNFα but increases intra-tissue uptake (Figs. 2, 3, 5, 7). TNFα monomers have to associate to the trimeric form in order to bind chondrocyte receptors, since the trimer is the bioactive form for ligand-receptor binding [12]. While transport of trimeric TNFα to cell receptors would not be slowed by the diffusion-binding kinetics relevant to the monomeric form, trimer diffusion within cartilage would be slower than monomer diffusion due to its larger size.

Trypsin treatment of cartilage did not significantly alter the uptake ratio of TNFα, suggesting that the binding of TNFα was not disrupted by trypsin treatment (Fig. 7). Trypsin treatment of cartilage is well known to cause extensive degradation and release of aggrecan and other proteoglycans. In addition, using chondroitinase-ABC treatment, we further confirmed that chondroitin sulfate GAGs were not major binding sites (Fig. S3). Among collagenous proteins, collagen type II is the most abundant form in cartilage, and collagen types IX, XI, VI, and X are present in relatively smaller amounts. TNFα is reported to bind weakly to collagen type II as well as I and IV [18, 19], suggesting that collagen molecules might be candidates for binding sites. Ongoing studies are focusing on the further identification of the matrix molecules that bind TNFα.

The uptake ratio of cross-linked (trimeric) ^{125}I-TNFα was close to ~ 0.98 (the average value of 5 different concentrations), and remained constant even in the presence of cross-linked unlabeled TNFα, up to 100 nM (Fig. 5). In this limit when solute binding is negligible, the equilibrium uptake ratio is theoretically identical to the solute partition coefficient [26]. The partition coefficient of similarly-sized molecules, such as 40 kDa dextrans, has been reported to be in the range ~ 0.03-0.3, depending on the detection methods used [32, 33]. Since the cross-linked ^{125}I-TNFα is trimeric (51 kDa, Fig. 4) and slightly negatively charged [14], the measured uptake ratio of ~ 0.98 was somewhat higher than expected. These results raise the possibility that trimeric TNFα might bind to the tissue, but the amount of binding would still be much less than that of the monomer, whose uptake ratio was ~ 6 without unlabeled TNFα (Fig. 5).

It must be considered that the cross-linking process could change the structure of TNFα, disrupting its native binding properties and, as a result, the cross-linked TNFα would not bind to cartilage tissue in the same manner as the native trimeric molecule. Smith and coworkers reported that cross-linked TNFα had a somewhat weaker binding to cell receptors and that in order to elicit the same level of cytotoxicity of TNFα, larger amounts of their cross-linked TNFα were needed [12]. While the activity of their cross-linked TNFα was weaker than untreated TNFα, receptor binding was not completely lost [12]. In the present study, we tested chondrocyte ligand-receptor binding by measuring the downstream catabolic effects of TNFα on cartilage explants. Cross-linked TNFα induced GAG release to the medium, but at a higher concentration than non-cross-linked TNFα (Fig. 6), suggesting that the receptor-binding properties of TNFα were not completely altered by cross-linking. Since GAG release is one of many responses that can be induced by TNFα acting on chondrocytes in cartilage tissue, the somewhat lessened GAG release induced by cross-linked TNFα suggests that cross-linked TNFα has bioactivity, but may not be as fully-functional as untreated TNFα.

In synovial fluid, a significant porti on of TNFα would be monomeric due to its low concentration *in vivo*. Soluble forms of TNFα receptor (sTNFR) are known to bind TNFα and inhibit its bioactivity [34, 35]. Increased levels of sTNFR have been reported in the serum and synovial fluid of patients with rheumatoid arthritis (RA) and OA [35]. Interestingly, in a cell-monolayer study, Aderka et al. showed that sTNFR could augment the effect of TNFα by stabilizing TNFα structure and prolonging TNFα activity when sTNFR existed at low concentrations [34]. These results suggested that sTNFR could function as a carrier of TNFα, maintaining the trimeric form of TNFα in the surrounding bath (analogous to synovial fluid). However, the size of the TNFα-sTNFR complex (75-100 kDa) [36] is too large to penetrate into the interstitial space of intact cartilage and, therefore, the potential role of sTNFR in mediating transport of trimeric TNFα into cartilage would seem limited.

In summary, our results suggest that TNFα can bind to matrix sites in cartilage, which can significantly alter the transport of TNFα into the tissue. The quaternary structure of TNFα appears to be a crucial factor in determining such binding interactions with cartilage matrix macromolecules and, hence, this structure would also affect the local concentration of TNFα in the tissue. Binding was not affected by trypsin treatment. Thus, even after significant GAG loss associated with joint injury or osteoarthritis, the binding of TNFα within cartilage matrix would presumably not be altered. In contrast, loss of tissue GAG would facilitate the uptake of TNFα by passive diffusion since GAG loss would diminish steric hindrance. In addition, Byun and co-workers recently showed that mechanical injury to cartilage in the presence of an inflammatory environment, in vitro, caused matrix degradation and increased the diffusive uptake of a 48 kDa protein (specifically, an antigen binding (Fab) fragment) into cartilage tissue [37]. This finding suggests that the diffusive transport of TNFα would be similarly affected following joint injury. After joint trauma and following initial cartilage degradation, increased uptake of monomeric TNFα into cartilage could occur more easily and associate to the trimer form; increased uptake of pre-existing trimeric TNFα could also be facilitated, leading to elevated bioactivity of TNFα. Further ECM loss during OA pathology that follows aggrecan degradation might alter the binding of TNFα. Therefore, further research is needed to identify matrix binding sites and binding mechanisms to better understand the feedback between TNFα stimulation and matrix remodeling in OA pathogenesis [4].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

TNFα	tumor necrosis factor-α
ECM	extracellular matrix
GAG	glycosaminoglycan

OA	osteoarthritis

5. References

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- TNFα bound to sites in cartilage matrix, enhancing its uptake into the tissue.
- Trimer-monomer conversion affected binding of TNFα in cartilage tissue.
- Binding of trimeric TNFα to sites in cartilage matrix was negligible.
- Only monomeric TNFα exhibited significant binding to cartilage matrix.
- TNFα binding in cartilage tissue was not disrupted by trypsin treatment.

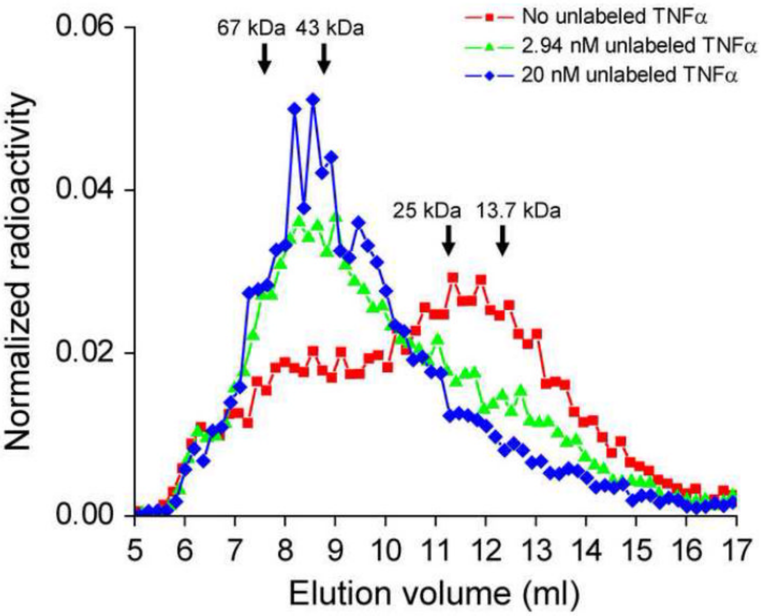

Figure 1. The effect of total concentration of TNFα on the quaternary structure of TNFα

A fixed amount of ^{125}I-TNFα (0.15 nM = 2.55 ng/ml) was incubated in the absence and presence of graded amounts of unlabeled TNFα (2.94 nM = 50 ng/ml, 20 nM = 340 ng/ml) for 48 hr at 4°C in 1×PBS supplemented with 0.1% BSA. After incubation, samples were analyzed by Sephadex G75 gel filtration chromatography. Arrows indicate the positions of molecular weight standards (albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa)).

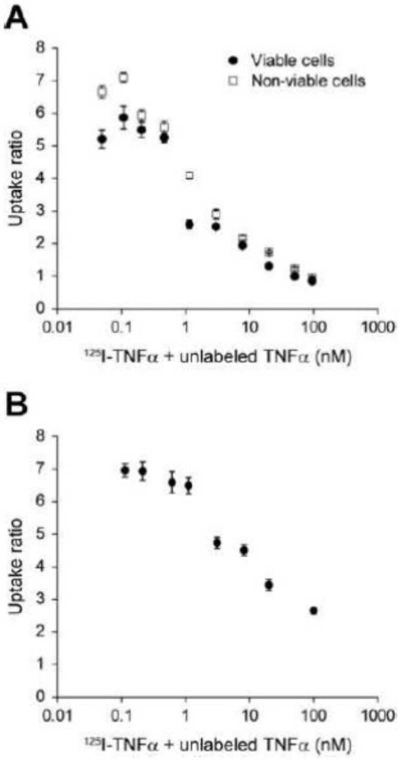

Figure 2. Concentration-dependent uptake ratio of ^{125}I-TNFα into bovine and human cartilage

Cartilage explants were incubated with a fixed amount of ^{125}I-TNFα and graded amounts of unlabeled TNFα. **A.** Equilibrium uptake ratio in bovine cartilage disks of ^{125}I-TNFα (49.2 pM = 838 pg/ml), incubated in DMEM with 1% ITS, and 0.1% BSA at 37°C for 48 hr in the absence (closed circle, viable cells) or presence (open square, non-viable cells) of 0.01% NaN_3 and protease inhibitors. Mean ± SEM (n = 6 disks per condition, harvested from 2 joints). **B.** Equilibrium uptake ratio in adult human knee cartilage disks of ^{125}I-TNFα (112 pM = 1910 pg/ml), incubated in 1×PBS with 0.1% BSA, 0.01% NaN_3 and protease inhibitors at 37°C for 48 hr. Mean ± SEM (n = 8 disks for each condition, harvested from the distal femur of a 44 year old, male grade 0-1 joint).

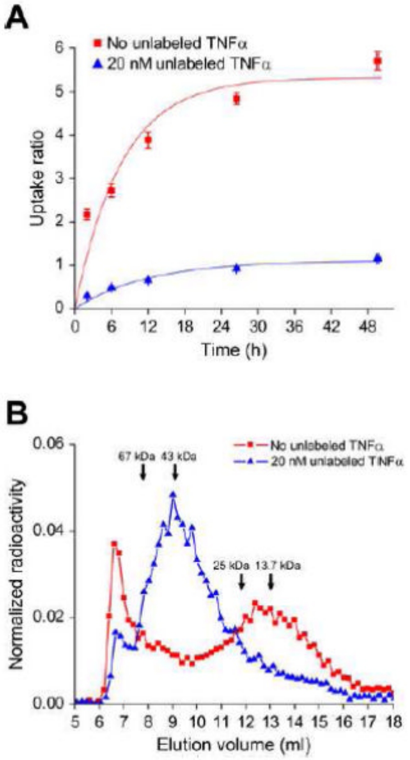

Figure 3. Transient uptake ratio of ^{125}I-TNFα into bovine calf cartilage

A. Transient uptake ratio of ^{125}I-TNFα (49.2 pM = 838 pg/ml) into bovine calf cartilage was measured in the absence and presence of unlabeled TNFα (20 nM = 340 ng/ml) in the bath. Cartilage disks were incubated in DMEM with 1% ITS, and 0.1% BSA at 37°C up to 48 hr. Mean ± SEM (n = 6 disks order kinetics model, $A(1 - e^{-t/\tau})$ (see Results). **B.** Sephadex G75 chromatograms of aliquots from equilibration baths at the end of 48 hr incubation. Arrows indicate the positions of molecular weight standard molecules, albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

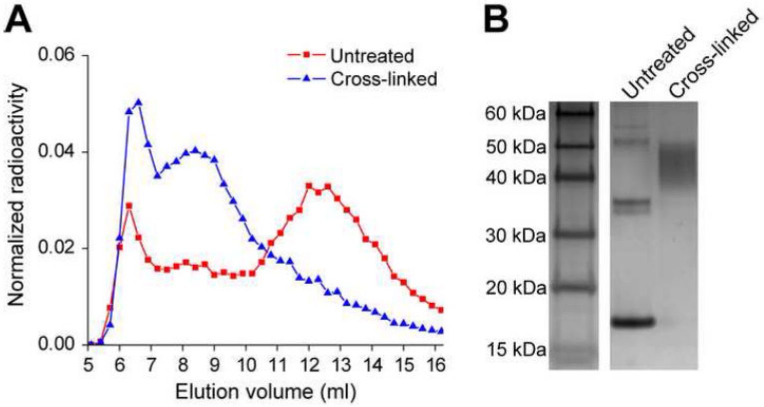

Figure 4. Cross-linking ^{125}I-labeled and unlabeled TNFα

A. Sephadex G75 chromatography of untreated and cross-linked ^{125}I-TNFα. ^{125}I-TNFα (5 nM = 85 ng/ml) was either untreated or cross-linked, and then diluted to a final concentration of 0.1 nM (1.7 ng/ml). After 24 hr at 37°C, each sample was run through a G75 column. **B.** Unlabeled TNFα was either kept untreated or cross-linked, filtered to remove species < 30 kDa, and the retentate run through SDS-PAGE (10% Bis-Tris gel) under non-reducing conditions and detected by silver staining.

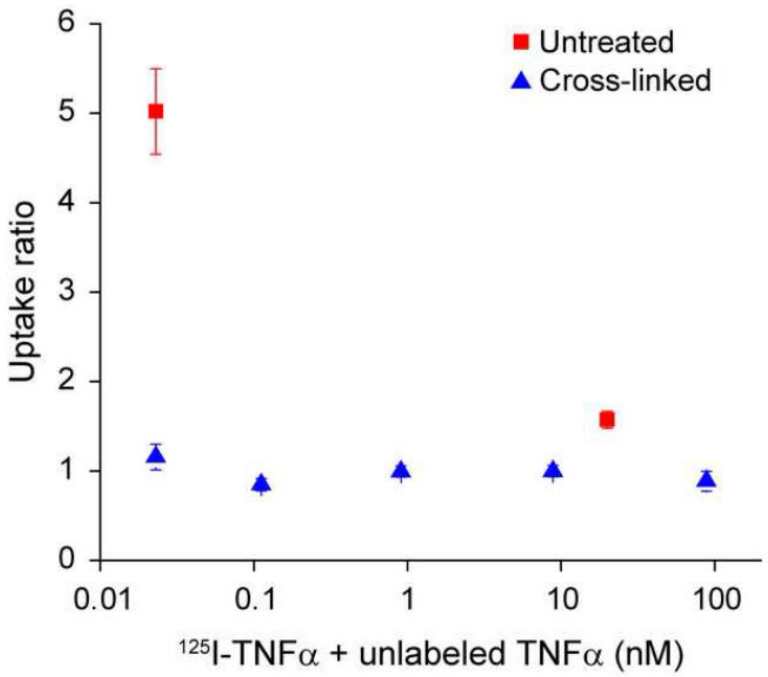

Figure 5. Equilibrium uptake ratio of untreated and cross-linked ^{125}I-TNFα into bovine calf cartilage

Untreated ^{125}I-TNFα (23 pM = 390 pg/ml) was incubated with or without untreated unlabeled TNFα (20 nM = 340 ng/ml). Cross-linked ^{125}I-TNFα (81 pM = 1.38 ng/ml) was incubated with graded amounts of cross-linked unlabeled TNFα. The uptake ratio of cross-linked ^{125}I-TNFα was not affected by varying the concentration of cross-linked unlabeled TNFα (1-way ANOVA, $p = 0.23$). Cartilage disks were incubated in 1×PBS with 0.1% BSA, protease inhibitors, and 0.01% NaN_3 for 24 hr at 37°C. Mean ± SEM (n = 6 disks for each condition, harvested from 2 joints).

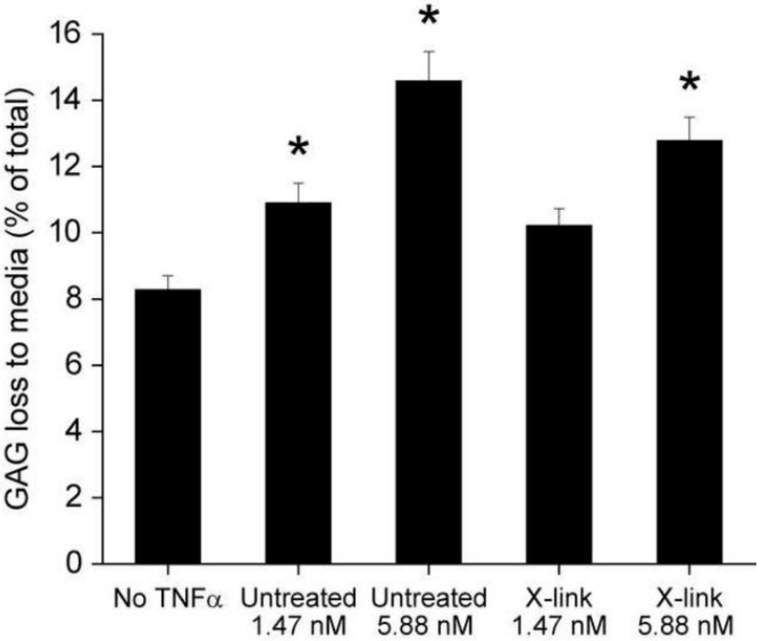

Figure 6. Catabolic effects of untreated and cross-linked TNFα on bovine calf cartilage

Cumulative GAG loss to medium during 6-day incubation with non-cross-linked (Untreated) and cross-linked (X-link) TNFα was measured (control = No TNFα, 1.47 nM = 25 ng/ml, 5.88 nM = 100 ng/ml, $*p < 0.05$, 1-way ANOVA, post-hoc Dunnett's test with control as a reference). Mean ± SEM (n = 6 disks for each condition, harvested from 1 joint).

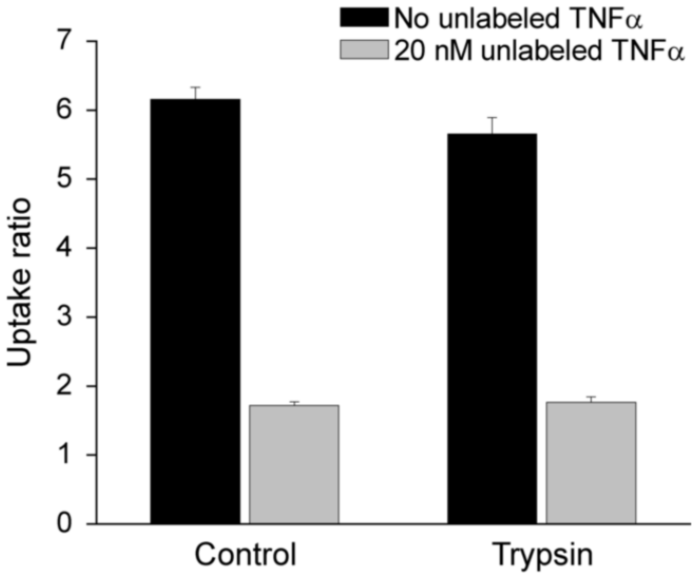

Figure 7. Effect of proteoglycan removal by trypsin on the equilibrium uptake of ^{125}I-TNFα

Bovine calf cartilage disks were either untreated (Control) or treated with 0.1 mg/ml trypsin for 48 hr at 37°C (Trypsin) prior to incubation with ^{125}I-TNFα (23 pM = 390 pg/ml) in the absence or presence of unlabeled TNFα (20 nM = 340 ng/ml) for 48 hr in 1×PBS with 0.1% BSA, protease inhibitors, and 0.01% NaN_3 at 37°C. The measured uptake ratio was only affected by addition of unlabeled TNFα and not by trypsin (2-way ANOVA, $p < 0.0001$ for the effect of unlabeled TNFα; $p = 0.164$ for the effect of trypsin). Mean ± SEM (n = 5 disks for each condition, harvested from 1 joint).