[Frontiers in Bioscience 9, 3257-3261, September 1, 2004]

TRANSGLUTAMINASE CONTRIBUTES TO CPPD CRYSTAL FORMATION IN OSTEOARTHRITIS

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TABLE OF CONTENTS

1. ABSTRACT

Calcium pyrophosphate dihydrate (CPPD) crystals are common components of osteoarthritic joints and correlate with a poor prognosis. Transglutaminase (Tgase) enzymes have been implicated in pathologic mineralization in cartilage; yet, definitive studies linking Tgase activity to CPPD crystal formation in osteoarthritic articular cartilage are lacking. We measured *in-vivo* Tgase activity in osteoarthritic and normal human cartilage, and explored the effect of Tgase inhibitors on CPPD crystal formation by normal chondrocytes. Osteoarthritic articular cartilage from was obtained from specimens discarded at the time of knee replacement surgery. Normal adult cartilage samples from a tissue bank were used as controls. Tgase-specific isopeptide (epsilon-(gamma-glutamyl) lysine) bonds were measured in cartilage extracts by HPLC. Tgase-specific crosslinks were localized in osteoarthritic cartilage by immunohistochemistry. The effect of Tgase inhibition was determined in an *in-vitro* model of CPPD crystal formation.

Tgase-specific crosslink levels were 1.55 ± 0.3 picomoles/ng protein in normal human adult articular cartilage and 4.74 ± 0.7 picomoles/ng protein in osteoarthritic human cartilage ($p<0.001$). Immunostaining confirmed the presence of Tgase crosslinks in the pericellular matrix of chondrocytes at potential sites of CPPD crystal formation. Tgase inhibitors suppressed CPPD crystal formation by porcine chondrocytes. These findings support a role for Tgase in CPPD crystal formation in aging or degenerated cartilage.

2. INTRODUCTION

Calcium crystals, including calcium pyrophosphate dihydrate (CPPD) crystals are common components of osteoarthritic synovial fluids (1) (2). Their presence correlates with increased radiographic severity of disease, with rapid progression of arthritis, and with a poor prognosis (2). Aging and osteoarthritis are major risk factors for CPPD crystal formation in cartilage matrix. Yet, the biochemical processes leading to pathologic calcification in the normally unmineralized articular cartilage matrix remain poorly understood.

The transglutaminases (Tgases) are protein crosslinking enzymes that catalyze the formation of unique, resilient, ε-(-γ-glutamyl) lysine bonds between or within proteins (3). Tgases were initially postulated to contribute to pathologic matrix mineralization in degenerative arthritis for several reasons. 1) They promote normal extracellular matrix mineralization in growth plate cartilage (4). 2) They are implicated in other degenerative processes of aging, such as cataract formation and Alzheimer's dementia (3). 3) They activate the crystal-promoting factor, transforming growth factor β (5).

Much circumstantial evidence implicates a role for Tgases in CPPD crystal formation. Articular chondrocytes have two Tgase enzymes. These include the ubiquitous type II (or tissue) Tgase and the more narrowly distributed Tgase, factor XIIIA (or plasma) Tgase (6) (7). Protein levels of these enzymes increase with age and osteoarthritis, mirroring the clinical pattern of disease (6) (8). Tgase activity correlates with calcium staining in chondrocyte extracellular matrix in fibrocartilage and mouse chondrocytes (9). Yet, proof of a direct link between Tgase activity and CPPD crystal formation in articular cartilage is lacking.

Here, we measure *in-vivo* Tgase activity in normal and osteoarthritic human cartilage, examine these

tissues for the presence of Tgase-specific crosslinks, and determine the effects of Tgase inhibitors on CPPD crystal formation in normal porcine articular chondrocytes.

3. MATERIALS AND METHODS

3.1. Cartilage

Human cartilage was obtained from samples discarded at the time of knee replacement surgeries from patients with osteoarthritis. Discarded normal articular cartilage from adult donors was obtained from the Musculoskeletal Transplant Foundation (Edison, NJ). Normal porcine articular cartilage was removed from the femoral and patellar surfaces of old (3-5 year old, Johnsonville Foods Company, Watertown, WI) or young (2 month old) animals. Chondrocytes were enzymatically isolated as previously described and plated at 4 x 10^5 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Herndon, VA) with 10 % fetal calf serum (10). These conditions maintain the differentiated chondrocyte phenotype during the short durations of the experiment (11). Experiments were performed in DMEM with 0.35 mg/ml bovine serum albumin after a 24 hour wash-out period in serum-free medium.

3.2. Assay for Tgase-specific crosslinks

The assay for Tgase-specific crosslinks was carried out as described (12). Cartilage pieces were homogenized, lyophilized and digested in N ethyl morpholine acetate buffer with collagenase for 8 hours, pronase for 18 and then 8 hours, aminopeptidase for 65 hours and carboxypeptidase A and B for 24 hours. After precipitation with trichloroacetic acid, supernatants were run on ion-exchange chromatography using AG 50 W 8X resin. Quantities of amino acids in the eluants were measured with the ninhydrin assay (Sigma Chemical Co., St Louis, MO), lyophilized, derivatized with phenyl isothiocyanate, and dried under nitrogen. Samples were run on a Hewlett Packard HPLC using a micro-PTH column with an organic gradient. A standard curve was generated with phenyl isothiocyanate-derivatized glutamine-lysine dipeptide (Sigma Chemical Co.). Results were corrected for protein levels in the sample using the Lowry assay (13).

3.3. Immunohistochemistry

To immunolocalize Tgase-specific isopeptide bonds in osteoarthritic cartilage, we fixed tissue specimens in 4% (W/V) paraformaldehyde for 2 hours at room temperature, and embedded them in paraffin. Two μm thick sections were cut and mounted on silane coated-glass slides (14) (15). After deparaffinization, sections were pretreated at 37 °C for 30 minutes with 40mU/ml of chondroitinase ABC (Seigagaku, Japan) diluted in 30mM sodium acetate and 10mM Tris-HCl at pH 7.4 (16). After washing, sections were treated with 0.3% (V/V) hydrogen peroxide for 30 minutes and with 10% (V/V) normal goat serum for 40 minutes at room temperature. Sections were incubated with anti- N ε-(γ-glutamyl) lysine isopeptide mouse monoclonal antibody (1:100, CovaLab, Lyon, France) overnight at 4°C. After washing, sections were treated with biotinylated goat-anti mouse IgG for 30 minutes at room temperature. Antibody staining was detected with avidin-biotin-antiperoxidase complex (ABC kit; Vector Laboratories Inc., Burlingame, CA,) according to manufacturer's directions. Sections were counterstained with methyl green, dehydrated in ethanol, cleared in xylene and mounted. As a negative control, non-immune mouse IgG was used in place of the isopeptide specific primary antibody.

3.4. CPPD model

This is a well-characterized model of CPPD crystal formation, based on the formation of spectroscopically proven CPPD crystals in adult porcine chondrocyte cultures exposed to ATP (17) (18). Chondrocytes were incubated with no additives (control) or one of the following Tgase inhibitors: 20 mM aminoacetonitrile, 125 μM cystamine, 5 mM monodansylcadaverine, or 2 mM N-ethylmaleimide for 48 hours. Media were trace-labeled with 1 μCi / mL ^{45}Ca. For each condition, half the wells were treated with 100 μM ATP added every 8 hours, and half were treated with no ATP. After 24 hours, cell layers were washed, and radioactivity in the cell layer was measured with liquid scintigraphy. Results were corrected for cell protein levels (13). Quantities of ^{45}Ca precipitated in the cell layer in the presence of ATP reflect the formation of CPPD crystals (17). The "no ATP" conditions control for non-specific calcium binding.

3.5. MTT toxicity assay

The 3-(4,5;dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide (MTT) assay was used to assess the toxicity of culture additives (19).

3.6. Statistics

The students two-tailed T-test was used to determine statistically significant differences between groups.

4. RESULTS

Tgase-specific crosslinks in human cartilage

We measured Tgase-specific crosslinks as evidence of *in vivo* Tgase activity in articular cartilage. Levels were 4.74±0.7 picomoles crosslink/ng protein in osteoarthritic cartilage (n=23), and 1.55±0.3 picomoles crosslink/ng protein in normal adult cartilage (n=11) ($p<0.0001$) (Figure 1). Immunohistochemical studies demonstrated the presence of Tgase-specific crosslinks in the pericellular matrix of osteoarthritic chondrocytes (Figure 2). Some cytoplasmic staining was also seen. No staining was seen in control sections treated with non-immune IgG.

Effect of Tgase inhibitors on CPPD crystal formation

Although much circumstantial evidence exists to implicate the Tgase enzymes in CPPD crystal formation, little direct evidence supports this hypothesis. We examined the effect of Tgase inhibitors on CPPD crystal formation in a well-characterized model. None of the Tgase inhibitors were toxic. (Data not shown.) In the presence of ATP, each Tgase inhibitor significantly

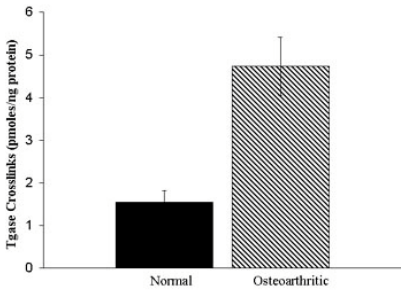

Figure 1. Tgase-specific crosslinks in articular cartilage. Cartilage explants were exhaustively digested with collagenase, pronase, aminopeptidase M, carboxypeptidase A, and carboxypeptidase B. Digests were collected by precipitation with trichloroacetic acid and ion-exchange chromatography. Amino acids were derivatized with phenol-isothiocyanate, and dried under nitrogen. Samples were run on a Hewlett Packard HPLC using a micro-PTH column with an organic gradient. Bars represent mean Tgase-specific crosslinks measured as picomoles/mg protein± standard deviation. Level of Tgase-specific crosslinks are higher in osteoarthritis (n=23) than in normal (n=11) cartilage samples ($p<0.0001$).

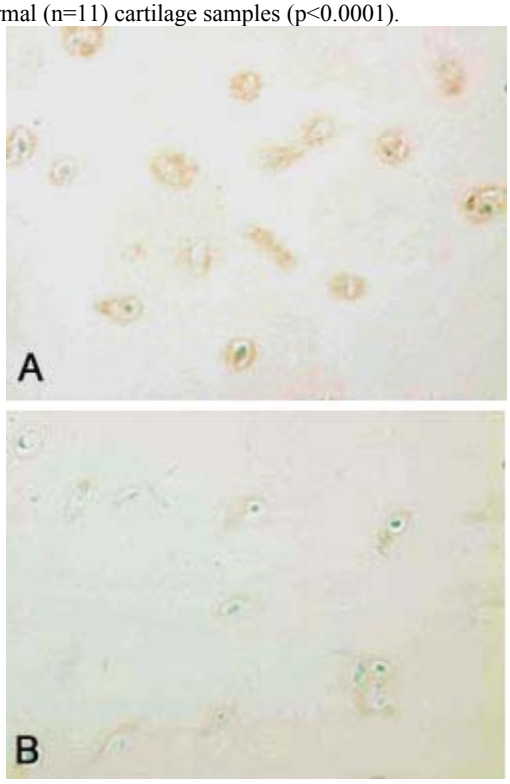

Figure 2. Tgase specific crosslinks in osteoarthritic cartilage. Fixed, paraffin- embedded osteoarthritic knee articular cartilage was incubated with anti- Nε-(γ-glutamyl) lysine isopeptide mouse monoclonal antibody (1:100, CovaLab, Lyon, France) (A) or non-immune serum (B) overnight at 4°C. After washing, sections were treated with biotinylated goat -anti mouse IgG for 30 minutes at room temperature. Antibody staining was detected with avidin-biotin-antiperoxidase complex (ABC kit; Vector Laboratories Inc.). Sections were counterstained with methyl green.

reduced CPPD crystal formation as measured by ^{45}Ca precipitation in old chondrocytes (Figure 3). In the absence of ATP, ^{45}Ca precipitation was unaffected by Tgase inhibitors. In young chondrocytes, very little CPPD crystal formation occurred, and Tgase inhibitors had no effect on ^{45}Ca precipitation in the presence or absence of ATP. The ability to form CPPD crystals correlates with measured Tgase activity, which is about 10 fold lower in young chondrocytes than in old chondrocytes (6).

5. DISCUSSION

These findings provide further support for a role for Tgase enzymes in CPPD crystal formation in aging and osteoarthritic articular cartilage. We show here that levels of *in vivo* Tgase activity, as measured by Tgase-specific crosslinks, are three fold higher in osteoarthritic cartilage than in normal human cartilage. This supports prior work showing increased potential Tgase activity and enzyme levels in osteoarthritic cartilage (8). However, as Tgase activity is so tightly regulated *in vivo*, protein levels and enzyme activity may not accurately reflect *in vivo* enzyme activities (3).

We also show that this activity is located in the pericellular matrix of chondrocytes. As CPPD crystals typically form in the pericellular matrix of aged or osteoarthritic chondrocytes, these findings suggest the presence of active enzyme at sites of CPPD crystal formation

A clear role for Tgase in CPPD crystal formation is demonstrated by the dramatic reduction in CPPD crystal formation in the presence of a variety of Tgase inhibitors. Using alizarin red-S, Johnson et al. previously showed that Tgase levels correlated with matrix calcification induced by interleukin 1 (8) (9). However, the type of mineral formed was not further characterized. While no perfect model of CPPD crystal formation exists, ATP-induced CPPD crystal formation by normal chondrocytes is well characterized (17). The dramatic difference in baseline CPPD crystal formation between young and old chondrocytes in this model mirrors the clinical pattern of CPPD deposition disease. These findings further validate the applicability of this well-established model to CPPD deposition disease.

The mechanism through which Tgase enzyme activity contributes to CPPD crystal formation remains unclear. The pericellular location of the Tgsase crosslinks support the theory that Tgase crosslinking modifies extracellular matrix proteins, such as calcium binding proteins or collagen, which then facilitate matrix mineralization. Additionally, much controversy exists as to which of the two Tgase enzymes in articular cartilage plays a role in CPPD crystal formation. Both type II Tgase and factor XIIIA are present in osteoarthritic cartilage (8) (9) (20). As Tgase inhibitors inhibit all active Tgase enzymes, this question is not addressed by the data presented here.

These studies are not without limitations. Previous literature suggests that Tgase activity increases

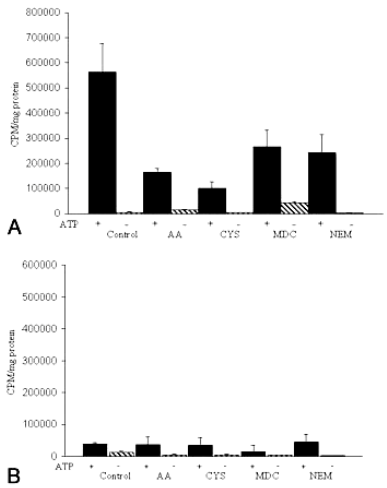

Figure 3. The effect of Tgase inhibitors on CPPD crystal formation in a porcine model. Old (A) and young (B) chondrocyte monolayers were incubated with no additives (Control), or one of the following Tgase inhibitors: 20 mM aminoacetonitrile (AA), 125μM cystamine (CYS), 5 mM monodansylcadaverine (MDC), or 2 mM N-ethylmaleimide (NEM) for 48 hours. Media were trace-labeled with ^{45}Ca. Some cultures were exposed to repeated additions of 100 μM ATP (solid bars). Some cultures had no ATP added (hatched bars). After 24 hours of incubation, ^{45}Ca in the cell layer was measured. The counts in the cell layer in the presence of ATP correlate with quantities of CPPD crystals. The "no ATP" conditions correct for non-specific binding. Tgase inhibitors suppress CPPD crystal formation in old chondrocytes ($n=6$, $p< 0.001$). Tgase inhibitors had no effect on CPPD crystal formation in young chondrocytes or in the absence of ATP ($n=6$, $p >0.05$).

with age in human articular cartilage (8). We were unable to ascertain the ages of the normal or diseased cartilage donors. Thus, some of the increase in Tgase-specific crosslinks in the osteoarthritic cartilage may be due to age. The use of enzyme inhibitors consistently raises concerns about toxicity and non-specific effects. The specificity of the Tgase inhibitor effect is supported by the observation that Tgase inhibitors significantly reduced CPPD crystal formation in old chondrocytes with high Tgase levels, but had little effect on CPPD crystal formation in young chondrocytes with low Tgase levels. Concerns about toxicity were addressed by using multiple inhibitors with different modes of action, comparing effects in old and young chondrocytes, and directly demonstrating lack of toxicity of these compounds.

In summary, we show here that Tgase enzymes are active in the pericellular matrix in cartilage from patients with osteoarthritis, and this activity promotes CPPD crystal formation.

6. ACKNOWLEDGEMENTS

We appreciate the generous donation of surgical specimens by Dr. James Ninomiya, MD and the helpful editorial comments of Dr. Lawrence M. Ryan.

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Key Words: Transglutaminase, Articular Cartilage, Calcium Pyrophosphate Dihydrate, Osteoarthritis

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