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Interleukin-1 Induces Pro-Mineralizing Activity of Cartilage Tissue Transglutaminase and Factor XIIIa

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Two transglutaminases (TGases), Factor XIIIa and tissue TGase (tTGase), are expressed in temporal-spatial association with matrix calcification in growth plates. Meniscal and articular cartilage matrix calcification are prevalent in osteoarthritis (OA) and aging. Here, we demonstrated up-regulation of tTGase and Factor XIIIa in superficial and deep zones of knee OA articular cartilage and the central (chondrocytic) zone of OA menisci. Transforming growth factor-β and interleukin (IL)-1β induced Factor XIIIa and tTGase expression in cartilage and meniscal organ cultures. Thus, we studied TGase activity. Donor age-dependent, OA severity-related, and IL-1-induced increases in TGase activity were demonstrated in both knee menisci and cultured meniscal cells. Meniscal cell TGase activity was stimulated by nitric oxide donors and tumor necrosis factor-α, but transforming growth factor-β did not stimulate TGase activity. The iNOS inhibitor N-monomethylarginine (NMMA) and an inhibitor of tumor necrosis factor receptor-associated factor (TRAF)2 and TRAF6 signaling (the zinc finger protein A20) suppressed IL-1 induction of TGase activity. Increased Factor XIIIa and tTGase activities, achieved via direct transfection of chondrocytic TC28 and meniscal cells, both induced matrix apatite deposition. Thus, Factor XIIIa and tTGase activities were increased in aging, degenerative cartilages and induced by IL-1. Because TGase activity promoted apatite deposition, our findings potentially implicate inflammation in the pathogenesis of cartilage matrix calcification. ***(Am J Pathol 2001, 159:149–163)***

Calcification of the pericellular matrix is a prevalent finding in aging and osteoarthritic articular cartilages and meniscal fibrocartilages.[1,2] Moreover, crystals of hydroxyapatite and calcium pyrophosphate dihydrate released from the cartilage matrix can activate resident intra-articular mononuclear leukocytes and synovial lining cells.[1,2] Consequent crystal-induced inflammation and expression of connective-tissue degrading enzymes can contribute to further cartilage degradation in degenerative joint disease.[1,2]

In contrast to the physiological mineralization that occurs in growth plate cartilage,[3] articular cartilage does not normally calcify.[1,2,4] Nevertheless, certain factors that modulate endochondral growth plate chondrocyte differentiation and mineralization also have the potential to modulate pathological calcification of articular and meniscal cartilages.[3] For example, PTHrP, a major mediator of temporal and spatial endochondral chondrocyte differentiation and matrix metabolism, is up-regulated in OA cartilage.[5,6] In addition, sequential chondrocyte hypertrophy and apoptosis develop adjacent to the mineralizing front in the growth plate.[3] Moreover, focal chondrocyte differentiation to hypertrophy and increased chondrocyte apoptosis are common findings in osteoarthritic (OA) cartilage.[7,8] Chondrocyte hypertrophy also is a frequent finding adjacent to articular cartilage deposits of calcium pyrophosphate dihydrate crystals.[9]

One of the features of growth plate chondrocyte differentiation proposed to promote matrix calcification is increased expression of certain transglutaminases (TGases) (EC 2.3.2.13) in the hypertrophic zone.[10,11] The central effect of TGases is induction of posttranslational protein cross-linking in cells and in extracellular matrices. In this calcium-dependent reaction, the γ-carboxyamide group of a peptide-bound glutamine residue and the primary amino group of either a peptide-bound lysine or a polyamine are covalently joined to form a γ-glutamyl-ϵ-lysine or polyamine bond.[12,13]

It has been proposed that TGase-induced polymerization of pericellular skeletal matrix calcium-binding proteins stabilizes the matrix and promotes nucleation and/or growth of calcium-containing crystals.[12–14] Skeletal matrix proteins with amine acceptor sites for TGases include collagens I and II, and fibronectin and a variety of calcium-binding proteins.[12,14,15] But it also has been demonstrated that TGases have the capacity to modulate processes that may indirectly affect matrix calcification in chondrocytes, such as signal transduction, cell adhesion, and activation of latent transforming growth factor

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(TGF)-β.[16–19] TGases also modulate the apoptotic process,[20–22] which is pro-mineralizing.[23] In this context, increased TGase expression has been used as a tissue marker of increased apoptosis.[23,24]

Seven distinct forms of TGase have been identified, the most widely expressed of which is tissue TGase (tTGase, or TG_C or type II TGase).[12,13] TGases with limited tissue distribution include epidermal, keratinocyte, osteoblast, and prostatic TGases.[12,13] A major circulating TGase is Factor XIII, a coagulation protein involved in clot stabilization.[12,13,25] The plasma form of Factor XIII is a latent (zymogen), soluble heterotetramer consisting of two a subunits (containing the catalytic site) and two b protein subunits.[25] Plasma Factor XIII zymogen requires thrombin for proteolytic activation to an active TGase.[25] Importantly, a latent tissue form of Factor XIII (factor XIIIa) also has been identified.[25] This form of Factor XIIIa, which consists only of two a subunits, is known to be expressed in not only platelets, monocytes, skin, placenta, and gut, but also in growth plate cartilage.[25]

In avian and nonavian skeletons, Factor XIIIa and tTGase expression have been observed to be temporally and spatially associated with terminal differentiation and matrix calcification in growth plate chondrocytes.[10,11,26] TGases are generally regulated not only at the level of gene expression but also by a variety of cell activation and differentiation-associated posttranslational changes that promote increased TGase enzymatic activity.[12] For example, hypertrophic chick chondrocytes have been demonstrated to express intracellular thrombin-like proteolytic activity with the capacity to activate the Factor XIIIa zymogen.[10]

Porcine articular chondrocytes have recently been observed to express tTGase, and porcine chondrocyte TGase enzymatic activity rises several-fold in aging.[27] Moreover, porcine articular chondrocyte TGase activity was implicated in augmenting extracellular PPi,[27] a major regulator of matrix calcification whose production by articular chondrocytes is TGF-β-inducible and rises in association with aging.[28] Thus, our objectives in this study were to explore TGase expression and activation in cells of human joint cartilages, to assess cartilage TGase activity in aging human joint cartilages, and to test the hypothesis that TGases directly promoted matrix calcification by chondrocytic cells.

Materials and Methods

Reagents and Antibodies

Human recombinant TGF-β1 and interleukin (IL)-1β were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibody to placental Factor XIIIa was from Calbiochem (La Jolla, CA), and goat polyclonal antibody to tTGase was obtained from Upstate Biotechnology (Lake Placid, NY). Murine monoclonal anti-A20 antibody was a gift from Dr. C. Vincenz (Dept. of Pathology, University of Michigan, Ann Arbor, MI).[29] All chemical reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

Meniscal Sections and Immunohistochemistry

Specimens of normal and degenerative articular cartilages and menisci were taken as full-thickness blocks (~1 mm in width and 2.5 mm in length) at autopsy or at the time of total knee replacement for advanced OA, as described previously and according to an institutionally approved protocol with appropriate informed consent.[5,30] In the case of meniscal samples, we studied the central (chondrocytic) region[31] of the medial meniscus, and 5-μm paraffin-embedded sections were evaluated. Meniscal specimens were blindly graded for the severity of OA as follows: grade 1, intact cartilage surface; grade 2, minimal fibrillation; grade 3, overt fibrillation; grade 4; cartilage erosion.[32]

Control specimens for normal human fetal growth plate tissue (160 days of gestation) were obtained from the University of Washington Tissue Bank via an institutionally approved protocol. The whole knee was removed and fixed in 10% neutral-buffered formalin. The nondecalcified tissues were embedded in paraffin and serial 5-μm sections were cut by microtome.

Immunohistochemistry was performed as previously described in detail.[30,33,34] In brief, immunohistochemical sections (5 μm) were pretreated with bovine testicular hyaluronidase (0.5 mg/ml at 37°C for 30 minutes) and incubated in 5% normal goat or rabbit serum for 20 minutes before avidin/biotin staining by the ABC method. Hematoxylin was used as the counterstain. Biotinylated anti-rabbit or anti-goat antibodies served as secondary antibodies. Levamisole was added to block endogenous alkaline phosphatase (AP). Negative controls were nonimmune rabbit or goat serum as a substitute for primary antibody.

Meniscal Cell Isolation and Culture

Meniscal cells were taken from tissue slices removed from the central regions of the medial and lateral menisci. Where indicated, meniscal organ culture was performed by incubating these slices for 48 hours in Dulbecco's modified Eagle's medium (DMEM)-high glucose containing 1% fetal calf serum (FCS) and 1% L-glutamine (and articular cartilage organ culture performed in the same manner, where indicated). Otherwise, we minced meniscal tissue with a scalpel, incubated in DMEM-high glucose containing 2 mg/ml clostridial collagenase, 5% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin (Omega Scientific, Tarzana, CA), and incubated on a gyratory shaker at 37°C until the tissue fragments were digested. Residual multicellular aggregates were removed by sedimentation (1000 $\times$ *g*), and cells were washed three times in DMEM containing 5% FCS.

Meniscal cells were maintained in DMEM-high glucose and supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin (Omega Scientific) and cultured at 37°C with 5% CO_2. In monolayer cell culture studies involving stimulation by TGF-β or IL-1, the cells were placed in DMEM-high glucose containing 1% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin. In all other studies, the cells were

cultured in complete medium (as described above). Only primary or first passage meniscal cells were studied. Type II collagen and aggrecan expression were confirmed in each meniscal cell preparation using reverse transcriptase-polymerase chain reaction, as previously described, and using G3PDH as a control.[28]

TC28 Cell Culture

Human immortalized juvenile rib chondrocyte cells (the TC28 cell line originally from Dr. M. Goldring, Harvard Medical School, Cambridge, MA) were maintained in DMEM/F12 (1:1) supplemented with 10% FCS, 1% L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin (Omega Scientific), and cultured at 37°C with 5% CO_2.[28] Only TC28 cells between passages 25 to 45 were used.

TGase Activity

TGase activity was measured by modifications to a previously described method.[35] Specifically, we coated 96-well ImmunoModule plates (Nunc, Rochester, NY) with 200 μl of 20 mg/ml *N*,*N*-dimethylcasein for 1 hour at 23°C. The *N*,*N*-dimethylcasein was removed and nonspecific protein binding was blocked by adding 3% bovine serum albumin in 100 mmol/L Tris, pH 8.5, 150 mmol/L NaCl, 0.05%Tween-20 (TBST) to each well for an additional 1 hour at 23°C. Then, aliquots of 5 μg of total cellular protein from meniscal or TC28 cells that had been lysed and sonicated (in 5 mmol/L Tris-HCl, 0.25 mol/L sucrose, 0.2 mmol/L $MgSO_4$, 2 mmol/L dithiothreitol, 0.4 mmol/L phenylmethyl sulfonyl fluoride, 0.4% Triton X-100, pH 7.5), were added to the plate in triplicate.

To measure TGase in extracts of whole menisci, 50 mg dry weight of tissue from the central zone of each medial meniscus was used as the source (after solubilization and sonication) for aliquots of 5 μg of soluble protein. Fifty μl of solution A (100 mmol/L Tris, pH 8.5, and 20 mmol/L $CaCl_2$) was added to all samples for TGase assay, followed by the addition of 50 μl of solution B (100 mmol/L Tris, pH 8.5, 40 mmol/L dithiothreitol, and freshly added 2 mmol/L 5-(biotinamido) pentylamine. The plates were incubated for 1 hour at 37°C. The wells were washed once with TBST containing 1 mmol/L ethylenediaminetetraacetic acid and then three times with TBST. One hundred μl of a 1:500 dilution of streptavidin-AP in 3% bovine serum albumin/TBST was added to each well for 1 hour at 23°C. The wells were washed twice with TBST, and 200 μl of solution C (100 mmol/L Tris, pH 9.8, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mg/ml of freshly added *p*-nitrophenylphosphate) was added to each well. Readings at OD_{410} were taken throughout 15 minutes. Purified guinea pig liver TGase (Sigma) was used to prepare a standard curve. TGase activity was designated as the amount of 5-(biotinamido) pentylamine incorporated into casein (per μg cellular DNA, determined chromogenically after precipitation in perchlorate,[28] or, where indicated, per μg protein, determined as previously described[28]).

Transfection Studies and Culture of Nonadherent Meniscal Cells and TC28 Cells to Measure Mineralizing Conditions

For transfection of meniscal cells, we used recombinant human Factor XIIIa, a gift from Dr. Dominic Chong (University of Washington, Seattle, WA). The cDNA insert was a 2.3-kb internal *Pst*I fragment of full-length cDNA containing 19 bp of the 5′ noncoding sequence, the entire coding region, and 140 bp of 3′ noncoding sequence, all cloned into the *Pst*I site of pUC18. A human 3.3-kb full-length tTGase cDNA construct, cloned into the *Eco*RI site of pSG5 was a gift of Dr. Peter Davies (University of Texas, Houston, TX).

To directly induce expression of each TGase in meniscal cells, 5×10^5 primary cells were plated in 60-mm dishes and allowed to adhere overnight. We modified[28] the manufacturer's protocol for the Lipofectamine Plus (Life Technologies, Grand Island, NY) methodology. To optimize the transfection of meniscal cells, we added 2.0 ml of serum-free DMEM/F12 containing 0.00015% digitonin to washed cells and incubated for 3 minutes at 23°C. Then, media was removed, and cells transfected at 37°C for 7 hours, followed by removal of the media and addition of complete DMEM-high glucose medium. Transfection of each TGase into TC28 cells was done by the same procedure, with the exception that the digitonin permeabilization step was omitted.

For A20 transfection studies, we used full-length human A20 cDNA (a gift of Dr. M. Jaattela, Danish Cancer Society Research Center, Copenhagen, Denmark)[36] subcloned in sense orientation into the *Xho*I site of pcDNA4/HisMax (Invitrogen, Carlsbad, CA). Transfection efficiency, estimated by control transfections of β-galactosidase and staining for β-galactosidase, was consistently >40% for meniscal cells and >50% for TC28 cells.

To promote matrix calcification in short-term cultures, we modified a nonadherent chondrocyte culture system[37] as described,[38] and assessed cells that formed calcifying nodules during 10 days in culture. In brief, meniscal and TC28 cells, at 24 hours after the transfection, were washed and removed from the dish using 0.2 mg/ml ethylenediaminetetraacetic acid, pH 8.0, then transferred to 6-well plates that had been previously coated with a 10% (wt/vol) in 95% ethanol solution of Poly (2-hydroxyethyl methacrylate) (polyHEME). Cells were then carried in complete DMEM-high glucose (for meniscal cells) or complete DMEM/F12 (for TC28 cells) supplemented with 10 mmol/L β-glycerophosphate, 50 μg/ml ascorbic acid, and 10^{-8} mol/L dexamethasone. Cells were cultured for 10 days in these conditions, with media replaced every 3 days.

Assessment of Matrix Calcification

To assay calcification of the pericellular matrix of meniscal cells and TC28 cells, we used a previously described quantitative Alizarin Red S binding assay.[38] In brief, the media and cells were removed from the polyHEME-

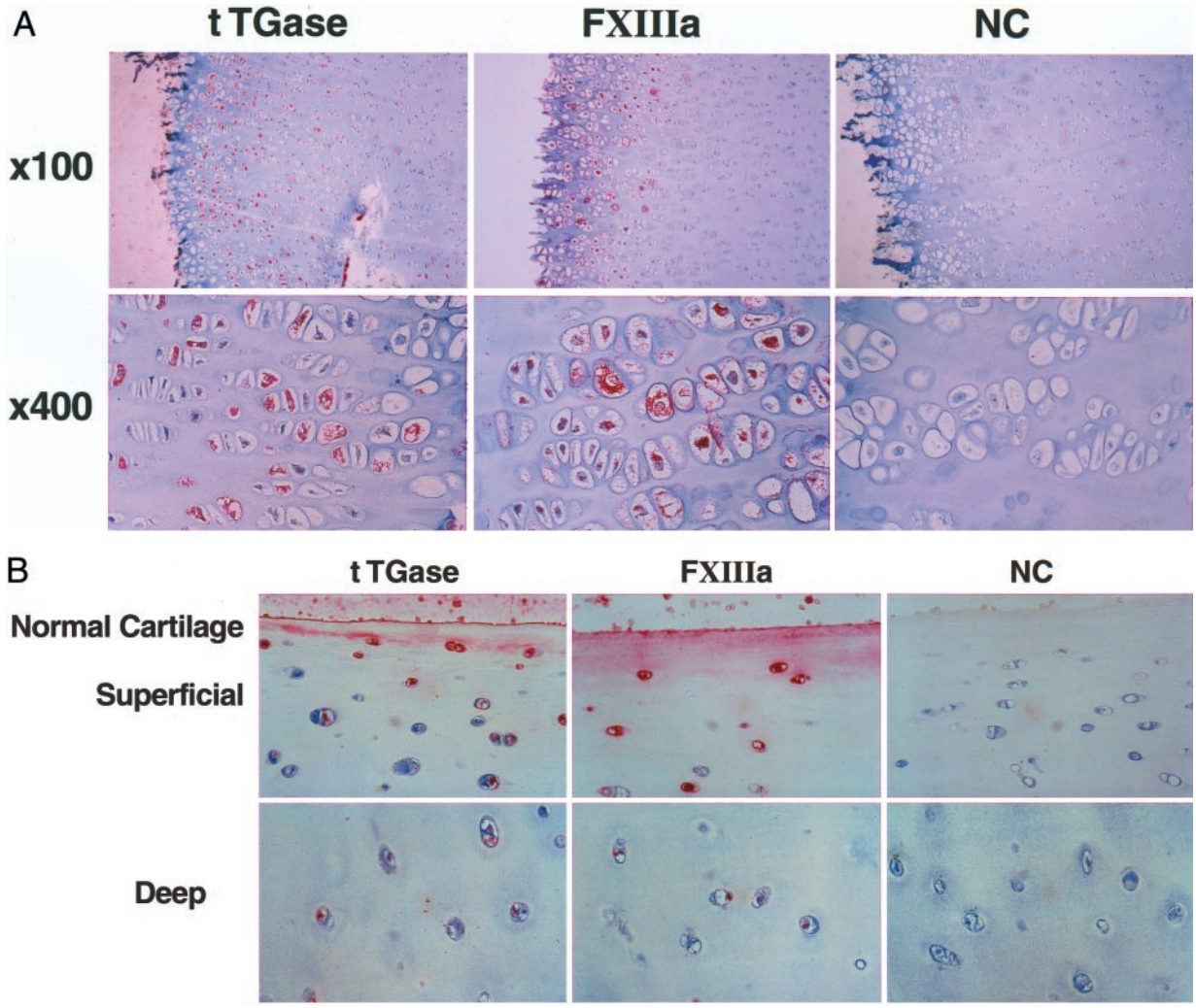


Figure 1. Comparison of Factor XIIIa and tTGase expression in normal and osteoarthritic (OA) knee articular cartilages and menisci. Normal human knees (obtained at autopsy) and OA knees (obtained at time of total knee replacement), as well as control normal tibial growth plates were sectioned and studied by immunohistochemistry using the avidin-biotin ABC method, as described in Materials and Methods. Hematoxylin was used as the counterstain. Brown-red staining was considered to be positive by this method. **A:** As a positive control in assessing chondrocytic Factor XIIIa and tTGase expression, we studied tibial growth plates of human fetal tissue (160 days gestation), as described in Materials and Methods [original magnifications: ×100 (**top**), ×400 (**bottom**)]. The results for Factor XIIIa and tTGase staining in the hypertrophic zone are representative of studies with two donors. Here and elsewhere in the figures, NC indicates negative control. **B:** Results are shown for normal adult articular knee cartilage specimens from one patient (original magnification, ×400; representative of studies with four different donors). **C:** Results are shown for human OA knee articular cartilage from one patient, sampled at the time of total joint replacement for the disease (original magnification, ×400; representative of studies with four different donors). **D:** Results are shown for the central zone of the medial meniscus of a normal knee and the medial meniscus of an OA knee (sampled at time of total knee replacement for severe OA), each from an individual patient (original magnification, ×200; each representative of four different patients).

coated dishes and the plates washed four times with phosphate-buffered saline (PBS), followed by addition of 1 ml of 0.5% v/v Alizarin Red S, pH 5.0, at 23°C for 10 minutes. The plates then were washed four times with PBS before the addition of 100 mmol/L cetylpyridium chloride for 10 minutes to release the remaining calcium-bound Alizarin Red. The solution was collected and read at OD_{570} on a SpectraMAX microplate reader (Molecular Devices, Sunnyvale, CA), with 1 OD_{570} = 1 U of Alizarin Red released per μg of DNA per culture dish. The extent of visualized Alizarin Red staining is confirmed to be consistent with quantitative Alizarin Red binding in each assay. No spontaneous crystal deposition is seen without the presence of cells in this system.

We extracted matrix crystals from plates under each condition using a papain-hypochlorite method,[38,39] and the crystals were embedded in Spurr epoxy resin, sectioned, viewed on a Philips EM340 transmission electron micrograph (Markham, Ontario, Canada), and analyzed by electron diffraction.[40,41]

Assays of PPi Metabolism, Cellular DNA, and Nitric Oxide (NO)

PPi was determined by differential adsorption on activated charcoal of UDP-D-[6-^{3}H] glucose (Amersham, Chicago, IL) from the reaction product 6-phospho-[6-^{3}H] gluconate.[28] Units of nucleoside triphosphate pyrophosphohydrolase (NTPPPH) and AP were designated as μmol of substrate hydrolyzed per hour (per μg DNA in each sample).[28] NO release by cultured meniscal cells was measured using the Greiss reaction.[33] Concentra-

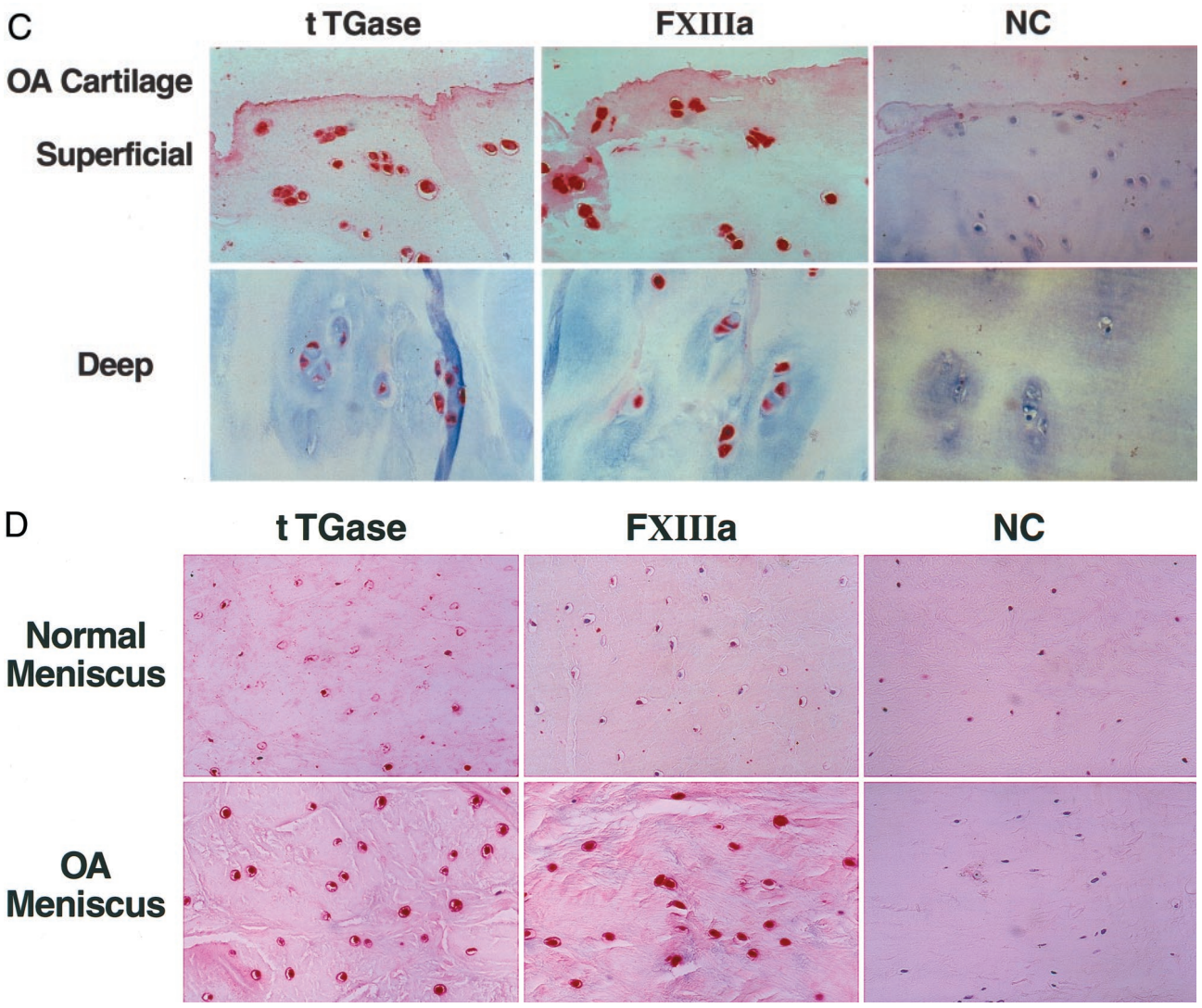


Figure 1. Continued

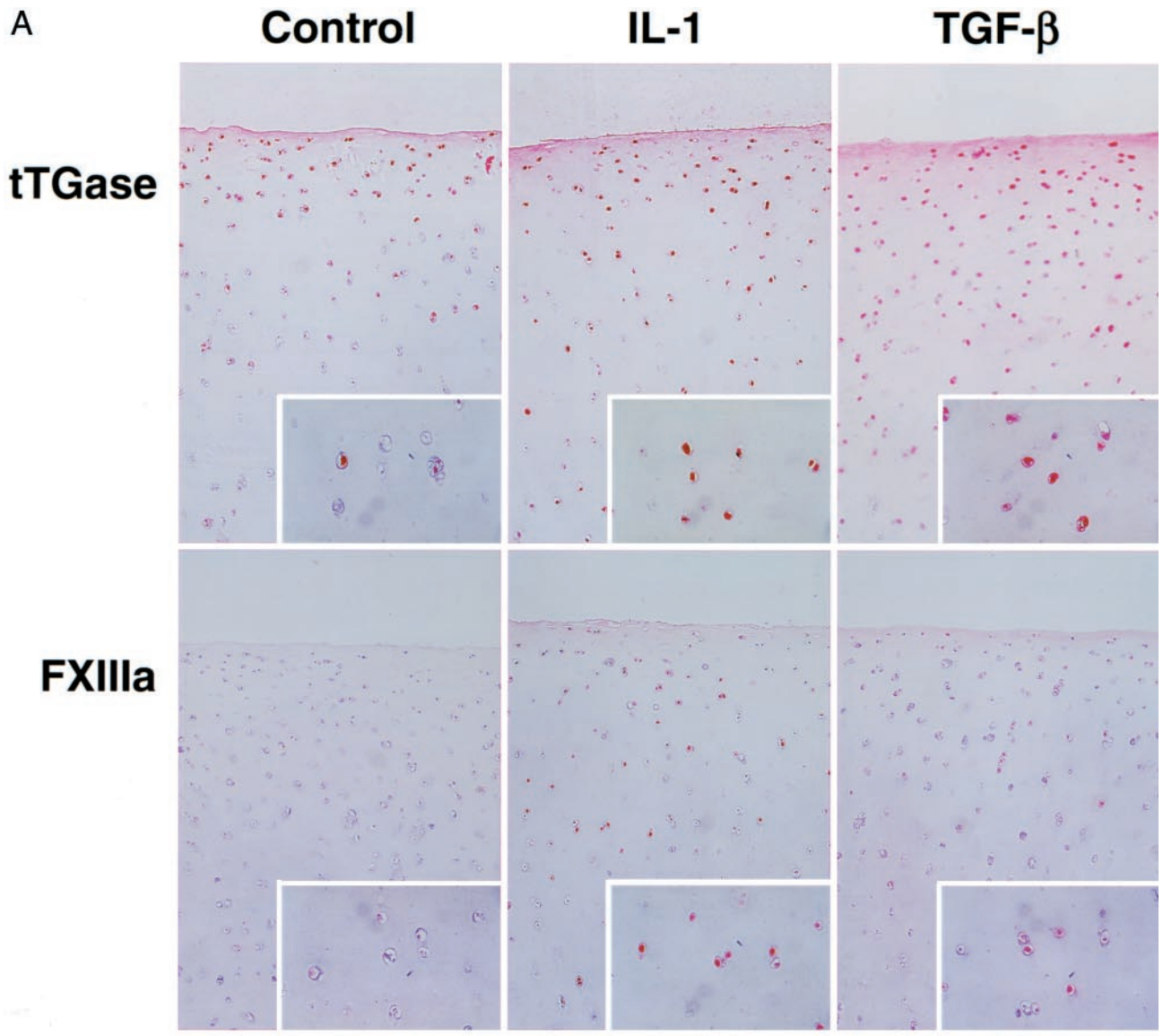
tions or specific activities of PPi, NTPPPH, and AP were equalized for cellular DNA concentrations in each well.

Western Blotting and Immunoprecipitation Studies

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were performed as previously described in detail,[28] using the antibodies to FXIIIa, tTGase, and A20 cited above. To immunoprecipitate tTGase and Factor XIIIa from meniscal cells, 100-μg aliquots of cell lysates were precleared with 1% of protein G Sepharose beads (Sigma). One μl (0.1 μg) of each antibody (FXIIIa, tTGase, and nonimmune controls) was added to the precleared extract. The samples were mixed at 4°C for 1 hour followed by the addition of protein G Sepharose beads to a final vol/vol ratio of 10%. The tubes were again mixed for 1 hour and then centrifuged at 14,000 $\times$ *g* for 1 minute. The beads were washed three times with PBS and resuspended in lysis buffer (5 mmol/L Tris-HCl (pH 7.5), 0.25 mol/L sucrose, 0.2 mmol/L $MgSO_4$, 2 mmol/L dithiothreitol, 0.4 mmol/L phenylmethyl sulfonyl fluoride, 0.4% Triton X-100). The total protein precipitated was quantified for each sample. Then, 5-μg aliquots were used for determinations of TGase activity, as above.

Caspase Activation Assays and Terminal dUTP Nick-End Labeling Staining of Cultured Cells

Caspase-1 and -3 activity was determined using the fluorescent substrates provided in the Promega (Madison, WI) caspase detection kit according to the manufacturer's instructions. In brief, cell lysates were incubated for 1 hour at 37°C in the provided buffer and then an additional 30 minutes with the substrates. Samples were analyzed at absorbance 360 nm, emission 410 nm. For terminal dUTP nick-end labeling staining, 3×10^5 cells were fixed with fresh 4% paraformaldehyde for 30 minutes at 23°C. Cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes on ice and then washed twice with PBS. The DeadEnd colorimetric apo-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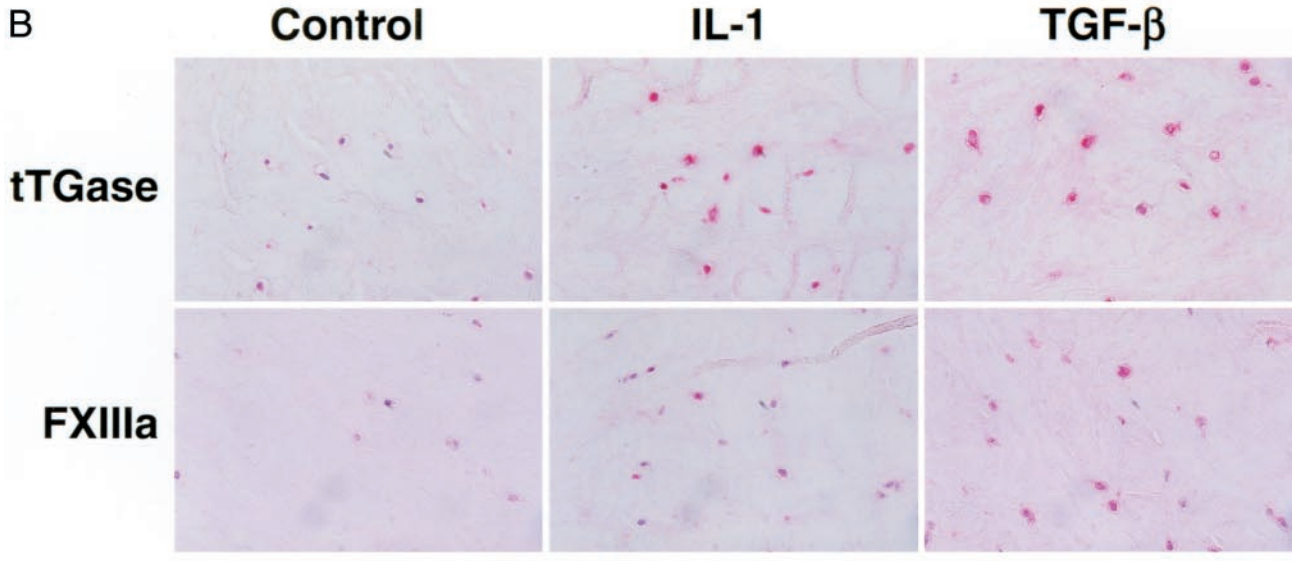
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FXIIIa

ptosis detection system (Promega) was used to stain the cells ($n > 200$ for each experiment), according to the manufacturer's instructions.

Statistics

Error bars represent SD. Statistical analyses were performed using the Student's *t*-test (paired 2-sample testing for means), and by analyzing correlation coefficients in linear regression studies, where indicated.

Results

Expression and Localization of Factor XIIIa and tTGase in Normal and OA Human Knee Cartilages

To assess Factor XIIIa and tTGase expression and localization in joint cartilages, we used an immunohistochemical approach that first confirmed physiological up-regulation of chondrocyte expression of Factor XIIIa and tTGase[11] in the hypertrophic zone of epiphyseal cartilage (Figure 1A). In normal knee articular cartilages, we detected some expression of Factor XIIIa and tTGase in flattened cells in the superficial zone, and trace expression of both TGases in the deep zone (Figure 1B). The articular cartilages of human knees with severe OA (sampled at the time of total joint replacement) demonstrated markedly up-regulated tTGase and Factor XIIIa expression by enlarged chondrocytes in the superficial and deep zones of articular cartilage (Figure 1C).

Similar to the findings in hyaline articular cartilages, trace expression of Factor XIIIa and tTGase was detectable in the central (chondrocytic) zone of knee medial menisci, (Figure 1D). Moreover, increased expression of both TGases was observed in enlarged cells in the central zones of medial meniscal cartilage sampled from severe OA, in specimens taken at the time of total knee joint replacement (Figure 1D).

Because expression of tTGase and Factor XIIIa was increased in OA, we next studied the effects on TGase expression of TGF-β and IL-1β, both of whose activities are up-regulated in OA.[42] Both TGF-β (10 ng/ml) and IL-1β (1 ng/ml) induced increased tTGase and Factor XIIIa immunostaining in knee articular cartilage (Figure 2A) and meniscal cartilage slices (Figure 2B) carried in organ culture for 48 hours. To better understand the potential functional implications of the TGase expression detected in joint cartilages, we proceeded to study regulation of enzyme activity of TGases in knee menisci and cultured meniscal cells.

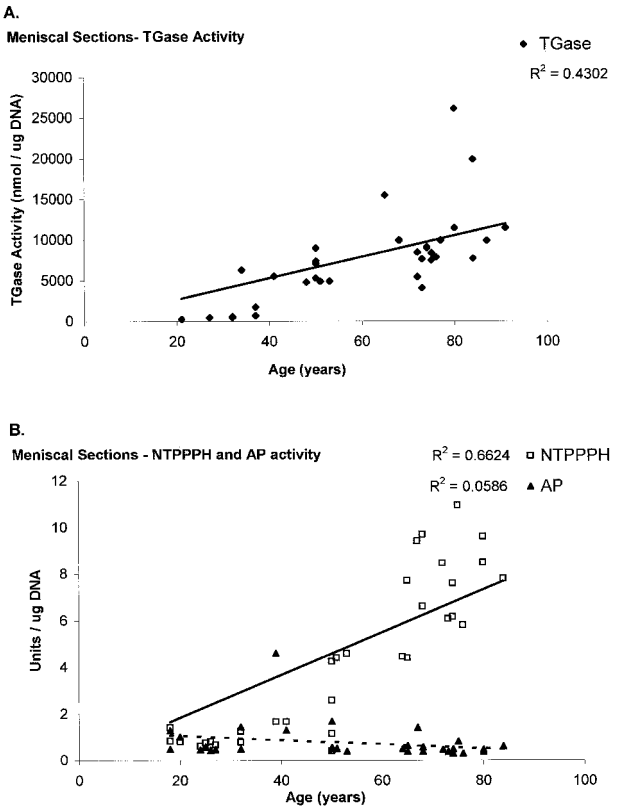

Figure 3. Comparison of specific activities of TGase with the matrix calcification-regulatory enzymes NTPPPH and AP in menisci of various ages. We studied samples taken from 50-mg of dry weight from the central zone of the medial menisci of adult donors of different ages. For the TGase assays, we prepared aliquots of 5 μg of soluble protein, as described in Materials and Methods, and added aliquots in triplicate to a plate previously coated with 20 mg/ml of *N,N*-dimethylcasein. For the TGase assays (**A**), performed as described in Materials and Methods, the results were expressed as total 5-(biotinamido) pentylamine incorporated into *N,N*-dimethylcasein (nmol/μg cartilage DNA). For NTPPPH and AP assays (**B**), aliquots of 5 μg of soluble protein were assayed in triplicate, as described in Materials and Methods. Results were analyzed by linear regression, as indicated.

TGase Activity in Menisci and Meniscal Cells

Knee meniscal TGase activity increased in a donor age-dependent manner in whole tissue extracts from a panel of meniscal specimens from adult donors (Figure 3A). As additional controls, we assessed and compared the activities of other types of matrix calcification-regulatory enzymes in menisci from these donors. Thus, we studied PPi-generating NTPPPH activity, because it rises significantly in association with both aging and chondrocalcinosis in articular cartilages, and is inducible by TGF-β in chondrocytes.[1,28] In addition, we studied PPi-degrading AP activity, because it does not rise in aging cartilages.[1] NTPPPH activity but not AP activity (Figure 3B) increased

Figure 2. Effects of IL-1β and TGF-β on expression of Factor XIIIa and tTGase in knee articular cartilage and medial meniscus in organ culture. Articular cartilage and medial menisci from normal donors were studied in cartilage organ culture as described in Materials and Methods, and analyzed after 48 hours of treatment with TGF-β (10 ng/ml), IL-1β (1 ng/ml), or medium alone. **A:** Knee articular cartilages were studied by immunohistochemistry for tTGase and Factor XIIIa expression, as described above [original magnification, ×100; insets, ×200] showing magnified areas in the superficial zone). The figure is from an individual patient (representative of studies with three donors). **B:** The figure shows the central zone of the medial meniscus from an individual patient (original magnification, ×200; representative of studies with three donors).

in an age-dependent manner in the same panel of specimens in which TGase activity was augmented.

We next examined the potential relationship between OA severity grade and TGase activity in a separate group of donors older than 60 years of age whose samples were graded for the degree of OA. We observed a direct correlation between the grade of OA and mean specific activity of TGase (per μg DNA) (Figure 4A). There also was a significant direct correlation between the severity of OA and the specific activity of NTPPPH in knee meniscal specimens, but, in contrast, there was no significant correlation between the grade of OA and AP activity (Figure 4, B and C).

Because TGase activity increased with more severe OA, we next studied the effects of IL-1 and TGF-β on meniscal cell TGase activity *in vitro*.[42] IL-1β (10 ng/ml) induced increased TGase activity in a donor age-dependent manner, and did so to a much greater degree than TGF-β (10 ng/ml) in cultured normal meniscal cartilage cells (Figure 5A). In contrast, TGF-β but not IL-1 stimulated increased NTPPPH activity in association with aging (Figure 5B), and AP activity did not significantly change in response to either IL-1 or TGF-β or alter with aging (Figure 5C).

We performed immunoprecipitation studies to determine whether IL-1 induced increased TGase activity attributable to each TGase. Antibodies to both Factor XIIIa and tTGase (but not nonimmune control IgG) removed TGase activity from cell lysates of IL-1-stimulated meniscal cells (Figure 6A). There was differential recovery of TGase activity in the washed immunoprecipitates (Figure 6A), in association with greater neutralizing activity for TGase of the antibody to Factor XIIIa than the antibody to tTGase (Figure 6B).

Mechanism of Induction of TGase Activity by IL-1

IL-1-induced NO generation mediates certain IL-1 effects in chondrocytes.[43] We observed that the NO donor Noc-12 (2.5 to 25 μmol/L), and the peroxynitrite generator Sin-1 (1 to 10 μmol/L)[44] shared the ability of IL-1 to induce TGase activity in cultured normal knee meniscal cells, although Noc-12 and Sin-1 were less effective than IL-1 at inducing TGase activity (Figure 7). Tumor necrosis factor (TNF)-α, which also acts on chondrocytes,[45,46] stimulated increased TGase activity in cultured normal knee meniscal cells (Figure 7). In contrast, TGF-β did not induce TGase activity under these conditions (Figure 7). The NOS inhibitor NMMA blocked the ability of both IL-1 and TNF-α to induce TGase activity (Figure 7). Thus, we further investigated the mechanism of induction of TGase activity.

IL-1 and TNF-α signaling both transduce signaling through TNF-α receptor-associated signaling factors (TRAFs), TRAF2 and TRAF6.[47–50] A20 inhibits both IL-1 and TNF-α signaling partly at the level of TRAF2 and TRAF6 action.[36,47,48,51,52] and A20 can suppress IL-1-induced NO production.[52] Resting meniscal cells in culture had weak or undetectable A20 expression, but when

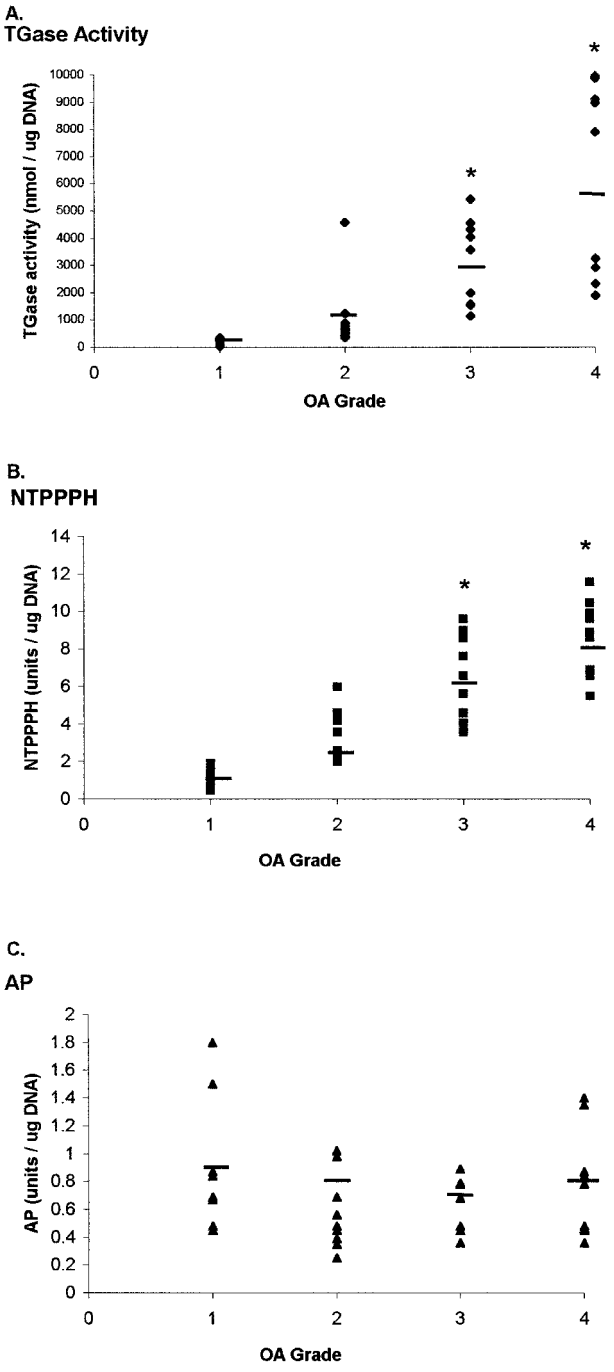

Figure 4. Association of increased TGase and NTPPPH but not AP-specific activities with increased severity of OA in the meniscus of aged donors. Meniscal specimens [50 mg blocks from the central (chondrocytic) region of the medial meniscus] were taken at the time of autopsy or total joint arthroplasty for OA in a panel of 45 donors older than the age of 60. This panel of donors was entirely separate from the panel of donors studied in Figure 3. Cartilage samples were graded in a blinded manner for degree of OA as follows: grade 1, intact cartilage surface; grade 2, minimal fibrillation; grade 3, overt fibrillation; grade 4, erosion. We then studied, in a blinded manner, TGase (**A**)-, NTPPPH (**B**)-, and AP (**C**)-specific activities (per μg cartilage DNA), performed as described above. *, $P < 0.05$.

we used a plasmid DNA transfection approach, as described in Materials and Methods, to efficiently express recombinant human A20 in cultured meniscal cells, we confirmed that transfection markedly up-regulated me-

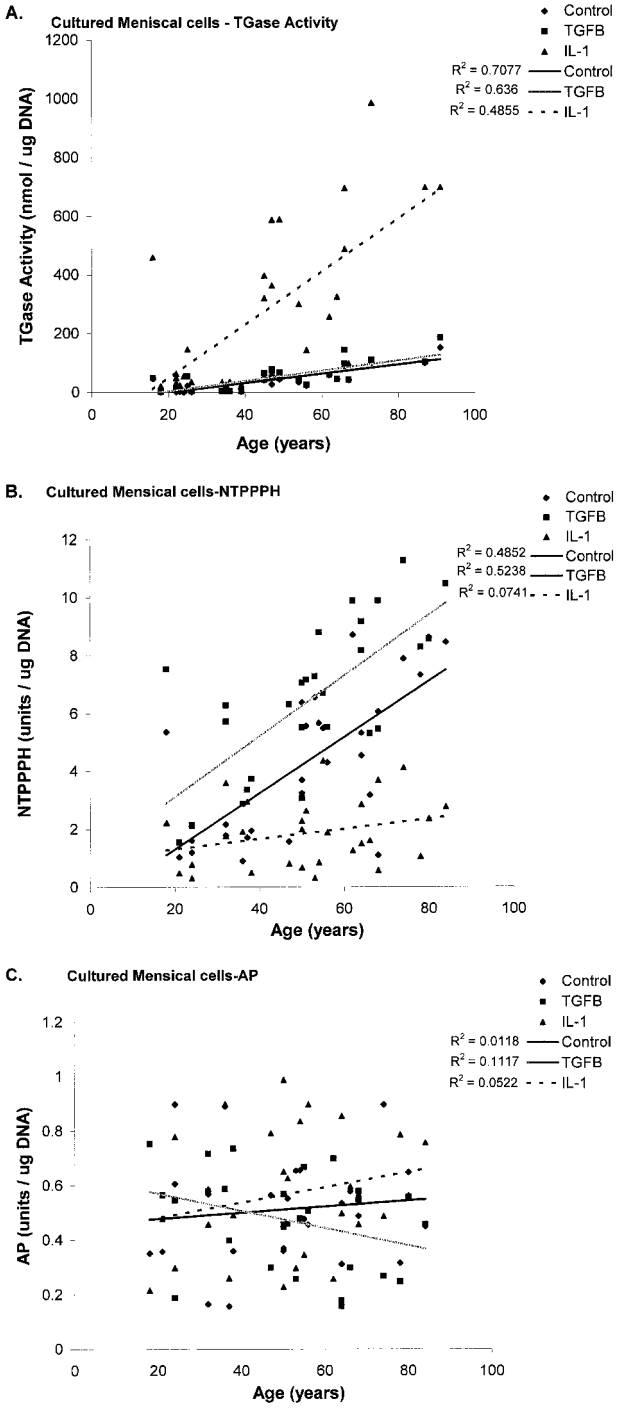

Figure 5. IL-1 selectively induced TGase activity (and not NTPPPH and AP activity) in meniscal cells in primary culture; association with aging. Meniscal cells (from the same donors used in Figure 3 above) were released from the matrix with collagenase digestion and maintained in monolayer culture for 72 hours. Cells were then replated in 35-mm dishes (containing 3×10^5 cells), followed by treatment for 48 hours with 10 ng/ml of TGF-β or IL-1β (10 ng/ml) in 1% FCS-containing DMEM high-glucose medium, and cells were collected and lysed for the TGase (**A**), NTPPPH (**B**), and AP (**C**) assays as described in Materials and Methods. Five μg of soluble protein were studied in triplicate for each assay performed as previously described. Results were analyzed by linear regression, as indicated.

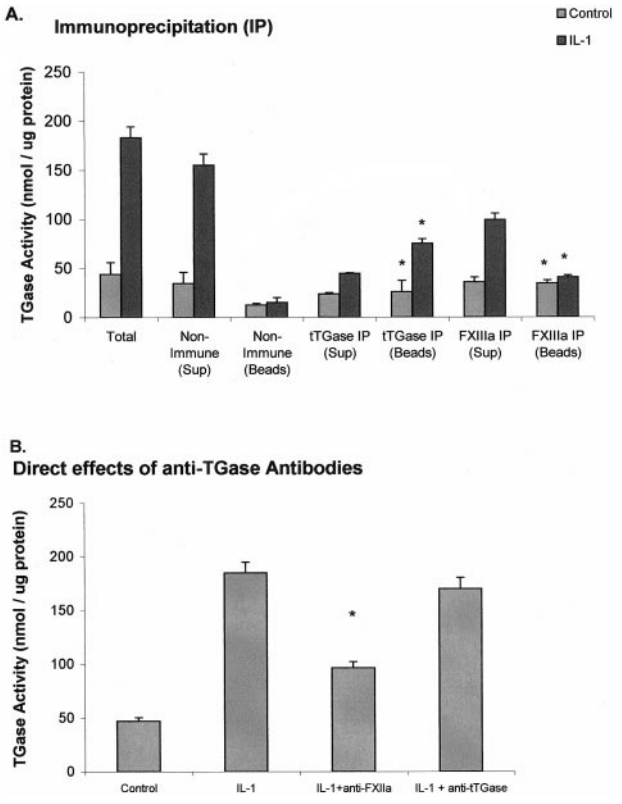

Figure 6. TGase activity induced by IL-1 in meniscal cells was attributable in part to both tTGase and FXIIIa. Meniscal cells (3×10^5) were cultured in 35-mm dishes for 72 hours in 1% FCS containing DMEM high-glucose media with or without IL-1β (10 ng/ml). **A:** To assess for possible immunoprecipitation of active tTGase and Factor XIIIa, 1 μl (0.1 μg) of antibody to FXIIIa or tTGase, or nonimmune IgG was added to equal aliquots (100 μg protein) of lysates of meniscal cells that had previously been treated with IL-1, as described above. This was followed by preclearance of the cell lysates that had been precleared with nonimmune IgG, as described in Materials and Methods. The samples were mixed at 4°C for 1 hour followed by the addition of protein G-Sepharose beads to a final ratio of 10% (v/v), followed by remixing for 1 hour, centrifugation at 14,000 $\times$ *g* for 1 minute, and washing of beads and resuspension in a Tris-HCl-containing buffer, pH 7.5, as described in Materials and Methods. Protein precipitated was quantified for each sample, and then 5-μg aliquots were used for determinations of TGase activity, as above. **B:** To assess for possible neutralization of TGase activity attributable to FXIIIa or tTGase, we incubated meniscal cell lysates (100 μg of protein from cells that had been treated with IL-1, as above) with 0.1 μg of antibody to FXIIIa or tTGase, or nonimmune IgG as a control in an equal volume for 2 hours at 4°C. The TGase activity of each cell extract was then determined as previously described. Data pooled from cell lysates of three normal donors studied in triplicate. *, $P < 0.05$.

niscal cell production of A20 as a 72-kd polypeptide by Western blotting (not shown). Under these conditions, A20, like NMMA, attenuated IL-1 and TNF-α-induced NO release (Figure 8), and A20 (Figure 9), like NMMA (Figure 7) attenuated IL-1 and TNF-α-induced TGase activity (Figure 9). However, A20 did not inhibit TGase activity induced by direct provision of the NO donor Noc-12 or the peroxynitrite donor Sin-1 (Figure 9).

Direct Effects of Factor XIIIa and tTGase on Matrix Calcification

Last, we evaluated and compared the direct functional effects of Factor XIIIa and tTGase in cultured meniscal cells. Because human articular chondrocytes are difficult

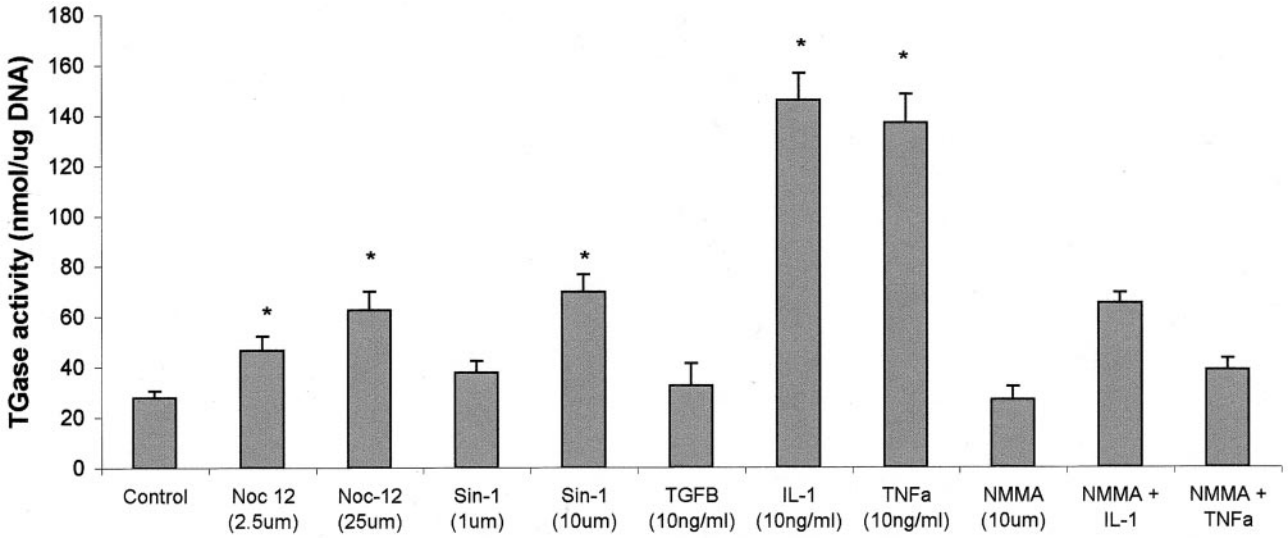

Figure 7. Peroxynitrite and NO donors, and TNF-α, but not TGF-β, induce TGase activity in cultured meniscal cells. Meniscal cells (3×10^5) were cultured in 35-mm dishes for 72 hours in the presence of the indicated concentrations of the peroxynitrite donor Sin-1, the NO donor Noc-12, TNF-α, and TGF-β. The cells were lysed and 5 μg of total protein was assayed for TGase activity as described previously. Data pooled from five normal donors studied in triplicate. *, $P < 0.05$.

to efficiently transfect, we additionally transfected TC28 cells,[28] an immortalized line of human juvenile costal chondrocytes that we confirmed to express collagen II and aggrecan (not shown). We studied cells in a system where matrix calcification was promoted in nodule-forming nonadherent chondrocytes in short-term culture[37] by the use of polyHEME-coated tissue culture plates and media supplemented with dexamethasone (10^{-8} mol/L), the phosphate source β-glycerophosphate, and ascorbate (50 μg/ml).[39] Transfection of both Factor XIIIa and tTGase markedly increased TGase activity in both cultured knee meniscal cells and TC28 cells (Figure 10). Treatment with IL-1 induced significant increases in TGase activity under these conditions (Figure 10). TGF-β did not significantly induce increased activity of TGases in chondrocytes cultured in this manner. Under these conditions, IL-1 but not TGF-β significantly increased matrix calcification (Figure 11).

Increased activity directly associated with transfection of either TGase was not associated with increased meniscal cell or TC28 cell apoptosis, measured by terminal dUTP nick-end labeling assay as well as by caspase-1 and caspase-3 activation (not shown). Augmented TGase activity, induced by either transfection of Factor XIIIa or tTGase, also failed to induce significant changes in extracellular PPi, or in PPi-regulating NTPPPH activity or AP activity (not shown). But, under these conditions, marked increases in TGase activity that followed forced expression of either Factor XIIIa or tTGase induced particularly marked increases in the amount of calcium precipitated in the matrix of cultured meniscal cells and TC28 cells (Figure 11). Transmission electron microscopy and electron diffraction analysis of the type of crystals deposited in this system, revealed, under all conditions, exclusively spherulitic HA, with D-spacings of the observed crystals on electron diffraction of 3.44, 3.08, 2.81, 2.78, and 2.63 Angstroms.

Discussion

In this study, we demonstrated up-regulated expression of two TGases, Factor XIIIa and tTGase, in knee cartilages with OA. The levels of TGase activity in tissues are modulated by both gene expression and by a variety of posttranslational events that regulate enzymatic activity of the translated TGases.[12] Thus, we focused on the potential relationships between OA, aging, and TGase activity, and we directly explored for direct regulatory functions of activated Factor XIIIa and tTGase in cartilage matrix calcification. Our results directly linked increased activity of TGase to aging, to increasing severity of OA of the knee, and to the capacity for cartilage matrix calcification. Our findings also established the potential for joint inflammation to increase both cartilage TGase activity and matrix calcification.

In specimens from knee OA, up-regulated chondrocyte expression of Factor XIIIa and tTGase was observed in the superficial and deep zones of articular cartilages, as well as the central zones of knee menisci, in association with cells that were grossly enlarged in size. Up-regulated chondrocyte expression of Factor XIIIa and tTGase in the hypertrophic zone of growth plate cartilage[11] was confirmed in control specimens in this study. It will be of interest to further examine the direct relationship between specific markers of chondrocyte hypertrophy, or chondrocyte apoptosis, and the expression of individual TGases *in situ* in knee meniscal or articular cartilage specimens.

In the avian skeleton, chondrocyte hypertrophy has been linked to the ability to convert latent Factor XIIIa to an active TGase.[10] Here, we observed that normal, cultured human knee meniscal chondrocytic (collagen II and aggrecan-expressing) cells transfected with tTGase or with the Factor XIIIa zymogen developed marked increases in TGase activity. In a previous study of chick

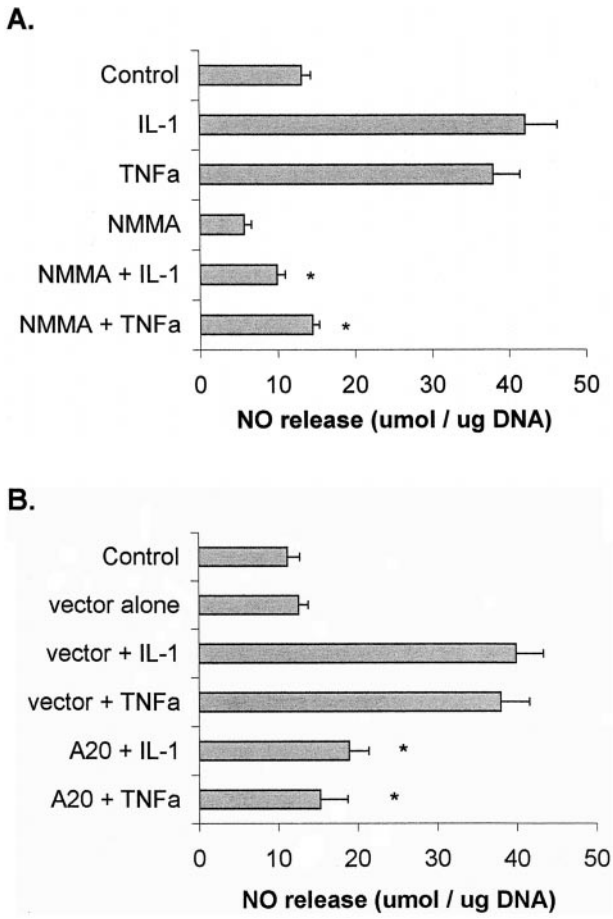

Figure 8. The zinc finger protein A20, like the NOS inhibitor NMMA, suppresses IL-1 and TNF-α-induced NO release in meniscal cells. We studied NO release by 3×10^5 meniscal cells, which were plated and stimulated with TNF-α (10 ng/ml) or IL-1 (10 ng/ml), with or without 10 μmol/L of NMMA (**A**), for 72 hours. Conditioned media were collected and 50-μl aliquots analyzed for NO release using the Greiss reaction, with results expressed as μmol released per μg DNA. For the studies in **B**, the meniscal cells were transfected with A20 or empty plasmid (where indicated), using the procedure described in Materials and Methods, which included use of lipofectamine plus and a previous 3-minute incubation with 0.00015% digitonin to optimize the transfection. After 72 hours of incubation with TNF-α or IL-1, the conditioned media were collected from the cultured, transfected cells and analyzed in triplicate for NO release, as above. Data pooled from four normal donors studied in triplicate for **A** and **B**. *, $P < 0.05$.

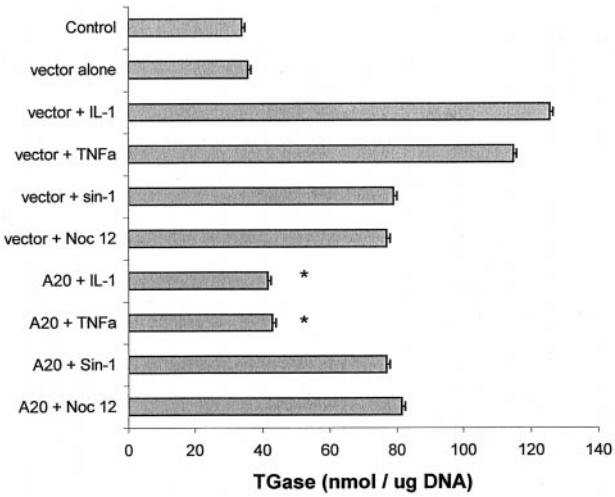

Figure 9. A20 suppresses the ability of IL-1 and TNF-α to induce TGase activity in cultured meniscal cells. We studied aliquots of 3×10^5 normal knee meniscal cells, which were transfected with empty vector or with the A20 expression plasmid, as described above, plated in 35-mm dishes and allowed to adhere overnight. Cells were then incubated an additional 72 hours in the presence of TNF-α (10 ng/ml), IL-1 (10 ng/ml), Sin-1 (10 μmol/L), Noc-12 (25 μmol/L), where indicated. The cells were lysed and 5-μg aliquots of total protein were assayed for TGase activity as described previously. Data pooled from four donors studied in triplicate for both **A** and **B**. *, $P < 0.05$.

sternal chondrocytes, nonhypertrophic cells did not effectively convert transfected latent Factor XIIIa to an active TGase.[10] In this study, activation of Factor XIIIa TGase after transfection might have been attributable in part to cell stress from our transfection approach. Alternatively, human meniscal and articular chondrocytic cells may have a different capacity than chick sternal chondrocytes to activate latent Factor XIIIa TGase activity.

We observed that two putative mediators of OA, TGF-β and IL-1,[42,45] induced Factor XIIIa and tTGase expression in articular cartilage in organ culture. We determined that IL-1 induced TGase activity in a manner mediated by NO production in cultured meniscal chondrocytic cells. We also demonstrated that NO donors, and TNF-α (in a NO-mediated manner) increased the meniscal chondrocytic cell TGase activity. Assessment of the TGase activity induced by IL-1 in cultured meniscal cells, using TGase-selective antibodies, identified contributions of both Factor XIIIa and tTGase to the increased TGase activity.

Possible factors in NO-induced, IL-1-induced, and TNF-α-induced increases in TGase activity would be anticipated to include posttranslational TGase phosphorylation, fatty acylation, and proteolytic cleavage.[12] Tissue forms of TGases are primarily cytosolic, but tTGase can concentrate in specialized areas on the inner leaflet of the plasma membrane.[12] In addition, tTGase and factor XIIIa can be partly extruded from cells,[10,12] and tTGase colocalization with pericellular fibronectin could modulate matrix assembly.[53] Thus, potential regulatory effects of alterations of TGase structure on TGase subcellular localization also will be of interest to investigate as potential modulators of TGase activity and functions in chondrocytes.

Effects of either IL-1 or TGF-β on TGase-specific activity were not directly correlated with effects on the specific activities of the matrix calcification regulatory enzymes NTPPPH and AP in the aging study described for meniscal cells from human donors. Our results suggest the possibility of a selective mechanism for TGase activity induction in association with aging in human knee mensical cells. Some induction mechanisms for TGase activity may be selective for subsets of cytokines and inflammatory mediators because TGF-β did not induce increased TGase activity in cultured meniscal cells. We speculate that TGF-β may have less of a capacity than IL-1, TNF-α, and NO donors to stimulate posttranslational activating modifications of TGases in chondrocytes because of a relatively lower capacity of TGF-β to stimulate signal transduction events that drive production of proteases and oxidants that modify TGases. However, there

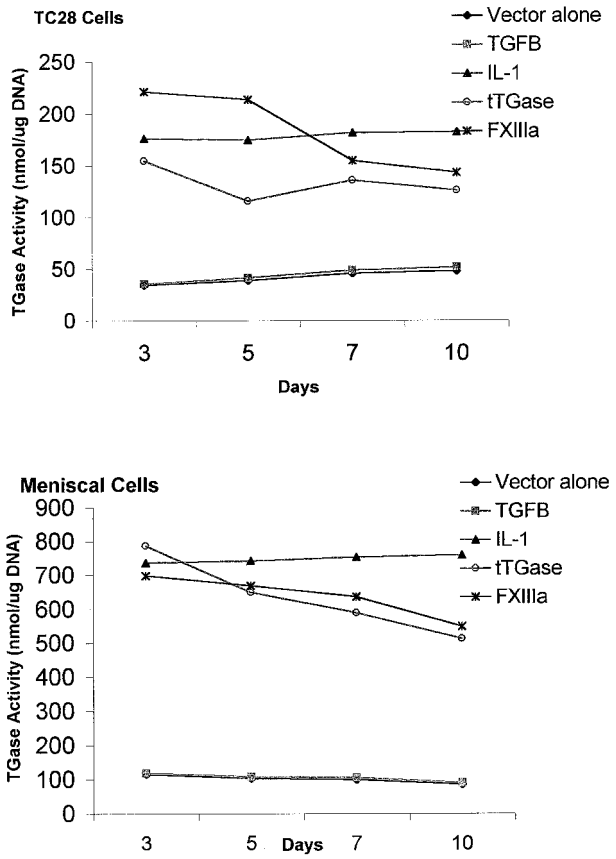

Figure 10. Effects of direct Factor XIIIa or tTGase expression on TGase activity in meniscal cells and TC28 cells. Human chondrocytic TC28 cells or normal knee meniscal cells (5×10^5), were plated in 60-mm dishes and allowed to adhere overnight, as described in Materials and Methods. We then transfected 2 μg of plasmid DNAs or empty plasmid vector, performed as described above. After 24 hours, the cells were removed from the dishes and plated on polyHEME-coated tissue culture plates and media supplemented with 10 mmol/L of glycerophosphate, ascorbate (50 μg/ml), and dexamethasone (10^{-8} mol/L), which stimulated the cells to form mineralizing nodules for 3 to 10 days in culture. For comparison purposes, cells were treated only with IL-1 or TGF-β (10 ng/ml each) for up to 10 days. At each time point designated, the cell nodules were collected and lysed, and TGase activity then determined as previously described. For meniscal cells, data pooled from six donors studied in triplicate. For TC28 cells, $n = 6$ each, studied in triplicate. Statistics not indicated on this graph, but $P < 0.05$ for the increases in TGase activities in response to both Factor XIIIa and tTGase transfection, and for IL-1 treatment, at all time points.

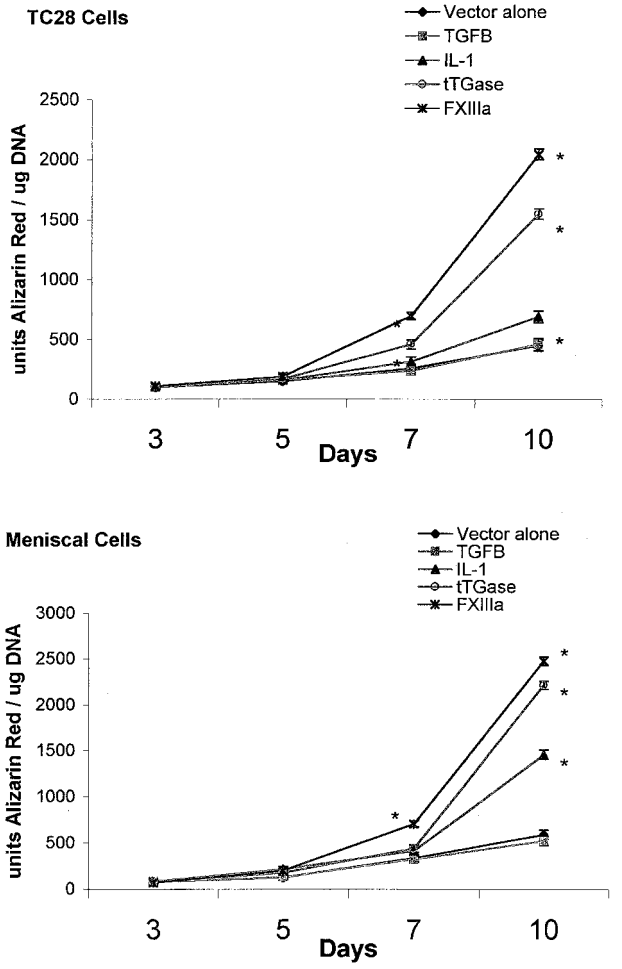

Figure 11. Effects of increased factor XIIIa or tTGase-associated TGase activities on matrix calcification in TC28 cells and meniscal cells. TC28 cells and meniscal cells were transfected with empty plasmid or either TGase expression construct, and then transferred to polyHEME and cultured for up to 10 days, as described above. For comparison purposes, control untransfected cells were treated with IL-1 or TGF-β (10 ng/ml each) for up to 10 days. To measure matrix calcification, we used a quantitative Alizarin Red-binding assay, as described in Materials and Methods, with 1.0 OD_{570} = 1 U of Alizarin Red released per μg of DNA per culture dish ($n = 6$ each, studied in triplicate, and using six different knee meniscal donors). *, $P < 0.05$.

may be other significant relationships between TGF-β and TGase activity in chondrocytes pertinent to pathogenesis in OA. Specifically, TGases in chondrocytes and other cells can promote the activation of TGF-β from the latent form.[12,19] Moreover, TGF-β expression increases in both the superficial and deep zones of articular cartilages in OA.[54] Thus, activation of Factor XIIIa and/or tTGase by inflammatory stimuli could modulate TGF-β activation in OA cartilage. Because TGF-β induces chondrocyte expression of the matrix metalloproteinase (MMP)-13,[54] the activation of Factor XIIIa and tTGase also could modulate cartilage matrix degradation in OA through this TGF-β-mediated pathway.

Although IL-1 markedly induced TGase activity in chondrocytic cells, the IL-1 treatment seemed to have a less marked enhancing effect on matrix calcification than did direct expression of Factor XIIIa and tTGase in this study. Preparation of the pericellular matrix for mineral deposition involves modulation of expression, synthesis, and degradation of the collagenous and noncollagenous matrix constituents. Therefore, it is possible that catabolic effects of IL-1 for matrix protein synthesis and degradation[42] imposed limits on the extent of any increases in matrix calcification attributable to TGase activity.

We observed that IL-1-induced TGase activity was under the regulatory control of the widely expressed cytosolic zinc finger protein A20.[47–49,51,52] A20 acts to limit apoptosis and the nuclear factor-κB-mediated expression of genes including iNOS *in vitro* and *in vivo*.[52] Although A20 is a broader inhibitor of TNF-α than IL-1 responsiveness,[51] A20 does suppress IL-1-induced NO production in cultured pancreatic β cells,[52] similar to our findings in chondrocytic cells in this study. A20 inhibits TRAF2 and TRAF6 signaling pathways used by both TNF-α and IL-1 receptors, but A20 also interacts with other cytokine-inducible signaling pathways that mediate

nuclear factor-κB activation.[47–50] Thus, it is possible that the ability of A20 to attenuate IL-1-induced TGase activity may have been mediated via effects that extended beyond suppression of IL-1-induced NO production.

Constitutive A20 expression is generally low,[51,52] a finding reiterated in cultured meniscal cells in this study. However, A20 is induced by a variety of cytokines and cell stressors (including IL-1, lipopolysaccharide, and CD40/CD40L ligation, and the Tax protein of HIV-1) in a manner mediated in part by two nuclear factor-κB binding sites in the A20 promoter.[52,55] It will be of interest to determine whether cartilage A20 expression is functionally altered *in vivo* in degenerative joint disease, cartilage aging, and chondrocalcinosis, and to determine whether targeted regulation of A20 can affect cartilage degradation and matrix calcification *in vivo*.

TGases have long been postulated to directly promote skeletal matrix calcification, in part by cross-linking calcium binding proteins in the pericellular matrix.[12,14,23,26] Chondrocalcinosis linked to cartilage aging is strongly associated with increased generation by chondrocytes of PPi,[27] and extracellular PPi is a major regulator of matrix calcification.[1,28] Loss of function of TGase induced by several classes of pharmacological inhibitors has been associated with decreased extracellular levels of PPi in chondrocytes,[27] but we observed no significant effects of increased TGase activity on PPi-generating NTPPPH activity, PPi-degrading AP activity, or extracellular levels of PPi in this study. Moreover, despite the potential for direct expression of TGases to promote apoptosis in cultured cells,[20–23,56,57] we did not observe significant induction of chondrocytic cell apoptosis, which promotes chondrocyte matrix calcification *in vivo* and *in vitro* in growth plate and articular chondrocytes.[4,58] Thus, the direct capacity of elevated Factor XIIIa and tTGase-associated TGase activities to promote chondrocytic cell matrix calcification in this study did not seem to be attributable to changes in PPi metabolism or apoptosis.

Extracellular TGase activity promotes polymerization of secreted calcium-binding proteins such as S-100 and osteonectin.[14,15,59] We speculate that effects of elevated TGase activities to stabilize pericellular calcium-binding proteins[12] promoted matrix calcification in this study, but intracellular TGase activities could also have been at play. Specifically, increased intracellular TGase activity can affect signal transduction[12–14,16] and promote extrusion of cytosolic contents (including TGases) in chondrocytes.[10]

Interestingly, tTGase exerts several unique intracellular regulatory effects on signal transduction,[12,16] yet Factor XIIIa and tTGase activities similarly promoted matrix calcification. This finding, and the co-localization of Factor XIIIa and tTGase in both growth plate and OA cartilage specimens argue for a potentially redundant, central mechanism for regulation of cartilage matrix calcification. The absence of clinically defined bone or joint pathology in Factor XIIIa-deficient humans lends further support to this notion.[25,60] However, it remains to be established if TGase activity is necessary for cartilage matrix calcification.

In conclusion, the results of this study established potential linkages between increased IL-1 and TNF-α expression, increased NO production, dysregulated TGase activity, and the assembly of a chondrocyte pericellular matrix that supports pathological calcification, particularly in aging joint cartilages. Cartilage Factor XIIIa and tTGase could to be molecular targets for the regulation of cartilage matrix calcification.

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