

matrix from proteolytic activity^{16,17}. Still, relatively little is known about protease inhibitors in articular cartilage ECM.

The trappin family distinguishes itself from other protease inhibitors by the presence of a 4-disulfide core motif containing the protease inhibitor function and several N-terminal TGase substrate domains¹⁸. The TGase domains anchor trappin-2 to ECM proteins through the protein crosslinking action of TGase. TGase enzymes catalyze the formation of ϵ -(γ -glutamyl) lysine bonds between or within proteins¹⁹. TGase enzyme activity levels are increased in aging²⁰ and OA²¹ cartilage. However, TGase substrates in cartilage remain poorly characterized.

The goals of this study were to identify and localize trappin-2 in OA articular cartilage and SF; to determine whether chondrocytes synthesize trappin-2; and to identify the ECM molecules to which trappin-2 binds in cartilage ECM.

MATERIALS AND METHODS

The study was approved by the Ethics Committees of both institutions.

Cartilage preparation. Normal human articular cartilage used to make cartilage extracts for Western blotting was obtained from frozen tissue discarded by the Musculoskeletal Transplant Foundation (Edison, NJ, USA). Donors were 18–35 years old. Normal human knee articular cartilages for ELISA were from cadavers of a similar age range. OA cartilage was obtained from discarded pathologic specimens removed at the time of knee replacement surgery, in accord with Institutional Review Board regulations. Cartilage was shaved from the patellae and femoral and tibial condyles. A portion of cartilage was processed for chondrocyte culture as described below. The remaining tissue was fixed for immunohistochemistry or frozen at -70°C for later isolation of mRNA and protein. No tissues were pooled.

Chondrocyte culture and cell lysates. OA chondrocytes were isolated by sequential enzymatic digestion as described²². Normal adult human articular chondrocytes and neonatal chondrocytes were purchased from Clonetics (Walkersville, MD, USA). Cells were plated at high density (4×10^5 cells/cm²) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Medium was replaced by serum-free medium one day prior to harvest. Only first-passage cells were used. Cell lysates were obtained with M-PER™ mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA). The protein content of each sample was determined by the method of Lowry, *et al.*²³. For some experiments, normal or OA cartilage extracts were obtained during TRIzol (Life Technologies, Grand Island, NY, USA) extraction following the manufacturer's instructions. After the last wash, the protein pellets were solubilized in sodium dodecyl sulfate at 50°C . Protein extracts used for ELISA were solubilized in phosphate buffered saline (PBS) and stored at -70°C until use.

SF collection. OA human SF was obtained during clinically indicated arthrocentesis or during total knee arthroplasty. SF was collected into Vacutainer® tubes containing sodium heparin. Hyaluronidase type I-S (0.83 mg/ml) and 0.1 mM PMSF (Sigma, St. Louis, MO, USA) were added. Fluids were spun to remove cells, and supernatants were stored at -70°C until use.

Western blotting. Western blotting was performed to detect trappin-2 protein in SF, chondrocyte lysates, chondrocyte conditioned media, and cartilage extracts. Gel electrophoresis was performed on either Tris-HCl 4%–15% or Tris-acetate 3%–8% gels. Trappin-2 runs closer to its true molecular weight under nonreducing conditions¹⁸. However, the quality of nonreduced Western blots was poor. Thus, all results shown utilize reducing conditions. The primary antibody was goat anti-human ELAFIN/SKALP antibody (Hycult Biotechnology, Uden, The Netherlands) used at 1:1000–1:2000 dilution. The secondary antibody was rabbit anti-goat. An enhanced chemiluminescence (SuperSignal West Pico kit; Pierce

Biotechnology) method was used to visualize immunoreactive protein. No staining was seen when nonimmune serum was used in place of the primary antibody (data not shown).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from normal adult and neonatal chondrocytes and OA cartilage was isolated by the acidified guanidinium isothiocyanate method, using TRIzol. Total RNA was reverse transcribed into complementary DNA (cDNA) in the presence of Moloney murine leukemia virus RNase H⁻ reverse transcriptase (SuperScript II, Life Technologies). cDNA was amplified using REDTaq™ DNA polymerase (Sigma). The trappin-2-specific primer pairs were SKA1 forward primer (5'-AGG GCC AGC AGC TTC TTG ATC-3') and SKA3 reverse primer (5'-CAG CAC TTC TTG A(C/T) (C/T)C CTG G-3')²⁴. The GAPDH primers were sense 5'-GGT GAA GGT CGG AGT CAACG-3' and antisense 5'-CAA AGT TGT CAT GGA TGA CC-3'. Cycling parameters for trappin-2 were (95°C for 60 s, 57°C for 60 s, 72°C for 120 s) \times 35 cycles. An additional elongation step at 72°C for 7 min was included. Transcripts were analyzed with 1.5% agarose/Tris-acetate-EDTA gel electrophoresis. The expected transcript size was 496 bp for GAPDH and 460 bp for trappin-2, accounting for introns and exons as described by Zeeuwen, *et al.*²⁴. For sequence confirmation, the PCR product was inserted into pCR®II-TOPO® plasmid vector using a TOPO TA® cloning kit dual promoter (Invitrogen, Carlsbad, CA, USA). The sequence was confirmed by dideoxy chain termination sequencing²⁵.

2-D gel electrophoresis and mass spectrophotometry. OA articular cartilage was homogenized, and an EGTA eluate was prepared. The EGTA elution buffer was exchanged with 8 M urea, 2% CHAPS, and traces of bromophenol blue. The final volume was reduced to 400 μl using Centricon 3 filters (Millipore, Billerica, MA, USA). IPG buffer (Pharmacia) was added to a final concentration of 0.5%, and 0.7 mg DTT was added to 250 μl of sample. The first dimension was run overnight using Immobilin strips (Pharmacia), pH 4–7, and the Protean IEF Cell (BioRad, Hercules, CA, USA). The second dimension was run using 10%–20% precast criterion gels (BioRad). Duplicate 1- and 2-dimensional gels were run. One gel of each was transferred to a polyvinylidene fluoride membrane. Blots were treated with goat anti-human SKALP/ELAFIN primary antisera, followed by alkaline phosphatase-conjugated rabbit anti-goat antibodies. The reactions were developed using the NBT/BCIP colorimetric method (Promega, Madison, WI, USA).

The protein bands and spots corresponding to the immunoreactive bands and spots were excised from the silver stained gels, washed twice with 50% acetonitrile and twice with 50% acetonitrile in 50 mM NH_4HCO_3 (pH 8.0). Gel pieces were dried and reswollen in the presence of 25 mM NH_4HCO_3 containing 1 μg trypsin per gel piece to a final volume of 50–100 μl per gel piece. Digestion was performed overnight at 37°C . Peptides were extracted first with 1% formic acid in water followed by 80% acetonitrile in 1% formic acid. Extracted peptides were dried and used for peptide mass fingerprinting as follows.

Peptides were resuspended in 20 μl 0.1% TFA and purified using ZipTip-C18 (Millipore). They were eluted off the tips in the presence of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile in 0.1% TFA. The peptide mixture was eluted directly onto the sample plate for the MALDI-TOF mass spectrometer (Voyager DE-PRO, PerSeptive Biosystems, Foster City, CA, USA). Peptide mass fingerprints were analyzed using MS-Fit software and the NCBIInr database.

Immunohistochemistry. OA cartilage pieces were fixed in 4% paraformaldehyde and embedded in paraffin. After deparaffinization, endogenous peroxidase activity was quenched with 1% hydrogen peroxide, followed by 500 U/ml testicular hyaluronidase. Primary antibody was polyclonal goat anti-human SKALP/ELAFIN at 1:500 dilution (Hycult Biotechnology). Secondary antibody was rabbit anti-goat. Nonimmune goat serum was used as a negative control. The Vectastain peroxidase goat ABC kit (Vector, Burlingame, CA, USA) was used to identify immunoreactive protein according to manufacturer's directions. Nuclei were stained with methyl green.

Sandwich ELISA. ELISA for trappin-2 was performed using a commercial kit (Hycult Biotechnology). The kit does not detect the 6 kDa form of trappin-2. Normal and OA cartilage protein extracts solubilized in PBS were used undiluted or diluted 1:2. Triplicate samples from each donor were assayed.

TGase substrate assay. TGase substrate proteins will incorporate polyamines in the presence of active TGase enzyme¹⁹. Fibromodulin (10 µg; Sigma) was incubated in a reaction mixture containing 50 mM Tris HCl, 100 mM NaCl, 10 mM CaCl₂, 0.5 mM DTT, and the fluorescent polyamine, 0.5 mM dansylcadaverine (Sigma) 37°C with or without 0.02 U type II TGase (Sigma). Controls included reaction mixture alone, and type II TGase in reaction mixture with no fibromodulin. After 30 min, aliquots of the mixtures were run on Tris-HCl 4%–15% gels. Gels were examined and photographed under UV light.

Statistical analysis. Trappin-2 levels from normal and OA cartilage were compared using Student's t test assuming unequal variance.

RESULTS

Trappin-2 protein in OA cartilage and chondrocytes. Trappin-2 protein is easily detectable in OA cartilage protein extracts (Figure 1A) and cell lysates (Figure 1B) from cultured OA human articular chondrocytes by Western blotting. The protein is also present in normal cartilage extracts and chondrocyte lysates, although levels appear lower than in OA samples.

The major immunoreactive band in chondrocyte lysates is found at roughly 40 kDa and corresponds to that seen in cultured keratinocytes under reducing conditions²⁶. Interestingly, in cartilage extracts, trappin-2 is seen primarily as a 100 kDa band. The explanation for this heterogeneity in molecular weight is not immediately apparent. Because of its strongly cationic properties, trappin-2 typi-

cally runs slower on gel electrophoresis than expected, particularly under reducing conditions¹⁸. It is highly protein-bound in most tissues, and its higher molecular weight in whole cartilage extracts likely represents covalent binding to other matrix constituents.

Trappin-2 protein was also measured in normal and OA cartilage protein extracts by ELISA (Figure 2). Normal cartilage specimens (n = 3, mean age 27.5 yrs, range 20–35) contain 14.97 ± 1.92 ng trappin-2/mg cartilage wet weight. OA specimens (n = 23, mean age 69.05 yrs, range 56–77) showed a mean trappin-2 level of 22.98 ± 1.28 ng/mg carti-

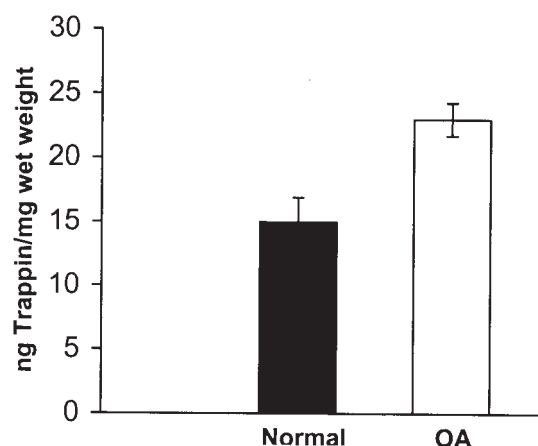


Figure 2. Trappin-2 ELISA. Trappin-2 protein was measured by sandwich ELISA in normal (n = 3) and OA (n = 23) cartilage protein extracts. Results represent mean trappin-2 concentrations in samples, corrected for wet weight of cartilage. OA samples contain significantly higher amounts of trappin-2 compared to normal samples (p < 0.01).

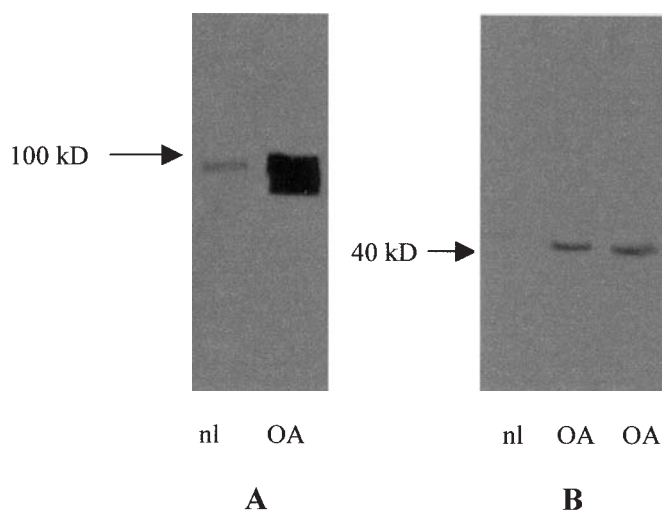


Figure 1. Western blotting of trappin-2 protein in cartilage and chondrocytes. **A:** Protein extracts of normal (nl) and osteoarthritic (OA) cartilage protein extracts run in a Western blot with anti-trappin-2 antibody (1:1000) under reduced conditions. An immunoreactive band is seen at 100 kDa. **B:** Lysates from normal and OA chondrocytes were prepared as described and run on Western blots with anti-trappin-2 antibody (1:1000) under reduced conditions. The major immunoreactive band is seen at 40 kDa. No staining was seen when nonimmune serum was used in place of the primary antibody (data not shown).

lage wet weight. OA cartilage contains a significantly higher amount of trappin-2 compared to normal cartilage ($p < 0.01$). *Trappin-2 in OA SF.* Trappin-2 was measured in SF by ELISA and Western blotting. OA fluids contain 75.4 ± 80.8 ng trappin-2/ml ($n = 19$). Western blotting confirmed the presence of trappin-2 in OA SF (Figure 3). The major immunoreactive band in SF was seen at 100 kDa, suggesting protein binding.

Immunolocalization of trappin-2 in OA articular cartilage. All layers of OA cartilage show some trappin-2 staining (Figure 4). Esophagus was used as positive control (Figure 4D). As with other small protease inhibitors, such as bovine pancreatic trypsin inhibitor¹⁵ and secretory leukocyte proteinase inhibitor²⁷, significant cytoplasmic staining was noted in chondrocytes. Very faint staining was noted in the chondrocyte pericellular matrix. No staining was seen when nonimmune serum was used in place of the primary antibody (Figure 4E).

Trappin-2 is synthesized and secreted by articular chondrocytes. To confirm that trappin-2 is synthesized by chondrocytes, we looked for the presence of trappin-2 mRNA in cultured chondrocytes. As shown in Figure 5A, trappin-2 mRNA was easily detectable in normal and OA articular chondrocytes. Figure 5B shows trappin-2 protein in conditioned media from OA chondrocytes. Its secretion into conditioned media also supports local production. Major immunoreactive bands are detected at 75 and 180 kDa. Trappin-2 in conditioned medium may be bound to proteins different from those in cartilage, resulting in immunoreactive bands of various molecular weights.

Trappin-2 binding partners in OA cartilage extracts. The presence of high molecular weight immunoreactive bands on Western blots of cartilage extracts (Figure 1A) suggests that trappin-2 is covalently bound to ECM proteins. To identify the proteins bound to trappin-2 in cartilage, we performed 2D gel electrophoresis on OA cartilage extracts.

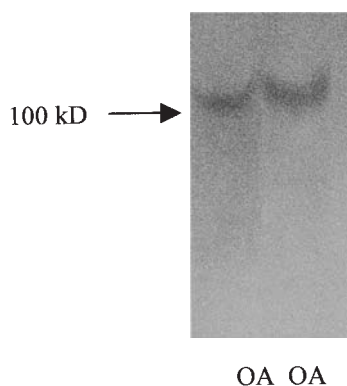


Figure 3. Trappin-2 in OA SF run on Western blot with anti-trappin-2 antibody (1:1000) under reduced conditions. A representative Western blot shows the major immunoreactive band at 100 kDa in 2 SF samples.

Trappin-2 immunoreactive spots were identified by Western blotting. Peptide fingerprinting of the trappin-2 immunopositive spots identified one of the trappin-2-bound proteins as fibromodulin, a 60 kDa proteoglycan known to be a significant component of cartilage ECM. This corresponds nicely to the 100 kDa band seen on Western blotting of OA cartilage extracts. While other binding proteins are certainly present in cartilage, they were not identified more specifically by this method.

Fibromodulin is not known to be a TGase substrate. To investigate whether it is capable of incorporating a polyamine such as dansylcadaverine in the presence of TGase enzyme, we incubated purified fibromodulin with dansylcadaverine in the presence and absence of TGase. Controls include TGase and dansylcadaverine alone, as type II TGase enzyme itself is a known TGase substrate. Unfortunately, the bands representing type II TGase and fibromodulin overlap in this system. However, as shown in Figure 6, there is a broadening of the 80 kDa band and appearance of a new lower molecular weight fluorescent band when dansylcadaverine is incubated with fibromodulin in the presence of TGase compared to the band seen with TGase and dansylcadaverine (Figure 6). These findings strongly suggest that fibromodulin is a TGase substrate.

DISCUSSION

We observed the presence of the serine protease inhibitor trappin-2 in osteoarthritic articular cartilage and chondrocyte cultures. Local synthesis is confirmed by the presence of trappin-2 mRNA in OA articular chondrocytes, and supported by its secretion into conditioned media. Higher concentrations of trappin-2 are found in OA cartilage than in normal articular cartilage when trappin-2 concentrations are measured by ELISA. Our data support results from studies of Molhuizen, *et al*, who found small amounts of trappin-2 in normal articular cartilage²⁸.

Trappin-2 can be induced by injury in skin²⁹ and by inflammatory stimuli, such as interleukins and tumor necrosis factor- α ³⁰, elastase³¹, or fetal calf serum². Similar regulatory factors in OA joints may induce the expression of this protein in chondrocytes. In addition, a role for age in increasing trappin-2 protein in cartilage cannot be excluded by our data, as we did not have age-matched controls to compare with our OA samples. Further experiments are under way to identify regulatory factors for trappin-2 in articular cartilage.

Trappin-2 functions as a serine protease inhibitor³². Its presence in articular cartilage is somewhat surprising, as it is typically considered an important defense mechanism in tissues that interface with the outside world, such as skin and esophagus. Trappin-2 inhibits the activities of elastase, proteinase 3, and some bacterial proteases³³. Although OA is not usually considered an inflammatory disease, neutrophil elastase is easily measurable in OA SF³⁴ and carti-

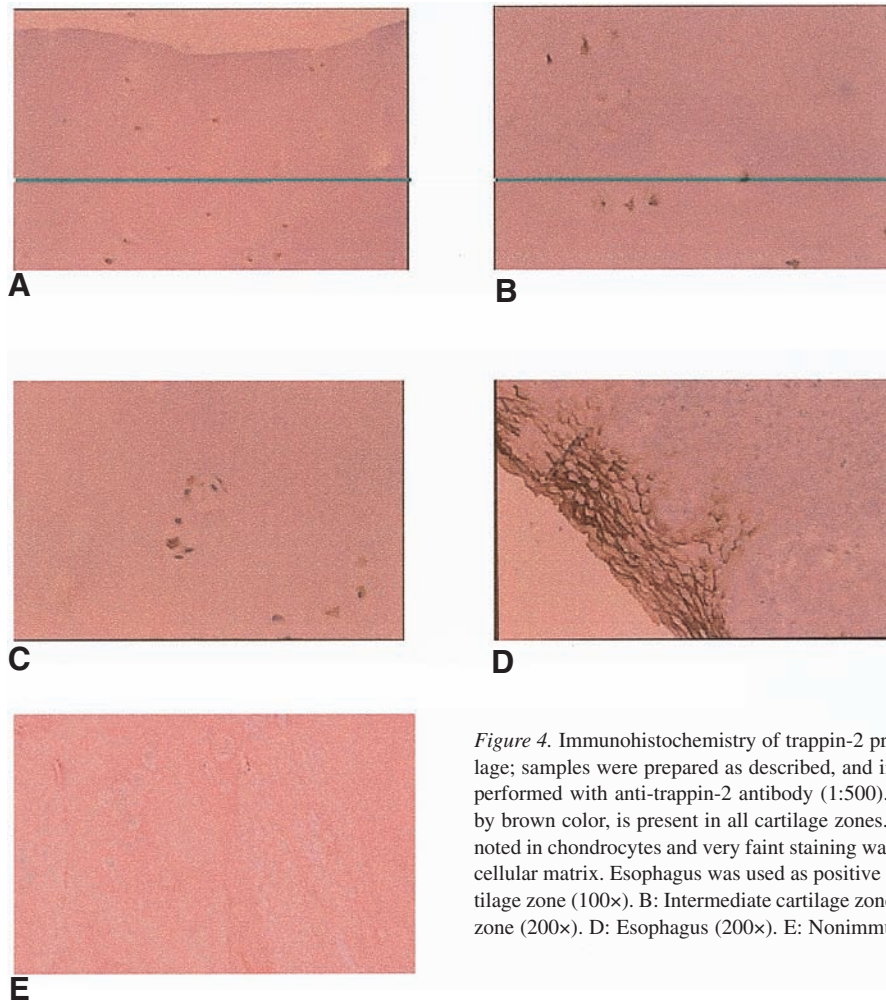


Figure 4. Immunohistochemistry of trappin-2 protein in OA articular cartilage; samples were prepared as described, and immunohistochemistry was performed with anti-trappin-2 antibody (1:500). Trappin-2 protein, shown by brown color, is present in all cartilage zones. Cytoplasmic staining was noted in chondrocytes and very faint staining was seen in chondrocyte pericellular matrix. Esophagus was used as positive control. A: Superficial cartilage zone (100 \times). B: Intermediate cartilage zone (200 \times). C: Deep cartilage zone (200 \times). D: Esophagus (200 \times). E: Nonimmune serum (200 \times).

lage⁶. Persistent low levels of neutrophil proteases may contribute to cartilage damage in OA. Synovitis in OA is well described and has recently been implicated as an important factor in cartilage degradation⁷. Because it is small and cationic, trappin-2 may penetrate cartilage matrix more effectively than higher molecular weight protease inhibitors. The large α 1-proteinase inhibitor, for example, fails to inhibit interleukin 1 α -stimulated cartilage damage¹¹, while low molecular weight elastase inhibitors reduce elastase-induced damage¹⁷. In addition, high molecular weight protease inhibitors are very susceptible to proteolytic degradation^{35,36}, while trappin-2 is relatively resistant to proteolysis³⁷. The function of trappin-2 in cartilage has not been studied.

Trappin-2 is found in multiple molecular weight forms in cartilage extracts and in chondrocyte conditioned media, suggesting that it is covalently bound to other proteins^{3,38}. However, multiple bands may also indicate the molecular heterogeneity of the trappin protein. In skin, trappin-2 is secreted as a 12.3 kDa preproelafin. After cleavage of the signal peptide, the 9.9 kDa form becomes bound to matrix proteins, and may be further processed to a 6 kDa form that

circulates in plasma. Further characterization of the various forms of trappin-2 in cartilage is warranted.

Although trappin-2 is likely bound to a variety of matrix proteins, we identified fibromodulin as one of its binding partners in cartilage by mass spectrophotometry. Fibromodulin is a member of the class II small leucine-rich proteoglycans. Like trappin-2, it is increased in aging and OA cartilage^{39,40}. It binds to collagen fibrils and aids in the formation of the collagen network in ECM^{41,42}. The higher levels of fibromodulin in OA cartilage matrix may be one mechanism responsible for the higher levels of trappin-2 found in OA. Although fibromodulin has not previously been identified as a TGase substrate, we found that it is capable of TGase-mediated polyamine incorporation. It is likely that trappin-2 is at least partially incorporated into cartilage matrix through the action of TGase enzymes, which are increased in OA²¹.

Immunohistochemical studies found intracellular trappin-2 protein staining in cartilage. Consistent with these data, immunohistochemical studies of other small serine proteases in cartilage^{15,27}, and of trappin-2 in other tissues³⁸, typically show intracellular staining, despite the clear extra-

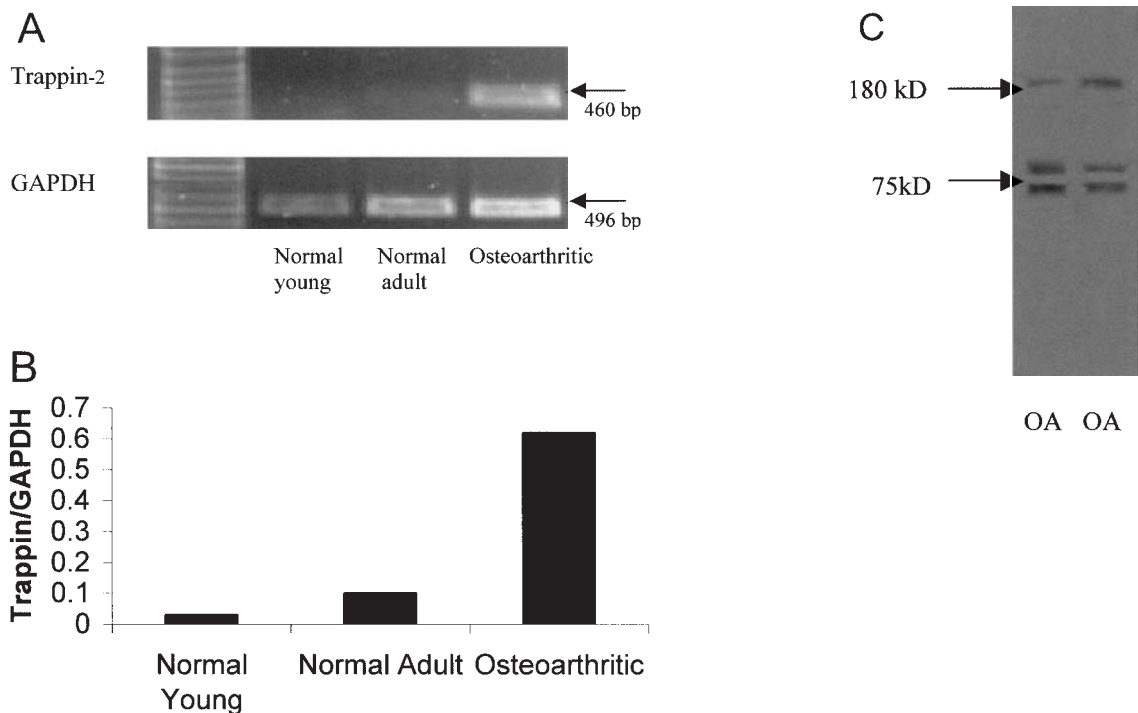


Figure 5. RT-PCR of trappin-2 in chondrocytes and Western blotting of chondrocyte-conditioned media. **A:** Trappin-2 mRNA was measured by RT-PCR. Normal and OA chondrocytes express trappin-2 mRNA. GAPDH was used as an internal control. **B:** Levels of trappin-2 mRNA were normalized to GAPDH levels by densitometry. **C:** Samples of conditioned media from OA chondrocyte cultures were run on Western blots with anti-trappin-2 antibody (1:1000) under reduced conditions. Immunoreactive bands were seen at 75 and 180 kDa.

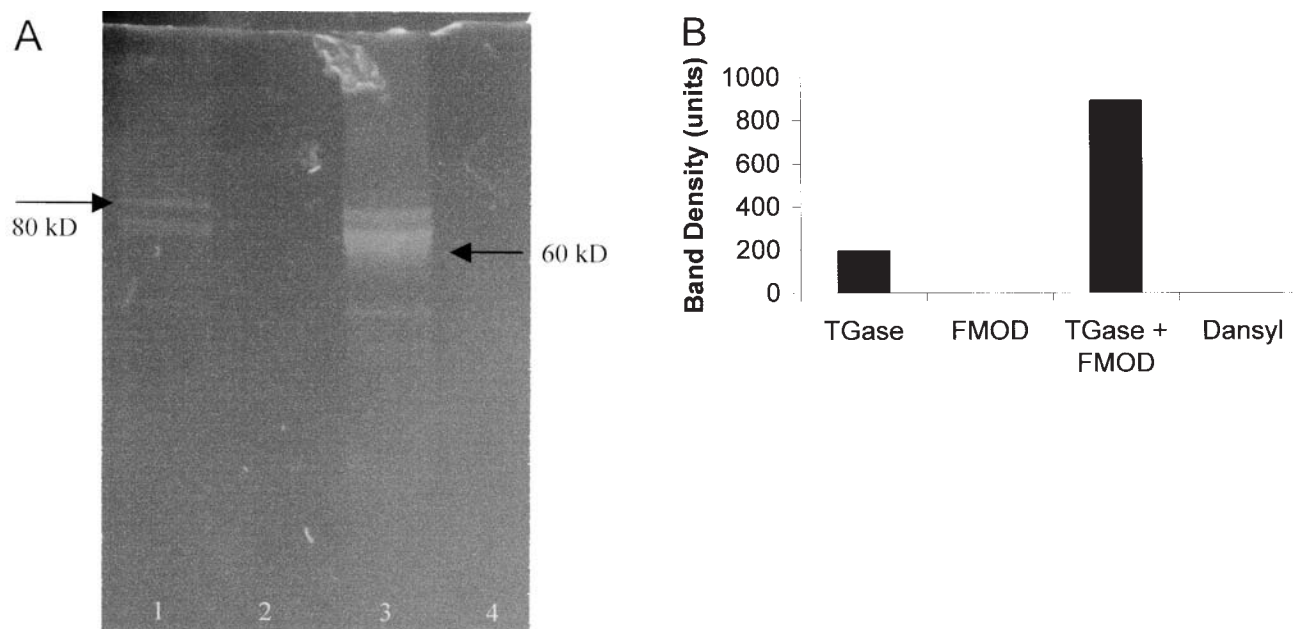


Figure 6. Determination of fibromodulin as a TGase substrate. Fibromodulin was incubated with the fluorescent polyamine dansylcadaverine in the presence and absence of active TGase. Aliquots of each sample were run on SDS-PAGE, and fluorescent bands were visualized and photographed under UV light. **A,** lane 1: TGase and dansylcadaverine (Dansyl); lane 2: fibromodulin (FMOD); lane 3: fibromodulin, dansylcadaverine, and TGase; lane 4: dansylcadaverine. High molecular weight fluorescent bands are seen in lanes 1 and 3, corresponding to incorporation of dansylcadaverine into proteins. **B:** Densitometry results showed 8-fold enhancement of a 60–80 kDa band in the presence of active TGase and fibromodulin compared to TGase alone, indicating that TGase is crosslinking fibromodulin to dansylcadaverine and is a TGase substrate.

cellular localization of these proteins. Trappin-2 is present in chondrocyte-conditioned media, thus demonstrating its ability to be secreted by chondrocytes. It is likely that major epitopes of trappin-2 and other small protease inhibitors are masked after their incorporation into ECM.

These studies are not without limitations. The lack of age-matched controls limits our ability to state with confidence that the increased trappin-2 concentrations in OA cartilage are a result of disease and not age. Further work is needed to differentiate these 2 possibilities. In addition, there is some discrepancy in trappin-2 concentrations measured by Western blotting compared to those measured by ELISA. We feel that the ELISA is a much more quantitative test for trappin-2. Finally, it is possible that fibromodulin and trappin-2 simply co-elute and are not covalently bound in cartilage. However, we feel that this is unlikely, given the strong evidence that fibromodulin is a TGase substrate.

The serine protease inhibitor and TGase substrate trappin-2 is present in cartilage affected by osteoarthritis. It is bound to other proteins, including fibromodulin, in cartilage, and can be synthesized by chondrocytes. Trappin-2 may prove to be an important natural defense against serine protease-mediated damage in cartilage.

ACKNOWLEDGMENT

The authors acknowledge the assistance of David Petsch, BS (Zablocki VA Medical Center, Milwaukee, Wisconsin), for his technical assistance.

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