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TYPE II COLLAGEN LEVELS CORRELATE WITH MINERALIZATION BY ARTICULAR CARTILAGE VESICLES

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Abstract

Objective—Pathologic mineralization is common in osteoarthritic cartilage and may be mediated by extracellular organelles known as articular cartilage vesicles (ACV). Paradoxically, ACVs isolated from osteoarthritic human cartilage mineralize poorly in vitro compared to those isolated from normal porcine cartilage. We recently showed that collagens regulate ACV mineralization. We sought to compare collagens and collagen receptors on human and porcine ACVs as a potential explanation of their different mineralization behaviors.

Methods—ACVs were enzymatically released from old and young human and porcine hyaline articular cartilage. Western blotting was used to determine the presence of collagens I, II, VI, and X, and various collagen receptors on ACVs. Type II collagen was quantified by ELISA. Biomineralization was assessed by measuring uptake of ^{45}Ca by isolated ACVs in agarose gels and ACVs *in situ* in freeze-thawed cartilage.

Results—As previously shown, isolated human ACVs mineralized poorly in response to ATP compared to porcine ACVs, but mineralized similarly *in situ* in freeze-thawed cartilage. Type II collagen levels were 100 fold higher in isolated human than in porcine ACVs. Type II collagen in human ACVs was of high molecular weight. Transglutaminase-crosslinking of type II collagen showed increased collagenase resistance, suggesting a possible explanation for residual collagen on human ACVs. Other collagens and collagen receptors were similar on human and porcine ACVs.

Conclusions—Higher levels of type II collagen in human ACV preparations, perhaps mediated by increased transglutaminase crosslinking, may contribute to the decreased mineralization observed in isolated human ACVs *in vitro*.

Keywords

collagen; osteoarthritis; matrix vesicles; mineralization; integrins; discoidin domain receptor

Pathologic calcification commonly occurs in the extracellular matrix of articular cartilage affected by severe osteoarthritis (OA). Two types of calcium-containing crystals dominate: calcium pyrophosphate dihydrate (CPPD) crystals, which are relatively unique to articular cartilage, and basic calcium phosphate (BCP) crystals, which are similar to the hydroxyapatite mineral of bone and other calcified tissues. While the mechanisms of BCP and CPPD crystal formation have not been fully elucidated, small extracellular organelles known as articular cartilage matrix vesicles (ACVs) have been implicated in this process (1). These organelles can be enzymatically isolated from both porcine and human articular cartilage; and when

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provided with calcium and a source of phosphate or pyrophosphate, they generate BCP and CPPD crystals identical to those found in human osteoarthritic joints (2) (3).

ACVs are easily isolatable from both normal and osteoarthritic cartilage (1). When removed from their extracellular milieu, normal porcine ACVs mineralize more effectively than ACVs derived from human osteoarthritic cartilage (1). As ACVs rarely mineralize in normal cartilage, this observation suggests a potential role for the normal extracellular matrix in suppressing ACV mineralization. Certainly, in other models, changes in extracellular matrix with osteoarthritis and aging facilitate pathologic crystal formation. For example, increased extracellular activity of the matrix-modifying transglutaminase enzymes promotes CPPD crystal formation in chondrocyte monolayers (4).

While there is ample evidence that factors such as levels of ATP or phosphate and levesl of enzymes regulating pyrophosphate/phosphate ratio affect mineralization (2), we recently showed that the ability of ACVs to generate pathologic calcium crystals was also affected by their extracellular milieu (5). In these studies, isolated ACVs were embedded in agarose gels containing various extracellular matrix components and their mineralization behavior was measured. We showed that the type II collagen found in normal cartilage suppressed ACV-induced CPPD crystal formation, while the combination of type I and II collagens seen in osteoarthritis stimulated both CPPD and BCP crystal formation. Collagens did not affect activity of mineralization-regulating enzymes on ACVs. Thus, how collagens regulate ACV mineralization remains unknown.

The behavior of growth plate matrix vesicles (GPMV) during normal bone growth has served as a paradigm for exploring the role of ACVs in pathologic articular cartilage mineralization. In contrast to type II collagen's effect on ACVs, Kirsch and Wuthier showed that mineral formation by GPMVs was stimulated by type II collagen, likely through annexin V-type II collagen interactions (6). GPMVs tightly bound type II and X collagens via annexins I, II and V, alkaline phosphatase, and link protein (7).

In contrast, we observed very little type II collagen in isolated porcine ACVs, while annexin V, CD44 and link protein were easily detectable (5). Collagens and collagen receptors on ACVs remain otherwise uncharacterized. Articular chondrocytes, from which ACVs are derived, contain multiple collagen receptors and binding proteins including integrins, annexins, and NG2, a proteoglycan which binds type VI collagen (8), a key collagen in the pericellular matrix. Recently, the discoidin domain receptors (DDRs) have joined this group. DDR2 preferentially binds type II collagen and is up-regulated in osteoarthritis, where receptor activation triggers catabolic protease release (9).

The purpose of the current work was to further characterize collagens and collagen receptors on porcine and human ACVs released enzymatically from whole cartilage and to determine if this might explain the differences in biomineralization of isolated ACVs. We show here that the persistence of type II collagen on isolated human ACVs could explain the seemingly paradoxical mineralization behavior of human ACVs ex vivo.

METHODS

Materials

All reagents were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO) unless otherwise stated.

Cartilage

Porcine cartilage from the knee joints of freshly killed adult (3–5 year old) pigs was generously provided by Johnsonville Foods, Inc, Watertown, WI. Young (6 months old) knee cartilage was harvested from pigs after euthanasia and use by other investigators. In accordance with the institutional review board, normal human articular cartilage was supplied as de-identified frozen samples from donors 18–32 years of age from the Musculoskeletal Transplant Foundation. Aged, normal -appearing human cartilage from patients age 65–80 originally obtained from the National Disease Research Interchange (NDRI) and after use by other investigators was donated as a kind gift from the Angel Donor Network and Carol Muehleman, PhD, of Rush Presbyterian St. Luke's Medical Center. All cartilage was maintained at −70° C until use. Prior work showed no differences in mineralization behavior between ACVs derived from fresh or frozen cartilage (1).

ACVs

Cartilage was thawed, minced, washed and weighed. Cartilage pieces were serially treated with 0.1% hyaluronidase, 0.5% trypsin, 0.2% trypsin inhibitor and 0.2% and then 0.05% bacterial collagenase (type 2 from *Clostridium Histolyticum*) as previously described (2). The mixture was filtered, centrifuged at 500 g for 15 minutes to remove cells, then at 37,000 g for 15 minutes to remove large cell fragments and organelles. The supernatant was then centrifuged at 120,000 g for 60 minutes to pellet the ACV fraction. The ACV-containing pellet was re-suspended in DMEM to a protein concentration of 12–15 mg/ml. Specific activities of nucleoside triphosphate pyrophosphohydrolase (NTPPPH) and alkaline phosphatase, which are the major regulators of pyrophosphate and phosphate metabolism in ACVs, were measured for each ACV fraction as previously described (2).

Western blotting

Thirty μg of ACVs in sample buffer were loaded and run on 10% Bis-Tris gels (NuPAGE®, Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose and then exposed to antibodies against type I collagen, DDR2, CD44, integrins α1 α2, β1, NG2, (Abcam Inc., Cambridge, MA), type II and VI collagens (Chemicon, Temecula, CA), Type X collagen (Sigma-Aldrich Chemical Co) and DDR1, Annexin V (R&D Systems, Minneapolis, MN) for 1.5 hours. After washing, the appropriate secondary antibody was added for one hour. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Hydroxyproline assay

Briefly, 500 μg of ACVs were homogenized in 2 ml distilled water and hydroxyproline was measured as described by Edwards and O'Brien (10).

Biomineralization

As previously described, one mg/ml of ACVs was added to 2 % warm agarose dissolved in calcifying salt solution (CSS) (5) Two hundred μl of the ACV/agarose mixture were added to each well of a 48-well tissue culture plate and solidified at room temperature. Five hundred μl CSS with 1μCi/ml ^{45}Ca were added to each well with or without 1 mM ATP or 1 mM β-glycerophosphate and ACVs were incubated for 3–7 days at 37° C. Every 48 hours, 10 μl of 5mM ATP or β glycerophosphate solution or 10 μl of CSS were added to maintain adequate levels of the original pyrophosphate and phosphate sources respectively. At the end of the experiment, overlying media were removed, and the gels were thoroughly washed with ice-cold CSS. After dissolving the agarose with 6.5% (v/v) sodium hypochlorite, and washing, radioactivity in the ACV fraction was measured by liquid scintigraphy.

ACVs-mediated mineralization was also measured *in situ* in whole freeze-thawed cartilage. All cartilage had been frozen for several months and did not contain viable chondrocytes. Thus, mineral formation in these tissues is presumed to be secondary to resident ACVs, which are very resistant to repeated freeze-thaw cycles. Whole cartilage was thawed, minced, weighed and incubated for 72 hours in weight-adjusted volumes ofDMEM, trace labeled with ^{45}Ca and containing no additives, 1 mM ATP or 1 mM β-glycerophosphate. It was washed thoroughly, treated with 6N HCL and ^{45}Ca was measured with liquid scintigraphy.

Collagenase susceptibility assay

Collagens are heavily post-translationally modified and are commonly crosslinked by transglutaminase enzymes and advanced glycation endproducts (AGEs) in aging tissues. We determined whether these modifications altered the susceptibility of type II collagen (from bovine articular cartilage, Elastin Products, Owensville, MD) to the broad spectrum bacterial collagenase used to isolate ACVs from whole cartilage. We cross-linked purified type II collagen with transglutaminase, and exposed type II collagen to glyoxylic acid to generate the AGE-modified collagen, N^{ε}- carboxymethyl-lysine (CML)- collagen (11). Identical quantities of cross-linked, AGE-modified or control collagens were embedded in agarose gels. These were overlaid with a solution containing no additives or 1 mg/ml bacterial collagenase. Hydroxyproline released into the media was quantified at 24 hours using the hydroxyproline assay described above. No hydroxyproline was present in the media in the absence of added collagenase.

Statistics

All experiments were repeated 3–5 times. The Student's t test was used to compare values between groups. Statistical significance was set at $p<0.05$.

RESULTS

Collagens associated with ACVs (Figure 1)

Easily detectable levels of types VI and X collagen were present on both human and porcine ACVs by Western blotting. No type I collagen was found in any ACV preparation (Data not shown). As we previously demonstrated (5), there was very little type II collagen in porcine ACVs. However, in human ACVs, type II collagen was easily detectable by Western blotting, and much of the type II collagen was of high molecular weight. The hydroxyproline content was also higher in old human ACVs than in old porcine ACVs, with levels of 5.3 ± 0.19 μg/mg protein in human and 3.78 ± 0.50 μg/mg protein in porcine ACVs ($p<0.001$). Attempts to remove type II collagen on human ACVs by an additional incubation with bacterial collagenase did not change the appearance of type II collagen on Western blots (Data not shown). Type II collagen levels measured by ELISA were 600–800 pg/mg protein on human ACVs and 4–14 pg/mg protein on porcine ACVs ($p<0.001$). Levels were modestly higher on old human ACVs than young human ACVs.

Biomineralization

To confirm differences in mineralization behavior between human and porcine ACV preparations, we embedded identical quantities of old ACVs in agarose gels and assessed their ability to mineralize in the presence of ATP or β- glycerophosphate. Levels of mineralization-regulating enzymes, and the ratio of NTPPPH/alkaline phosphatase (2) activities were similar (0.005 ±.002 in human ACVs and 0.004 ±.002 in porcine ACVs). As shown in Figure 2A, old porcine ACVs responded vigorously to both ATP and β-glycerophosphate by increasing ^{45}Ca uptake. When human ACVs were mineralized under similar conditions, there was no increase in ^{45}Ca uptake in the presence of either ATP or β-glycerophosphate. These result confirm our

earlier studies showing that ACVs embedded in gels containing type II collagen respond poorly to ATP and β-glycerophosphate compared to ACVs in collagen-free gels (5).

To determine if these differences in mineralization behavior were seen only with isolated ACVs, we mineralized ACVs *in situ* in thawed freeze-thawed cartilage without viable cells. ACVs are extremely hardy and are resistant to repeated freeze-thaw cycles. As shown in Figure 2B, human and porcine ACVs mineralized similarly in their native environment (Figure 2B). Taken together, these data suggest that human and porcine ACVs have similar mineralizing capacity *in situ*, which may be altered by residual matrix components, such as type II collagen, present in isolated human ACVs.

Collagen Receptors on ACVs

We wondered if the nature of the binding proteins or receptors for collagens on human and porcine ACVs could explain the difference in ACV-associated levels of type II collagen. Using Western blotting, and loading identical quantities of proteins, we saw very similar profiles of collagen receptors and binding proteins on human and porcine ACVs. Annexin V, CD44, DDR2, NG2, integrins α1, α2 and β1 were easily detectable on all ACV preparations (Data not shown). No DDR1 was present in any type of ACV preparation. Thus, the types of collagen receptors on old and young human and porcine ACVs were similar and were not likely to explain differences in type II collagen levels.

Collagenase resistance

Post-translational modifications may alter the resistance of type II collagen to collagenase digestion and explain the persistence of type II collagen in human ACV preparations. Both transglutaminase (12) and AGE-crosslinked collagens (13) may display increased collagenase resistance. We cross-linked purified type II collagen with transglutaminase (12) or the AGE, N^{ε}- carboxymethyl-lysine (CML) (11), and measured the ability of the bacterial collagenase used in ACV preparation to degrade collagen. As shown in Figure 3, transglutaminase-treated type II collagen was less effectively degraded by bacterial collagenase ($p<0.001$), while no changes in collagenase susceptibility were noted between CML-modified type II collagen and controls.

DISCUSSION

In summary, we show here that ACVs from human and porcine cartilage show significant differences in levels of type II collagen, which may be one of the factors that accounts for the difference in their mineralization behaviors *ex vivo*. The abundance of type II collagen in human ACVs can not be explained by differences in collagen binding proteins or receptors in human and porcine ACVs. Transglutaminase treatment of type II collagen does increase its resistance to bacterial collagenase. As transglutaminase activity increases with age in articular cartilage and these enzymes are present in ACVs(14), the residual type II collagen on human ACVs may persist due to age dependent modification by transglutaminases.

When human and porcine ACVs are mineralized *in situ* in their native matrix they demonstrate similar mineralization behavior. This observation strongly supports the hypothesis that the behavior of isolated ACVs is affected by matrix components, and this difference in mineralization behavior likely results from residual type II collagen adhered to the ACV fraction. It also reinforces the importance of careful attention to both species and tissue type in vesicle-based mineralization models. We continue to find important differences between vesicles derived from growth plate and those from articular cartilage. For example, type II collagen is found tightly bound to GPMV from immature chickens and stimulates rather than inhibits GPMV mineralization (6).

We were intrigued by the persistence of type II collagen on human ACVs despite significant and prolonged exposure to a broad spectrum bacterial collagenase. Attempts to retreat human ACVs with collagenase resulted in persistence of type II collagen, and suggested that this collagen was resistant to enzymatic digestion. The intensity of the high molecular weight bands in human cartilage suggested crosslinkage, and indeed treatment of type II collagen with the crosslinking enzyme transglutaminase resulted in less cleavage by bacterial collagenase, suggesting a possible role for the transglutaminase enzymes in this effect. The potential differences in transglutaminase enzyme activity in human and porcine cartilage warrant further exploration, but do not appear to be solely age-related.

We noted the presence of Types VI and X collagens on all types of ACVs. Type VI collagen is a heterotrimeric collagen found in the pericellular matrix of normal articular cartilage, where it may a play a role in cell attachment. To our knowledge, it has not been previously described in ACV or GPMV preparations. It's presence on ACVs supports the observation that ACVs as well as crystal formation occur in the pericellular matrix near chondrocytes. Type X collagen is associated with mineralizing extracellular matrices and is well characterized on GPMV (15). Its presence in ACVs is not surprising as it likely plays a role in matrix mineralization, although its exact function remains unknown. Because Western blots are semi-quantitative at best, it is not possible to detect small differences in quantities of these collagens in porcine and human ACVs, and further studies of type X collagen on ACVs are necessary.

Whether collagens are bound to specific proteins or receptors on ACVs remains uncertain. We show here that multiple collagen receptors and binding proteins are present on human and porcine ACVs, and that the profile of these collagen binding molecules is remarkably similar to that of chondrocytes and does not differ significantly with age or between humans and pigs. The presence of these receptors and binding proteins on ACVs may serve to anchor ACVs to the surrounding matrix or they may simply reflect the composition of the chondrocyte cell membrane from which they are derived. Alternatively or in addition, they may assist in communicating matrix changes to the ACV through outside-in signaling. Further work to identify the mechanism through which type II collagen inhibits ACV mineralization and whether it is mediated by specific receptor or protein binding is warranted.

These studies are not without limitations. Certainly, adherent matrix proteins are only one of the many factors affecting mineralization. We did not succeed in removing type II collagen from human ACVs to show a difference in mineralization. In addition, it is possible that factors other than transglutaminase crosslinking contribute to the collagenase resistance seen in human collagen. Thus, these associations, while intriguing, remain correlative.

We show here that isolated human ACVs contain considerable quantities of type II collagen not seen on porcine ACVs and that the poor mineralization capacity of isolated human ACVs may be an artifact stemming from the persistence of type II collagen during preparation. This work emphasizes the importance of recognizing extracellular matrix components in ACV fractions isolated from different species and tissue types as additional variables in biomineralization behavior.

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REFERENCES

1. Derfus BA, Kurtin SM, Camacho NP, Kurup I, Ryan LM. Comparison of matrix vesicles derived from normal and osteoarthritic human articular cartilage. Connective Tissue Research 1996;35(14):391–6.

2. Derfus B, Rachow J, Mandel N, Boskey A, Buday M, Kushnaryov V, et al. Articular cartilage vesicles generate calcium pyrophosphate dihydrate-like crystals in vitro. Arthritis Rheum 1992;35:231–40. [PubMed: 1734912]

3. Rosenthal A, Mattson E, Gohr C, Hirschmugl C. Characterization of articular calcium-containing crystals by synchrotron FTIR. Osteoarthritis Cartilage 2008;16:1395–1402. [PubMed: 18472285]

4. Heinkel D, Gohr C, Uzuki M, Rosenthal A. Transglutaminase contributes to CPPD crystal formation in osteoarthritis. Frontiers in Bioscience 2004;9:3257–61. [PubMed: 15353354]

5. Jubeck B, Gohr C, Fahey, Muth E, Matthews M, Mattson E, et al. Promotion of articular cartilage vesicle mineralization by type I collagen. Arthritis Rheum 2009;58:2809–17. [PubMed: 18759309]

6. Kirsch T, Wuthier R. Stimulation of calcification of growth plate cartilage matrix vesicles by binding to type II and X collagens. J Biol Chem 1994;269:11462–9. [PubMed: 8157677]

7. Wu L, Genge B, Lloyd G, Wuthier R. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. J Biol Chem 1991;266:1195–1203. [PubMed: 1845989]

8. McGlashan S, Jensen C, Poole C. Localisation of extracellular matrix receptors on chondrocyte primary cilia. J Histochem Cytochem 2006;54:1005–14. [PubMed: 16651393]

9. Xu L, Peng H, GLasson S, Lee P, Hu K, Ijiri K, et al. Increased expression of the collagen receptor discoidin domain receptor 2 in articular cartilage as a key event in the pathogenesis of osteoarthritis. Arthritis Rheum 2007;56:2663–73. [PubMed: 17665456]

10. Edwards C, O'Brien WJ. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. Clinica Chemica Acta 1980;104:161–7.

11. Alikhani M, Alikhani Z, Boyd C, Maclellan C, Raptis M, Lui R-Q, et al. Advanced glycation end products stimulate osteoblast apoptosis via the MAP kinase and cystolic apoptotic pathways. Bone 2007;40:345–53. [PubMed: 17064973]

12. O'Halloran D, Collighan R, Griffin M, Pandit A. Characterization of a microbial transglutaminase cross-linked type II collagen scaffold. Tissue Engineering 2006;12:1467–74. [PubMed: 16846344]

13. Reddy G. Cross-linking in collagen by nonenzymatic glycation increases the matrix stiffness in rabbit Achilles tendon. Experimental Diab Res 2004;5:143–53.

14. Rosenthal AK, Derfus BA, Henry LA. Transglutaminase activity in aging articular chondrocytes and articular cartilage vesicles. Arthritis Rheum 1997;40(5):966–70. [PubMed: 9153560]

15. von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, et al. Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 1992;35:806–11. [PubMed: 1622419]

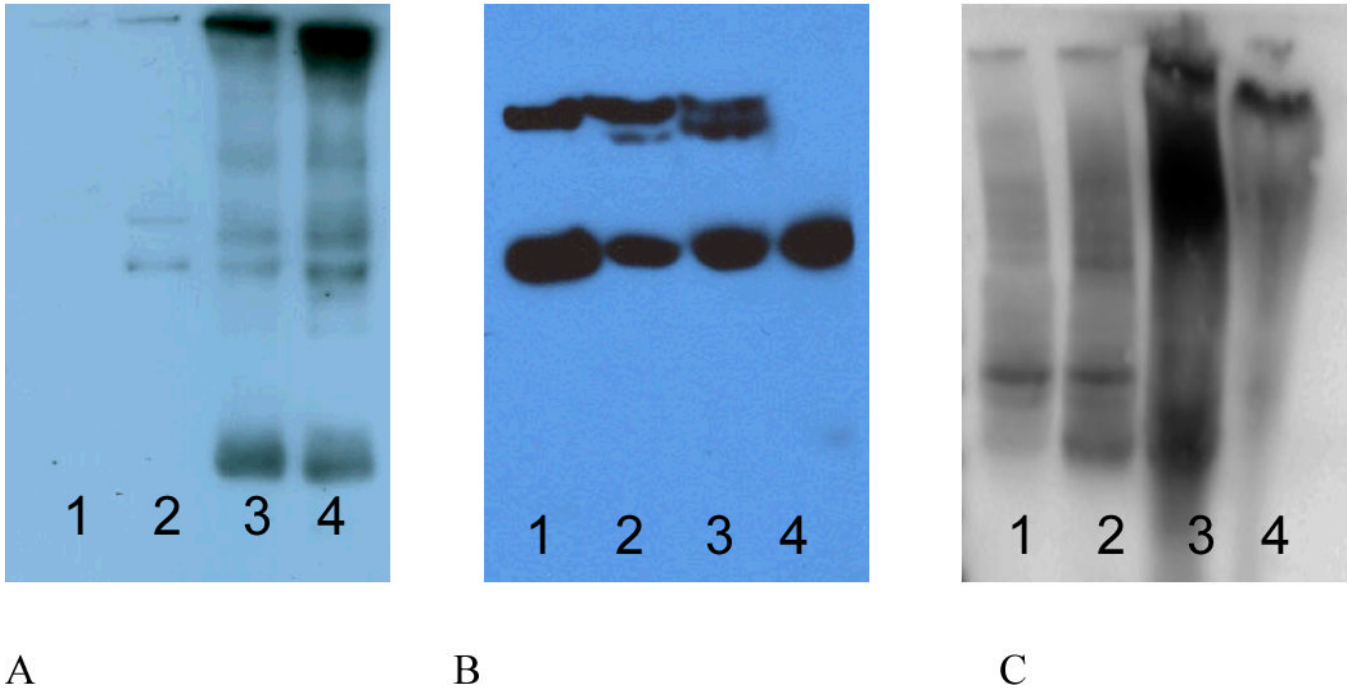
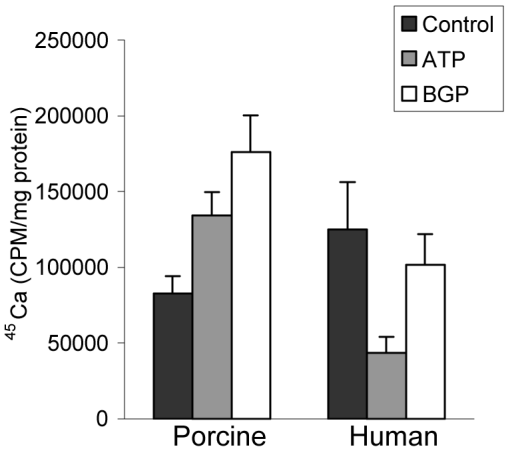


Figure 1. Collagens present in ACVs

ACVs were isolated from young porcine (lane 1), old porcine (lane 2), young human (lane 3) and old human (lane 4) cartilage and identical quantities of protein were loaded onto SDS-PAGE gels. After transfer to nitrocellulose membranes, Western blotting was performed with antibodies against three types of collagen; panel A: type II collagen (1:1000), panel B: type X collagen (1:1000) and panel C: type VI collagen (1:1000).



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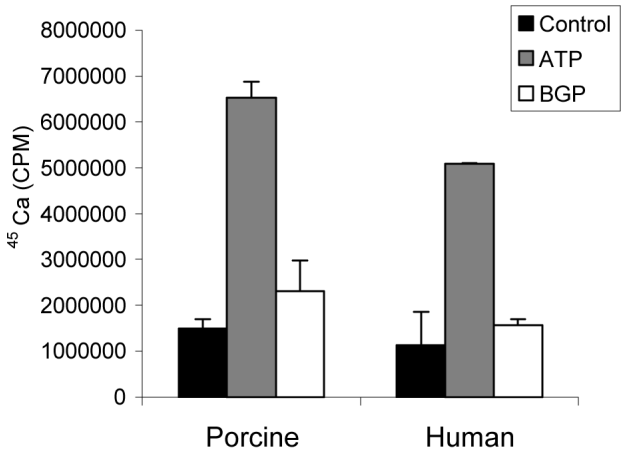

B

Figure 2. Calcification of ACVs in agarose gels and in native cartilage matrix

A. ACVs isolated from old porcine and human articular cartilage were embedded in 2% agarose (200 mg protein/ml). The agarose plugs were incubated with no additives (control), 1mM ATP or 1mM β-glycerophosphate (BGP) and trace labeled with 1μCi/ml ^{45}Ca. After 72 hours at 37° C, the agarose plugs were washed with PBS and then dissolved in 1% bleach. The pellets were dissolved in 1 N NaOH and ^{45}Ca in the vesicle pellet was determined by liquid scintigraphy and corrected for protein. **B**. Frozen old human and porcine articular cartilage pieces were thawed, weighed and incubated in weight-adjusted volumes of media trace labeled with ^{45}Ca and containing no additives (control), 1 mM ATP or 1 mM β-glycerophosphate (BGP) for 72 hours. Cartilage pieces were washed and treated with 6N HCl. ^{45}Ca was measured with liquid scintigraphy.

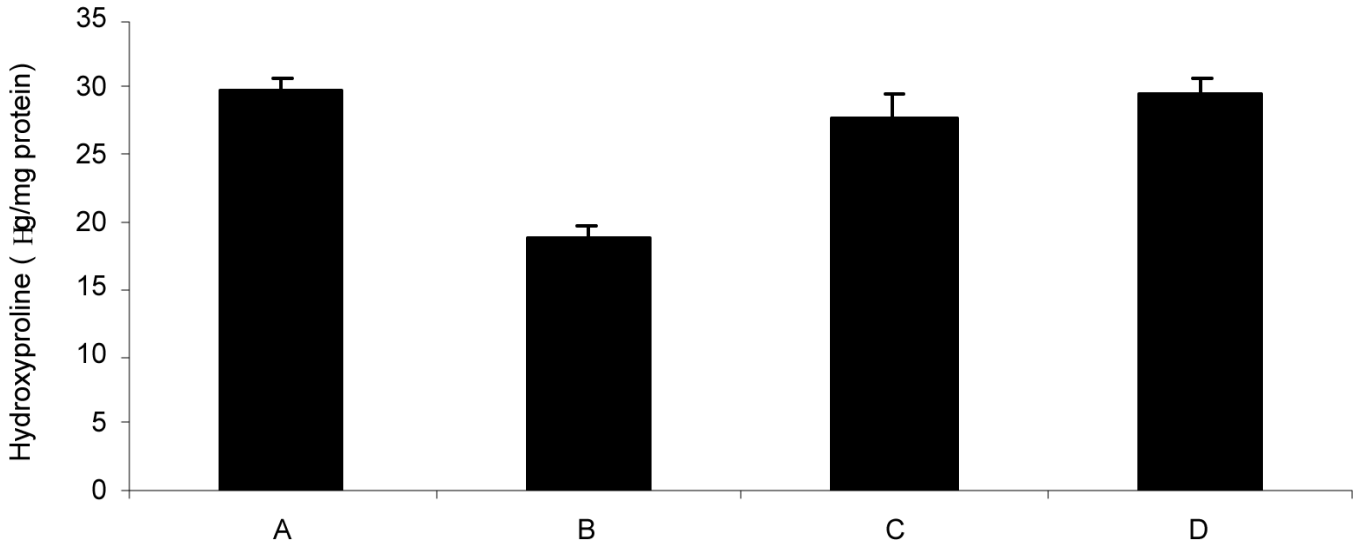

Figure 3. Collagenase resistance of modified type II collagen

Type II collagen (1.6 mg) alone (A), type II collagen treated with 20 Units type II transglutaminase (B), type II collagen treated with glyoxylic acid to generate the AGE-modified collagen, CML-collagen (D) or type II collagen treated with the reaction mixture for CML-modification without glyoxylic acid (C) were embedded in 2% agarose in a 48 well plate. After treatment with 1 mg/ml collagenase for 24 hours, the supernatants were collected and hydroxyproline levels were determined. There was significantly less hydroxyproline released from transglutaminase-treated collagen samples than controls ($n=8$, $p \leq 0.001$).