The Nucleoside Triphosphate Pyrophosphohydrolase Isozyme PC-1 Directly Promotes Cartilage Calcification Through Chondrocyte Apoptosis and Increased Calcium Precipitation by Mineralizing Vesicles

KRISTEN JOHNSON, KENNETH PRITZKER, JAMES GODING, and ROBERT TERKELTAUB

ABSTRACT. ***Objective***. Aging associated elevations of cartilage extracellular inorganic pyrophosphate (PPi) and PPi-generating nucleoside triphosphate pyrophosphohydrolase (NTPPPH) are linked with degenerative arthritis in chondrocalcinosis. Increased chondrocyte apoptosis and expression of annexin V occur at sites of matrix calcification in degenerative arthritis, and membrane limited chondrocyte apoptotic bodies containing NTPPPH may promote chondrocalcinosis by acting as mineralizing matrix vesicles (MV). Because chondrocytes express 3 related NTPPPH isozymes [PC-1, autotaxin (ATX), and B10/PDNP3], we evaluated the effects on apoptosis and MV mediated calcium precipitation of direct expression of NTPPPH isozymes.

Methods. To achieve "gain of function" of NTPPPH isozymes, we expressed the isozymes in cultured chondrocytic cells.

Results. Plasmid cDNA transfection of PC-1, but not ATX or B10/PDNP3, markedly increased apoptosis of cultured chondrocytic knee meniscal cells and increased calcium precipitation by MV fractions. The capacity of PC-1 to increase chondrocyte and meniscal cell apoptosis, and calcium precipitation by MV, further analyzed using adenoviral gene transfer in cultured meniscal cells and articular chondrocytes, was shown to be dependent on integrity of the PC-1 NTPPPH catalytic site. The MV-containing fraction released from meniscal cells and chondrocytes that overexpressed wild-type PC-1 had increased annexin V. Use of antibodies to annexin V and PC-1 revealed that both annexin V and PC-1 directly mediated the elevated calcium-precipitating capacity of MV. The increased ability of MV to precipitate calcium from PC-1-overexpressing cells did not require exogenous ATP.

Conclusion. Upregulated expression of enzymatically active PC-1 directly promotes apoptosis, increased MV annexin V, and an increased capacity of meniscal cell and articular chondrocyte MV to precipitate calcium. These results suggest a direct link between increased PC-1 expression and the pathogenesis of chondrocalcinosis. (J Rheumatol 2001;28:2681–91)

Key Indexing Terms:

B10/PDNP3 AUTOTAXIN/PD-Iα OSTEOARTHRITIS
CHONDROCALCINOSIS PYROPHOSPHATE

In idiopathic calcium pyrophosphate dihydrate (CPPD) crystal deposition disease of aging, mean articular cartilage extracellular inorganic pyrophosphate (PPi) levels and PPi-generating nucleoside triphosphate pyrophosphohydrolase (NTPPPH) activity both increase markedly in an age dependent manner[1-3]. PPi supersaturation in the pericellular matrix of articular and fibrocartilaginous chondrocytes, and certain other changes in chondrocyte differentiation and matrix composition, promote CPPD crystal deposition in the pericellular matrix in aging and osteoarthritic (OA) meniscal fibrocartilages and hyaline articular cartilages[1,2]. Under differing conditions, partly dependent on the amount of available extracellular ATP, the deposition of CPPD crystals, hydroxyapatite crystals, or both are favored in degenerative cartilage[1,4,5]. The level of extracellular PPi is believed to critically affect not only CPPD crystal deposition but also hydroxyapatite deposition in degenerative cartilage, as PPi suppresses hydroxyapatite crystal deposition and propagation[1,5,6].

The initiation of matrix calcification in articular cartilage may be mediated by mineralizing vesicles commonly termed matrix vesicles (MV), membrane limited, cell derived frag-

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ments that bear enzymes and matrix components that alter the extracellular matrix[7-9]. The interior of MV also serves as a sheltered environment for seeding of hydroxyapatite crystals[6], mediated by 2 MV associated inorganic phosphate (Pi) -generating enzymes, ATPase and alkaline phosphatase (AP)[7]. In addition, certain MV associated molecules, including annexin V, promote calcification by binding calcium and/or promoting the influx into the vesicles of free calcium[7,8].

The composition and mineralizing function of MV appear to directly reflect the differentiation and/or metabolic status of cells from which the MV are released[2,8,9]. For example, hypertrophic growth plate chondrocytes shed more mineralizing MV than resting or proliferating cells[8]. In addition, chondrocyte apoptosis is associated with the release of membrane limited cell fragments ("apoptotic bodies") that can precipitate calcium, which has suggested that apoptotic bodies, under some circumstances, can function as a form of MV capable of promoting calcification[9]. Moreover, 1,25 dihydroxyvitamin D3, transforming growth factor-ß (TGF-ß) and interleukin 1 (IL-1), and nitric oxide are among the agonists that regulate the composition and mineralizing activity of MV derived from both viable and apoptotic cells[6,7].

Potent regulators of calcification include MV associated PPi, whose MV concentration is also regulated by calciotropic hormones, as well as cytokines, and differentiation and growth factors acting on MV-producing cells[2,6,10,11]. PPi is generated by multiple biosynthetic reactions[1,2,10]. Certain growth factors, including TGF-ß, regulate PPi production, in part by modulating enzymes with PPi-generating NTPPPH/phosphodiesterase nucleotide pyrophosphatase (PDNP) activity (EC 3.6.1.8, EC 3.1.4.1)[2], and in part by NTPPPH-independent effects[12].

Articular chondrocytes, which constitutively elaborate relatively large amounts of extracellular PPi[2,12], also have the highest levels of specific activity of NTPPPH of all tissues studied to date[13]. NTPPPH activity is a function of closely related isozymes of the PDNP family[14]. A substantial fraction of cell associated NTPPPH activity of cultured resting human articular chondrocytes appears attributable to PC-1, the first described PDNP family member[14]. PC-1 translocates to the chondrocyte plasma membrane[2], and TGF-ß stimulated PC-1 expression and PC-1 translocation to the plasma membrane appear to play a co-ordinate role in raising the extracellular PPi concentration in chondrocytes[2]. In contrast, IL-1, which suppresses chondrocyte PC-1 expression, also suppresses chondrocyte PPi generation[15].

Some NTPPPH activity in cultured human articular chondrocytes is attributable to a PDNP family isozyme alternatively termed B10 or PDNP3, which remains predominantly intracellular in cultured articular chondrocytes, and apparently plays little role in regulation of chondrocyte extracellular PPi concentration[2]. Additional NTPPPH activity in cultured articular chondrocytes has been attributed to one or more products of the gene for autotaxin (ATX)[2], another PDNP family isozyme whose mRNA is alternatively spliced into several distinct isoforms[14,16].

However, ATX, unlike PC-1 or B10/PDNP3, has been observed to have threonine-type ATPase activity[16], which may interfere with PPi generation by reducing levels of the substrate ATP. In further distinction to PC-1 and B10/PDNP3, most ATX appears to be secreted[17].

We recently observed that robust PC-1 expression (but not B10/PDNP3 expression, and to only a modest degree ATX expression) increased the ability of meniscal cells to calcify their matrix *in vitro*, and that of these 3 isozymes, only PC-1 was detected in matrix vesicles of meniscal cells[18]. In this study, we further investigated the potential functions of increased expression of the PDNP family NTPPPH isozymes PC-1, ATX, and B10/PDNP3 by cultured primary meniscal cells. In this regard, enhanced apoptosis is seen in degenerative articular cartilages[19,20], and is spatially associated with both articular cartilage crystal deposits[21] and with foci of knee meniscal cartilage calcification[18]. Because apoptosis appears to promote chondrocytic mineralization *in vivo*[21], we further assessed and compared the relationships between individual NTPPPH isozymes, chondrocytic cell apoptosis, and MV mediated mineralization. We determined that increased PC-1 expression was unique (among NTPPPH isozymes) in being strongly associated with meniscal cell apoptosis. Thus, we went on to further characterize the mineralizing activity of MV from PC-1-overexpressing meniscal cells and articular chondrocytes.

MATERIALS AND METHODS

Reagents. Human recombinant TGF-ß1 and IL-ß were purchased from R&D Systems (Minneapolis, MN, USA). All chemical reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

Meniscal cell isolation and culture. Human knee menisci were taken from donors at autopsy, where samples were normal, i.e., graded as having no evidence of OA. Meniscal cells were taken from tissue slices removed from the central regions of the medial and lateral menisci. Tissue was minced with a scalpel, incubated in Dulbecco's modified Eagle's medium (DMEM) high glucose containing 2 mg/ml clostridial collagenase, 5% fetal calf serum (FCS), 1% L-glutamine, 100 units/ml penicillin, and 50 µg/ml streptomycin (Omega Scientific, Tarzana, CA, USA), and incubated on a gyratory shaker at 37°C until the tissue fragments were digested. Residual multicellular aggregates were removed by sedimentation (1000× g), and cells were washed 3 times in DMEM containing 5% FCS.

Meniscal cells were maintained in DMEM high glucose and supplemented with 10% FCS, 1% L-glutamine, 100 units/ml penicillin, and 50 µg/ml streptomycin (Omega Scientific) and cultured at 37°C with 5% CO_2. In cell culture studies involving TGF-ß or IL-1, the cells were placed in DMEM high glucose containing 1% FCS, 1% L-glutamine, 100 units/ml penicillin, and 50 µg/ml streptomycin. In all other studies the cells were cultured in complete medium (as described above). Type II collagen and aggrecan expression were confirmed in each meniscal cell preparation using reverse transcription-polymerase chain reaction, as described, and employing G3PDH as a control[2].

Articular chondrocyte isolation and culture. Chondrocytes were isolated from normal human knee articular cartilages at autopsy, using methods for isolation and culture of knee articular chondrocytes as described[2]. As before, adherent articular chondrocytes were grown in DMEM high glucose with 10% FCS, 1% glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin[2]. Only first passage cells were studied.

Plasmid transfection and adenoviral gene transfer. Plasmid DNA constructs for full length human PC-1 (in pcDNA3) and B10/PDNP3 (in pBKCMV, from Dr. K. Sano, Kobe University, Japan) were as described[2]. The construct encoding full length human ATX was from Dr. M. Stracke (NCI, Bethesda, MD, USA)[16]. Empty pcDNA3 vector was used as a control, where indicated. Meniscal cells (5×10^5) were plated in 60 mm dishes and allowed to adhere overnight. Using the Lipofectamine Plus kit (Life Technologies, Grand Island, NY, USA), 2 μg of plasmid DNA in serum-free (S-F) DMEM/F12 was added to 8 μl of the "Plus" reagent and incubated at 23°C for 15 min. Twelve microliters of lipofectamine was added to this DNA complex and incubated another 20 min at 23°C. The cells were washed once with phosphate buffered saline (PBS) and once with S-F DMEM/F12. Two milliliters of S-F DMEM/F12 containing 0.00015% digitonin was added to the cells and incubated 3 min at 23°C. The media was removed and 2 ml of S-F DMEM/F12 was added to each plate. The DNA-"Plus"-lipofectamine reagent complexes were added to the cells and incubated at 37°C for 7 h. The media was removed and complete DMEM high glucose medium was added. Transfection efficiency, measured using a beta-galactosidase reporter system in control samples as described[18], was > 35% under these conditions. For adenoviral gene transfer of PC-1, the construction, characterization and protocol using recombinant replication defective E1 mutant adenovirus 5 encoding wild-type human PC-1, and enzyme-inactive murine PC-1 and source of the control "empty" adenovirus were as described in detail[6,12]. In brief, meniscal cells or articular chondrocytes (3×10^5) were plated in 35 mm dishes and incubated overnight. The cells were washed twice with PBS and then incubated with 5×10^3 PFU/ml of empty virus or recombinant viruses encoding cytomegalovirus promoter-driven wild-type and enzyme inactive PC-1 in DMEM high glucose containing 2% FCS, 1% L-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin for 6–8 h. The cells were then washed again with PBS and cultured an additional 72 h prior to collection. Efficiency of gene transfer, also measured using a beta-galactosidase reporter system in control samples as described[2], was > 55% under these conditions.

Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described[2], using the antibodies described above. For detection of annexin V, we used an affinity purified IgG from a rabbit polyclonal antiserum (from Dr. J. Mollenhauer, Rush University, Chicago, IL, USA)[22].

Assays of PPi metabolism, cellular DNA. PPi was determined by differential adsorption on activated charcoal of UDP-D-[6-^{3}H] glucose (Amersham, Chicago, IL, USA) from the PPi stimulated reaction product 6-phospho [6-^{3}H] gluconate[2]. PPi was equalized for the DNA concentration in each well, determined chromogenically following precipitation in perchlorate. Units of NTPPPH and AP, measured as described[18], were designated as moles of substrate hydrolyzed per hour (per μg protein in each sample). Intracellular ATP concentration was measured by luciferase assay as described[2].

Caspase activation assays and TUNEL staining of cultured cells. Cells (5×10^6) were plated in a 10 cm dish and were allowed to adhere overnight. The cells were then infected with adenovirus as described above, or stimulated with 10 ng/ml of TGF-ß or IL-1 for 72 h or 24 h with a positive control for apoptosis induction such as 1 mM sodium nitroprusside (SNP). The cells were washed once with PBS and scraped into a microfuge tube. The cells were pelleted and then lysed in hypotonic lysis buffer [1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT), 2 mM PMSF, 10 μg/ml leupeptin, 25 mM HEPES], frozen and thawed quickly 4 times, and then stored at –70°C until use. Caspase-1 and -3 activity was determined using the fluorescent substrates provided in the Promega (Madison, WI, USA) Caspase Detection kit according to the manufacturer's instructions. Briefly, cell lysates were incubated for 1 h at 37°C in the provided buffer and then an additional 30 min with the substrates. Positive and negative controls were included and the results were read on a 96 well fluorescent plate reader (absorbance 360 nm, emission 410 nm).

For TUNEL staining, cells (3×10^5 cells) were plated on 18 mm^2 coverslips placed in 35 mm dishes. The cells were then infected with adenovirus as described above, or stimulated with 10 ng/ml of TGF-ß or IL-1 for 72 h, or 24 h with 1 mM SNP. The cells were then fixed with fresh 4% paraformaldehyde for 30 min at 22°C. Cells were permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice and then washed twice with PBS. The Boehringher Mannheim TUNEL detection kit was used to stain the cells, according to the manufacturer's instructions.

MV isolation and MV ^{45}Ca precipitation assay. For assays of MV precipitation of insoluble calcium, conditioned media from cultured cells were collected at 72 h after transfection, adenoviral infection, or initial treatment with TGF-ß or IL-1ß in culture. The conditioned media was first centrifuged at 20,000 × g for 20 min at 4°C to pellet the cellular debris. This was followed by centrifugation at 100,000 × g for 1 h to isolate the MV fraction, which was resuspended in Hanks' balanced salt solution (HBSS)[6].

All MV preparations were verified to have an enrichment of AP specific activity of at least 10-fold relative to cell lysates before being employed in the studies described. MV fractions (0.04 mg protein in 0.025 ml) were added in triplicate to 0.5 ml of calcifying medium [2.2 mM $CaCl_2$ (1 Ci/ml ^{45}Ca), 1.6 mM KH_2PO_4, 15 mM KCl, 10 mM $NaHCO_3$, 50 mM N-tris (hydroxymethyl) methyl-2 aminoethanesulfonic acid, with or without 1 mM ATP disodium salt, pH 7.6], vortexed, and incubated at 37°C for 24 h[6]. The precipitate was washed 3 times in calcifying media without ATP, resuspended in HCl, and then counted in scintillation fluid. The results are expressed as a percentage of ^{45}Ca precipitated per μg of protein. Where indicated, we added, to the 40 μg MV in HBSS, 1 μl of ascites containing the 3E8 antibody to PC-1 or an isotype-specific (IgG2a) nonimmune ascites control, or alternatively, 1 μl rabbit IgG (from a 4 mg/ml solution in PBS) specific for annexin V or the same volume (and concentration) of nonimmune rabbit IgG (purchased from Sigma and placed in PBS). The samples were then mixed at 4°C for 24 h at 37°C prior to assessing MV calcium precipitation as described above.

Crystal analysis from the MV preparations studied was performed as described, via electron diffraction and energy dispersive x-ray spectroscopy[6,23,24].

Statistics. Where indicated, error bars represent standard deviation. Statistical analysis was performed using the Student t test (paired 2 sample testing for means).

RESULTS

Divergent effects of direct NTPPPH isozyme expression on apoptosis and MV mediated calcium precipitation in cultured meniscal cells. To identify and compare potential effects on apoptosis of NTPPPH isozymes, we used our recently validated method for plasmid DNA transfection of NTPPPH isozymes in meniscal cell preparations, which routinely produces 4 to 6-fold increase in NTPPPH activity in response to transfection of each NTPPPH isozyme by 72 h[18]. Directly upregulated expression of PC-1 but not ATX by plasmid transfection induced markedly increased apoptosis (Figure 1). Increased B10/PDNP3 expression by plasmid transfection was associated with a modest although statistically significant rise in meniscal cell apoptosis, as measured by both TUNEL assay and by caspase-1 activation (Figure 1A-B).

MV-containing vesicular fractions from transfected meniscal cells were previously observed to have increased NTPPPH activity in isolates from cells transfected with PC-1 but not ATX or B10/PDNP3[18]. We demonstrated here that only MV fractions from cells transfected with PC-1 were associated with increased calcium precipitation (Figure 2).

PC-1 induced effects on apoptosis and MV mediated calcium precipitation require PC-1 enzyme activity in meniscal cells and articular chondrocytes. Because of the unique association of the NTPPPH isozyme PC-1 with apoptosis and calcium

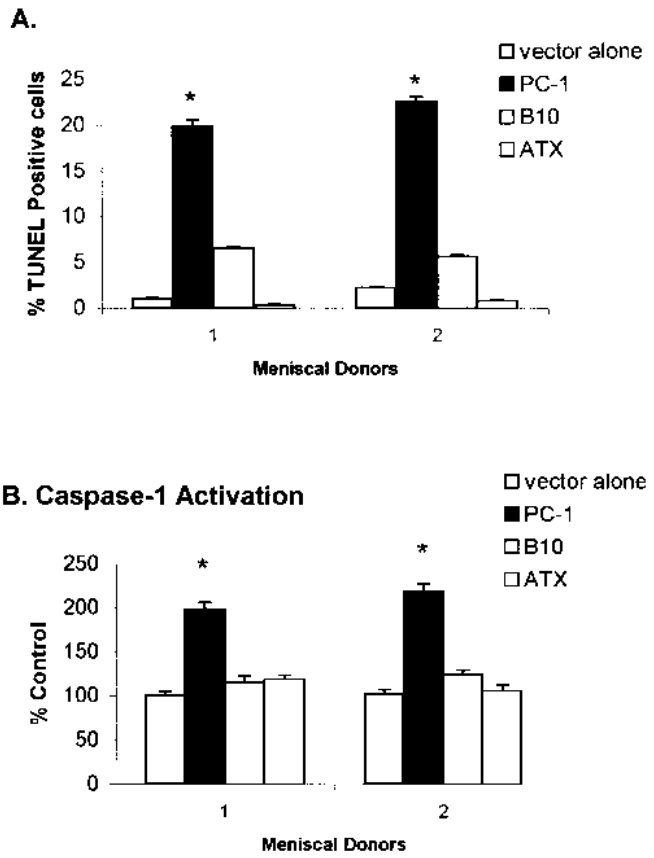

Figure 1. Differential effects of 3 NTPPPH isozymes on meniscal cell apoptosis. We expressed plasmid DNA for PC-1, autotaxin, and B10/PDNP3 in cultured knee meniscal chondrocytes from the central regions of normal medial and lateral knee menisci by a Lipofectamine Plus based transfection approach, using 5×10^5 cells in first passage culture, as described in detail in Materials and Methods. We assessed TUNEL staining in triplicate (A) and caspase-1 activation (B) at 72 h after transfection. The donors here (and in Figure 2), designated 1 and 2 (a 25-year-old and a 64-year-old man, respectively), and these results are representative of studies using 3 separate donors, performed on primary, first passage, and second passage meniscal cells in culture. *$p < 0.05$.

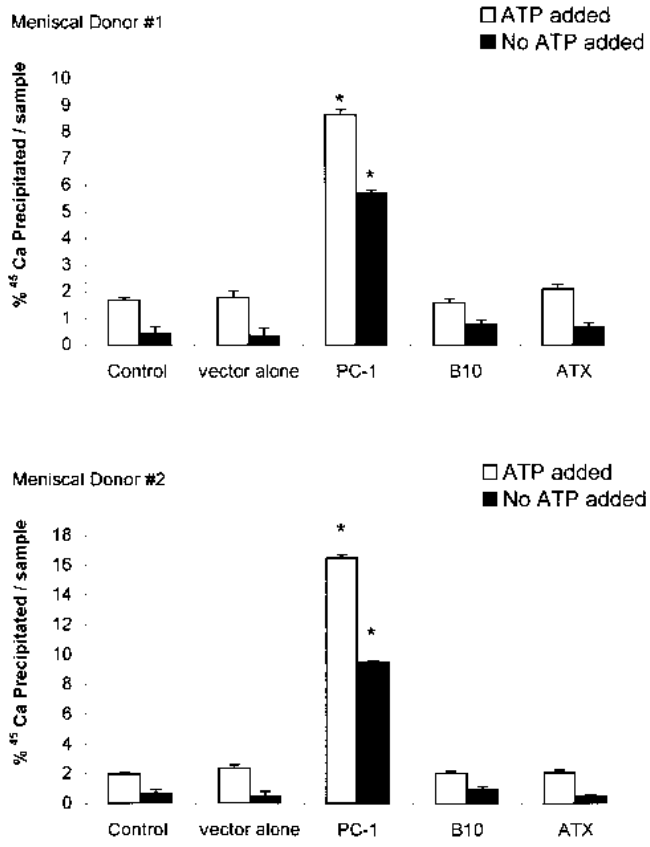

Figure 2. Calcium precipitation by MV from meniscal cells transfected with 3 NTPPPH isozymes. Meniscal cells (5×10^5) from donors 1 and 2 (as in Figure 1) were transfected with 2 μg PDNP cDNA using the Lipofectamine Plus based approach described for Figure 1. An additional set of cells (labeled control) was untransfected. The transfection of empty plasmid served as another control as described in Materials and Methods. At 72 h after transfection, the MV fractions were collected by differential centrifugation. The MV fractions (40 μg protein in 25 ml) collected from each group of treated cells were added in triplicate to 0.5 ml of the calcification-promoting buffer (with or without ATP) and incubated 24 h at 37°C. The precipitate was washed 3 times in the calcification-promoting buffer without ATP, resuspended in HCl, and then counted in scintillation fluid. The results are expressed in percentage of ^{45}Ca precipitated per each 40 μg MV protein sample (n = 9 from each donor). Representative of results using 3 separate donors, and performed on primary, first passage, and second passage meniscal cells in culture. *$p < 0.05$.

precipitation by MV, we further examined the mechanism for the observed effects of robust expression of PC-1. In doing so, we expanded our studies to cultured human articular chondrocytes from normal human knees. Because articular chondrocytes are difficult to efficiently transfect with plasmid DNA, we used a validated adenoviral gene transfer approach[2,6] to more efficiently express both wild-type and enzyme inactive PC-1 in the articular chondrocytes and cultured meniscal cells.

Adenoviral gene transfer of wild-type PC-1 achieved significant upregulation of both cellular NTPPPH and PPi generation (Figure 3). Under these conditions, a robust direct elevation of expression of wild-type PC-1, but not enzyme inactive PC-1, was associated with apoptosis, assessed by caspase-1 and caspase-3 activation and also by TUNEL staining (Figure 4). Under the conditions where apoptosis was induced, adenoviral gene transfer of PC-1 did not significantly alter the concentration of intracellular ATP (data not shown).

Because excess expression of wild-type PC-1 promoted apoptosis, we tested the hypothesis that direct upregulation of wild-type but not enzyme inactive PC-1 expression altered MV composition and mineralizing activity in cultured meniscal cells and articular chondrocytes. We induced expression of wild-type and enzyme inactive PC-1 by adenoviral gene transfer, and verified by Western blotting that immunoreactive PC-1 translocated in MV under both conditions (data not shown). We confirmed[18] that forced expression of wild-type PC-1 but

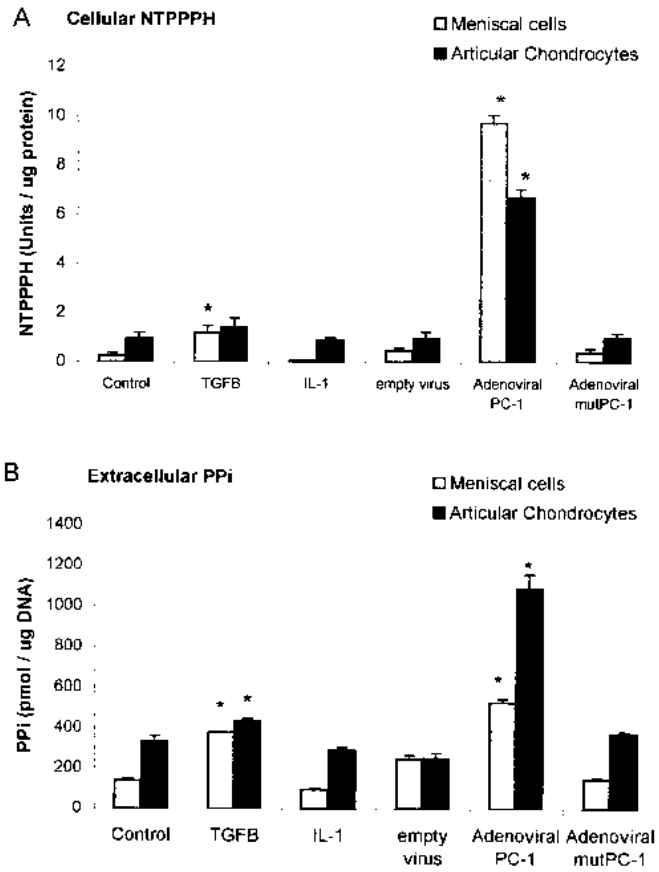

Figure 3. NTPPPH and extracellular PPi in cultured meniscal cells and articular chondrocytes in response to adenoviral gene transfer of PC-1. Cultured meniscal cells (3×10^5 cells) isolated and from the central regions of normal donor medial and lateral knee menisci (as in Figures 1 and 2) and normal knee articular chondrocytes (3×10^5 cells) were adenovirally infected in triplicate with (5×10^3 PFU in 1 ml) for 6 h, using empty virus or recombinant virus containing wild-type PC-1 cDNA, followed by one change of medium and additional incubation of cells for 72 h, as described in Materials and Methods. For comparative purposes, cells were incubated with 10 ng/ml TGF-ß or IL-ß (A). The cells were collected and lysed in 0.2 M Tris, pH 8.0, 1.6 mM $MgCl_2$, and 1% Triton X-100. Five micrograms of total protein were used for NTPPPH assays (B). The media was removed from the cells, and PPi was measured, with results expressed as pmol/μg of DNA. Representative of 5 donors. *$p < 0.05$.

not the enzyme inactive mutant PC-1 induced marked elevations of MV NTPPPH-specific activity and PPi (Figure 5A-B) without significantly altering the level of MV PPi-degrading AP-specific activity (Figure 5C). In addition, forced expression of wild-type PC-1, studied in meniscal cells, was associated with the production of MV containing more annexin V (Figure 6). In comparison studies, enrichment of annexin V in meniscal cell derived MV was not observed after adenoviral gene transfer with enzyme inactive PC-1 or simple treatment with TGF-ß or IL-ß (data not shown). In contrast, increased MV annexin V detection occurred after induction of meniscal cell apoptosis using staurosporine (Figure 6).

Adenoviral gene transfer of wild-type but not enzyme inactive PC-1 induced a marked increase in calcium precipitation by meniscal cell and articular chondrocyte MV, and addition of exogenous ATP was not necessary to see increased calcium precipitation by the MV (Figure 7). In these experiments, we noted a markedly enhanced ability of MV from TGF-ß stimulated articular chondrocytes but not meniscal cells to precipitate calcium when exogenous ATP was provided (Figure 7), despite the absence of significant changes in MV NTPPPH, PPi, or AP in MV fractions from the TGF-ß treated cells (Figure 5A-C).

Staurosporine induced apoptosis (Figure 8C) was confirmed to increase the calcium-precipitating activity of MV (Figure 8A). However, the increased MV mineralizing activity induced by the forced PC-1 expression was much greater than that induced by apoptosis in response to staurosporine (Figure 8A). Under these conditions, forced wild-type PC-1 expression, but not the staurosporine treatment, significantly increase MV NTPPPH-specific activity (Figure 8B). The calcium-containing crystals deposited by MV under these conditions were analyzed as described in Materials and Methods, and were determined to be hydroxyapatite (data not shown).

The 3E8 monoclonal antibody to native PC-1, but not an isotype-specific control antibody, significantly interfered with the majority of the activity of NTPPPH present in MV from meniscal cells adenovirally infected with PC-1 (data not shown). Under the same conditions, incubation of MV with the anti-PC-1 antibody significantly suppressed the calcium-precipitating activity of MV derived from meniscal cells that overexpressed wild-type PC-1 (Figure 8A). Antibody to annexin V also markedly suppressed the calcium-precipitating activity of MV derived from meniscal cells that overexpressed wild-type PC-1 (Figure 8A). However, antibody to annexin V but not PC-1 attenuated the increase in MV calcium-precipitating activity that occurred in association with staurosporine induced apoptosis (Figure 8A).

DISCUSSION

We demonstrated that a direct, marked increase in expression of the NTPPPH isozyme PC-1 promoted apoptosis of cultured meniscal cells and articular chondrocytes. Nonspecific cell stress associated with transfection likely contributed in part to enhanced apoptosis in association with increased PC-1 expression. However, the association of increased wild-type PC-1 expression with meniscal cell and chondrocyte apoptosis was not wholly attributable to nonspecific effects of transfection or altered protein expression. Specifically, directly upregulated expression of the NTPPPH isozyme ATX, or expression of an enzyme-inactive mutant of PC-1, were not associated with the same effect. Direct expression of the NTPPPH isozyme B10/PDNP3 promoted a relatively modest, although not statistically significant, increase in apoptosis of meniscal cells.

Apoptosis can be induced by factors that include cytokine induced nitric oxide (NO) production, dysregulation of mito-

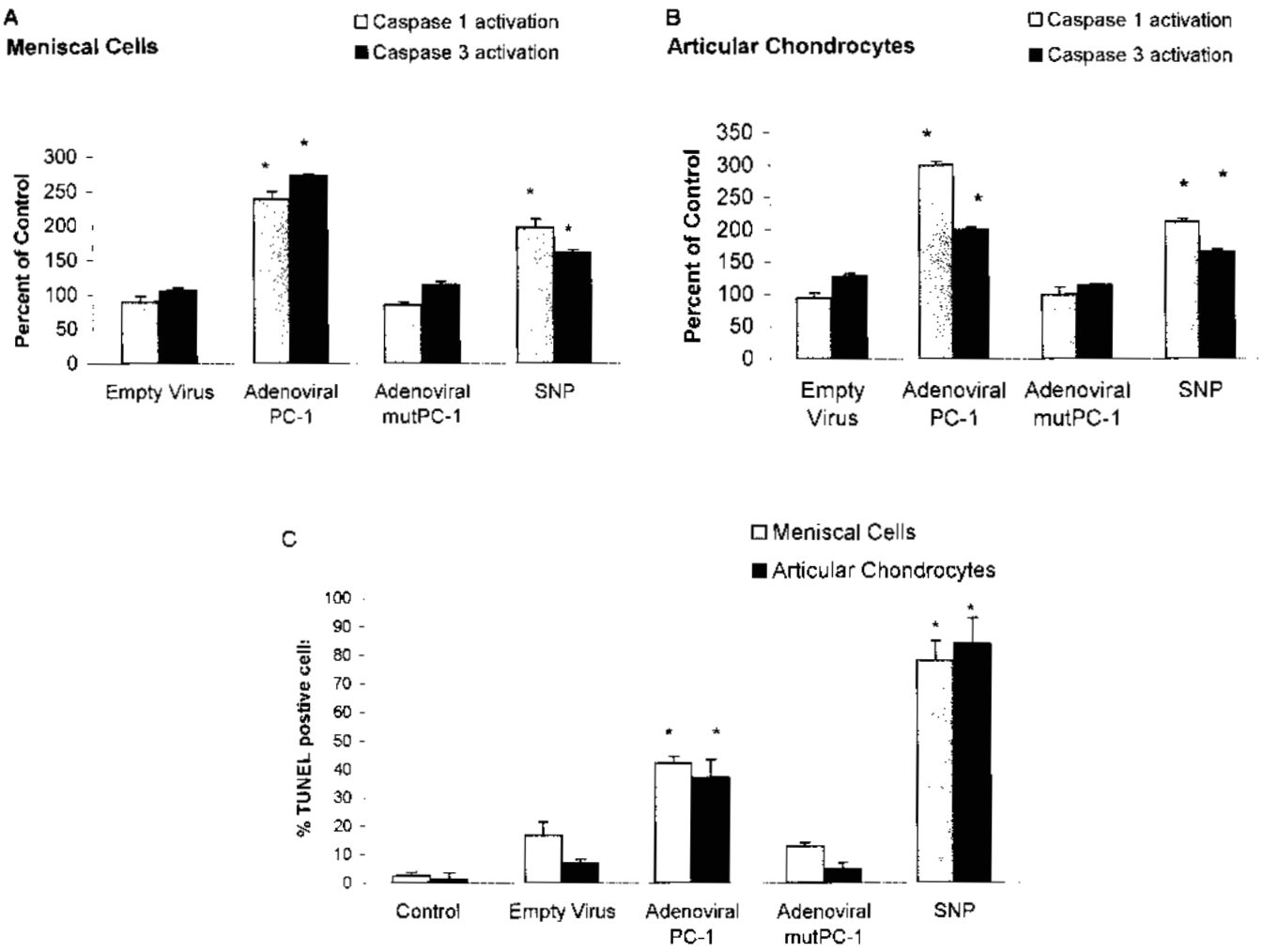

Figure 4. Effects of direct elevation of PC-1 expression (by adenoviral gene transfer) on apoptosis in meniscal cells and articular chondrocytes. Normal knee meniscal cells and articular chondrocytes were cultured as above and infected with 5×10^3 PFU/ml of empty virus or adenoviral PC-1 in 5 ml for 6 h, as described in Materials and Methods. After the medium was changed to DMEM (high glucose) with 10% FCS, the cells were incubated an additional 72 h. As a positive control for apoptosis induction, we incubated 1 mM sodium nitroprusside (SNP) with cells for the last 24 h. Cells were collected, lysed in hypotonic lysis buffer, frozen and thawed 4 times, and then assayed for enzymatic activity of caspases 1 and 3 (A, B) using the Promega caspase activation kit. C. Results for parallel studies of apoptosis by TUNEL assay, in which cells were fixed, permeabilized, and TUNEL stained 72 h after adenoviral gene transfer. Data for the caspase activation studies were pooled from results with 5 donors, each assayed in triplicate. Data for TUNEL staining (expressed as percentage TUNEL staining cells) pooled from 4 donors, n = 250 cells each. *$p < 0.01$.

chondrial function, denial of cell adhesion, or withdrawal of growth factor signaling[25-27]. We have observed that PC-1 does not affect NO production by chondrocytes (K. Johnson, *et al*, unpublished observations), and PC-1 did not diminish total intracellular ATP in meniscal cells in this study. PC-1 and B10/PDNP3, but not the predominantly secretory NTPPPH ATX, induce elevated intracellular PPi in chondrocytic cells[18]. Excess free PPi is believed to interfere with DNA replication and other general functions of cells, including protein biosynthesis[28,29], but it has not been established that intracellular PPi levels modulate apoptosis. In contrast, increased provision of extracellullar PPi, which is produced in part by PPi hydrolysis, has been repeatedly shown to stimulate apoptosis of both chondrocytes and osteoblasts, an effect mediated in part through the membrane sodium phosphate transporter and in part by Pi induced mitochondrial dysfunction[30-32]. Because elevated expression of PC-1, but not B10/PDNP or ATX, significantly increases extracellular PPi in chondrocytes[2,18], it will be of interest to assess if increased Pi generation mediates the pro-apoptotic effects of excess PC-1 expression in chondrocytes, and to assess if increased PC-1 expression regulates meniscal cell and articular chondrocyte apoptosis via effects on mitochondrial dysfunction.

Chondrocyte apoptosis is temporally and spatially associated with cartilage matrix calcification[21], and the apoptosis promotes MV mediated mineralization[9,33]. Further, the composition and mineralizing activity of MV reflects the underlying metabolic and differentiation status of the cells producing

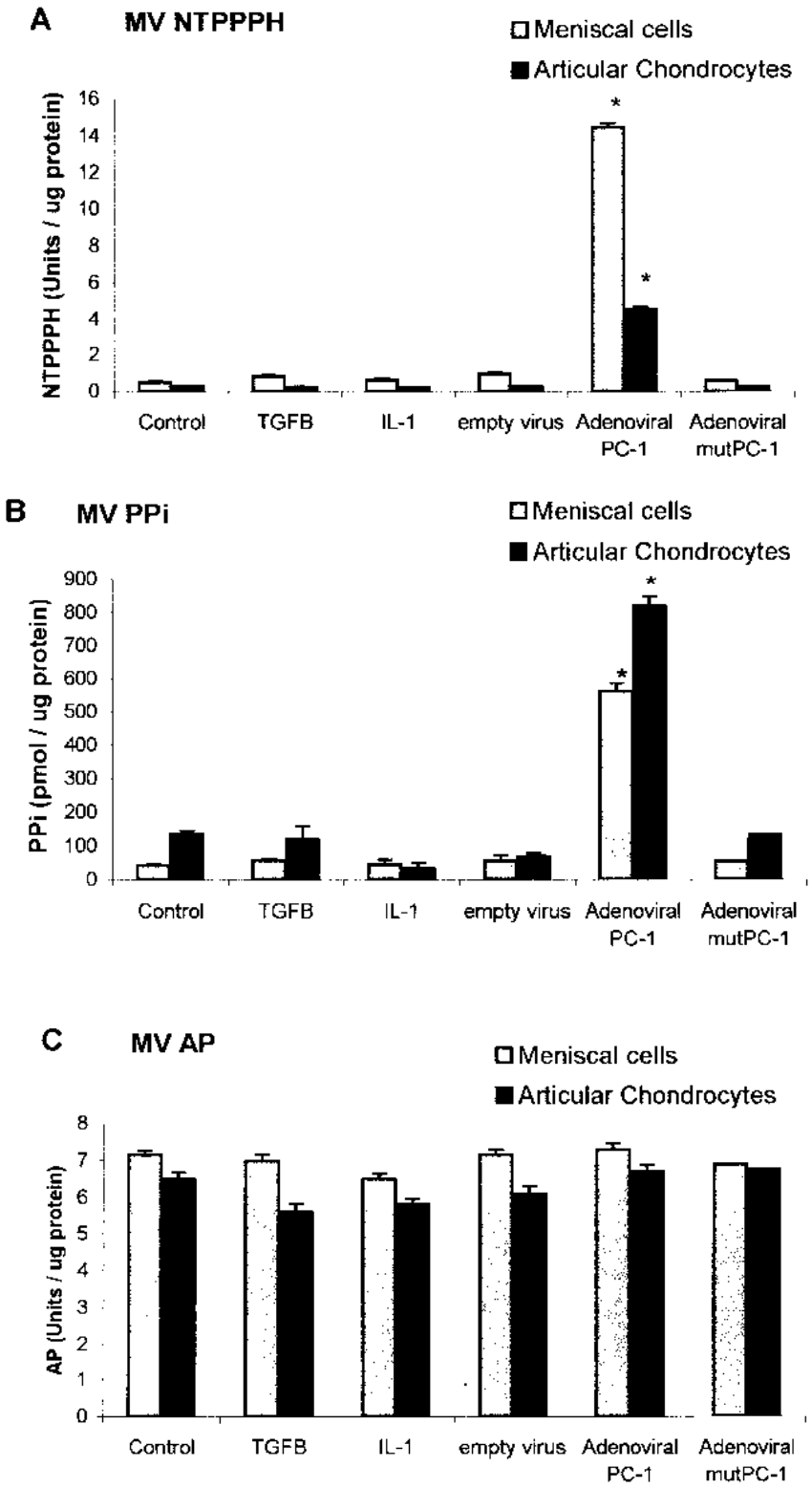

Figure 5. Effects of direct elevation of PC-1 expression (by adenoviral gene transfer) on meniscal cell and articular chondrocyte MV NTPPPH activity and PPi. Meniscal cells and articular chondrocytes (3×10^5 cells) were virally infected with 5×10^3 PFU empty virus or recombinant virus containing wild-type or enzyme-inactive PC-1, as described in Materials and Methods. Alternatively, cells were incubated with 10 ng/ml of TGF-ß or IL-ß. After 72 h further culture, MV fractions were collected from the media by differential centrifugation as described above. Five micrograms of protein were used to determine NTPPPH activity (A) as well as AP activity (C). The results are expressed in units/μg protein and representative of 5 donors each. PPi was measured (B) on aliquots of MV fractions that contained 40 μg of protein. The results for PPi are expressed in pmol/μg protein and are representative of 5 donors each. *$p < 0.01$.

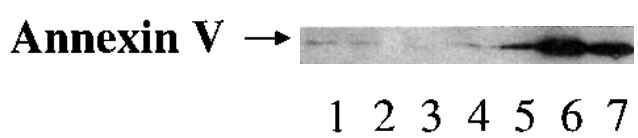

Figure 6. Effects of direct PC-1 expression (by adenoviral gene transfer) on annexin V detection in MV derived from meniscal cells. Meniscal cells (5×10^5 per 60 mm plate), cultured as described above, were adenovirally infected with empty virus, or enzyme-active PC-1, or treated with escalating doses of staurosporine, a known inducer of apoptosis. MV were then isolated after 72 h. Shown are results of SDS-PAGE and Western blotting for annexin V (molecular weight 34 kDa) using 20 μg aliquots of MV protein. Lane 1 negative control, Lane 2 empty virus, Lanes 3–6 ten nM, 100 nM, 500 nM, 1 mM staurosporine, respectively; Lane 7 adenoviral PC-1.

the MV[6,9,33]. In this study, we observed that direct and robust upregulation of PC-1 expression stimulated ATP independent calcium precipitation by MV. PC-1 induced increases in MV calcium precipitation were not totally dependent on provision of exogenous ATP as a NTPPPH substrate, suggesting that endogenous NTPPPH substrates associated with MV were regulating calcium precipitation. Using antibody to annexin V, we demonstrated that the stimulatory effects of wild-type PC-1 on MV mediated mineralization appeared to be a consequence, in part, of the increased concurrent localization in MV of annexin V. The observed effects of PC-1 expression on calcium precipitation through enhanced MV annexin V localiza-

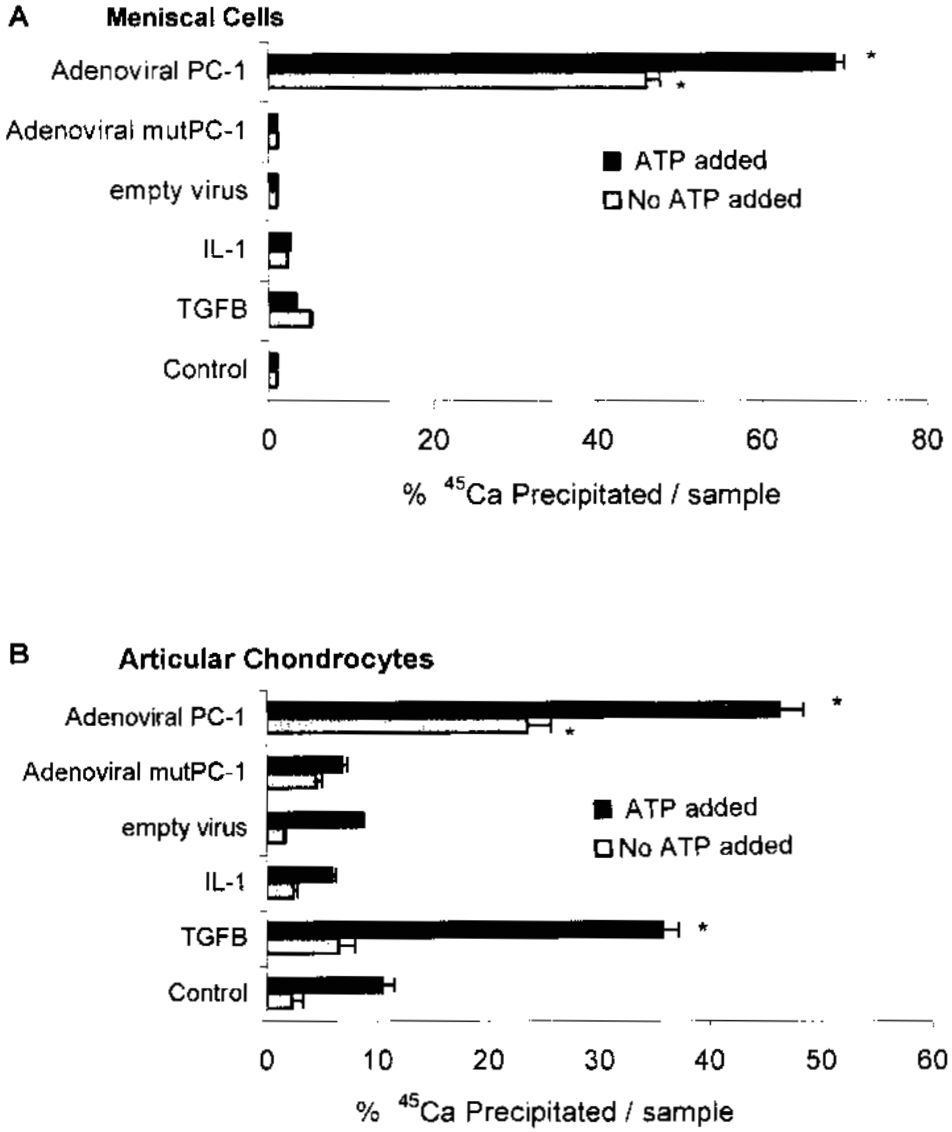

Figure 7. Effect of direct elevation of PC-1 expression (by adenoviral gene transfer) on calcium precipitation by meniscal cell derived MV. Meniscal cells (A) or articular chondrocytes (B) were treated as described, with adenoviral gene transfer used to induce expression of wild-type or enzyme inactive PC-1. MV fractions (40 μg protein in 0.025 ml) collected from each group of treated cells were added in triplicate to 0.5 ml of the calcification-promoting buffer (with or without ATP) described in Materials and Methods and incubated 24 h at 37°C. The precipitate was washed 3 times in calcification-promoting buffer without ATP, resuspended in HCl, and then counted in scintillation fluid. Results are expressed in percentage of ^{45}Ca precipitated per 40 μg MV protein sample. Data are pooled from 5 donors, each studied in triplicate. *$p < 0.05$.

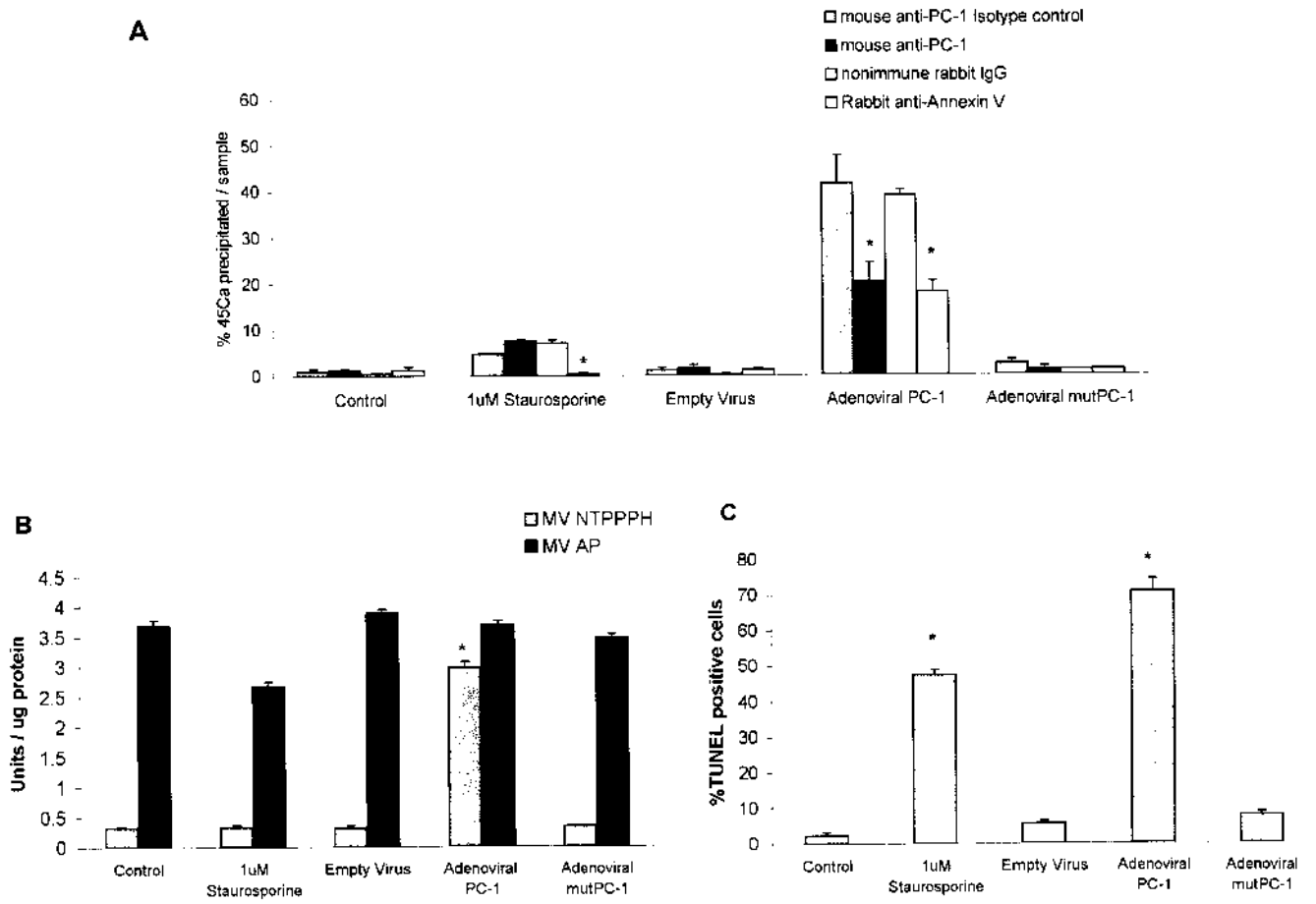

Figure 8. Effects of antibodies to PC-1 and annexin V on calcium precipitation by MV derived from meniscal cells. Meniscal cells were treated (as described previously) by adenoviral gene transfer of empty virus, wild-type or enzyme-inactive PC-1, or with 1 μM staurosporine for 72 h. A. MV fractions were collected and aliquots of MV (40 μg protein) in HBSS were mixed with 1 μl 3E8 ascites against human PC-1, or an equal volume of ascites isotype-specific control, or 1 μl rabbit anti-annexin V IgG, or 1 μl nonimmune rabbit IgG for 24 h at 4°C. Each fraction was then incubated 24 h with 0.5 ml of calcification-promoting buffer containing ATP, as described above. The fraction of applied ^{45}Ca precipitated was determined as described above. B. Five micrograms of protein from each MV fraction were used to determine MV NTPPPH and AP-specific activity as described above. C. Aliquots of the same cells as above were permeabilized and TUNEL stained for apoptosis (n = 250 cells from each donor). All data pooled from 5 donors; A and B were done in triplicate for each donor. *$p < 0.05$.

tion are noteworthy, because annexin V upregulation is not only a marker of metabolic alterations and changes in cellular phenotype (not limited to apoptosis)[22], but also a transitional marker of functional MV in mineralization[7]. Annexin V promotes calcium flux across the MV membrane[34], and annexin V is a component of the acid labile nucleationally active core (ALNAC) in the MV inner leaflet[35]. The ALNAC is essential for mineral formation by MV[35].

Using a monoclonal antibody to PC-1 that does not have PC-1 neutralizing ability in solution, we interfered with the majority of solid phase NTPPPH activity in MV, which we therefore speculate to reflect a steric effect exerted by 3E8 antibody seeing PC-1 in the milieu of the MV. Results with anti-PC-1 antibody revealed a direct role of increased MV PC-1 in the enhanced precipitation of calcium by MV from meniscal cells overexpressing PC-1. Direct expression of enzyme deficient PC-1 (which also translocated into MV) did not stimulate calcium precipitation by MV of meniscal cells. Although the precise molecular and physical mechanisms by which PC-1 increases calcium precipitation by MV remain to be defined, it is clear that NTPPPH enzyme activity is essential.

Taken together, the *in vitro* findings of our study suggest that the combination of apoptosis and elevated PC-1 expression is a particularly potent stimulus for MV to precipitate calcium. Using distinct methods involving culture of nonadherent chondrocytes, we also recently observed that PC-1 stimulates calcification of the pericellular matrix by meniscal cells[18]. The crystals deposited *in vitro* by MV in this study were determined to be hydroxyapatite. However, CPPD crystals, which are the predominant mineral phase deposited in calcified menisci of the knee, only form under a very narrow range of ionic conditions in a manner suppressed by ambient Mg^{2+}, phosphate, and AP activity[23,36]. These factors, along with the ability of PC-1 to provide more PPi to drive Pi formation, most likely explain why hydroxyapatite was the only

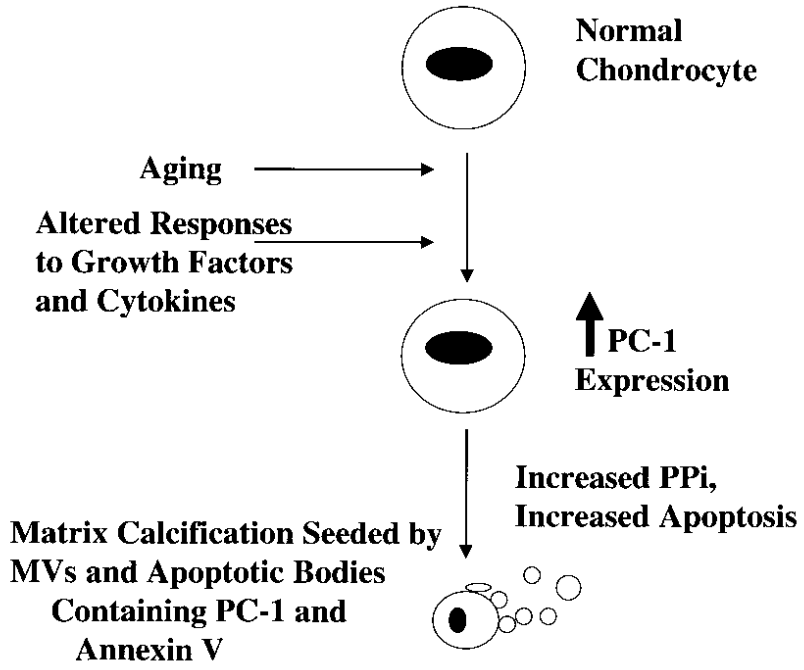

Figure 9. Hypothetical model for relationship between increased PC-1 expression and articular and meniscal cartilage calcification in degenerative joint disease in aging. The proposed model attempts to apply the findings of our study to the pathogenesis of articular cartilage and meniscal calcification in degenerative joint disease in aging.

type of crystal precipitated by MV in this *in vitro* study, and in a separate analysis of pericellular matrix calcification triggered by excess PC-1 expression in chondrocytic cells[18].

In situ studies of human knee meniscal fibrocartilages have indicated that PC-1 expression is sparse in normal menisci, but that PC-1 expression becomes upregulated in degenerative, calcified menisci at the sites of apoptotic cells (many in clusters) bordering calcifications[18]. Thus, a robust increase in PC-1 expression may be a direct determinant of heightened apoptosis and matrix calcification in cartilage.

PPi restrains hydroxyapatite formation, and at least some PC-1 expression has been established to be a physiologic necessity to suppress calcification of articular cartilages and menisci[37]. Further, the stimulatory effects of increased PC-1 expression on MV calcium precipitation in meniscal cells and articular chondrocytes found in our study are in direct contrast to previous findings in osteoblasts, where PC-1 inhibited MV mediated calcium precipitation by osteoblasts[6]. In addition, we did not previously observe an increase in ATP independent calcium precipitation by MV in response to forced expression of PC-1 in osteoblasts[6].

We speculate that differences in matrix proteins, and presumably MV proteins, released by osteoblasts and chondrocytic cells likely account for the contrasting roles of overexpression of PC-1 on MV mineralization. Meniscal cells and articular chondrocytes (but not osteoblasts) can promote CPPD deposition in association with matrix PPi supersaturation[1]. Therefore, it is likely that pathologic excess PC-1 expression only plays a stimulatory role for calcium-containing crystal deposition within articular fibrocartilages and hyaline cartilages.

PC-1, NTPPPH activity, and the annexin V content of MV are clearly not the only factors governing calcium precipitation by MV, as illustrated by the incidental findings with TGF-ß in our study. Specifically, under the conditions employed in our study, TGF-ß stimulated the release from articular chondrocytes but not meniscal cells of MV with a markedly increased capacity for ATP dependent calcium precipitation, and TGF-ß did so without inducing significant changes in MV NTPPPH and PPi. Pro-mineralizing factors regulated by TGF-ß in chondrocytes other than PPi metabolism[38] were most likely responsible for this observation.

In conclusion, despite the fact that constitutive PC-1 expression is necessary to prevent articular and meniscal cartilage calcification, marked, direct upregulation of PC-1 expression directly promoted both apoptosis and particularly active precipitation of calcium by MV of meniscal cells and articular chondrocytes. Increased chondrocyte apoptosis, NTPPPH activity, and PPi production all have been linked to cartilage calcification in aging meniscal fibrocartilages and articular hyaline cartilages. The results of our study, superimposed on previous immunohistochemical analyses[18], suggest that pathologic upregulation in expression of one of the 3 known cartilage NTPPPH isozymes, PC-1, can directly contribute to the pathogenesis of both decreased cell viability and matrix calcification in degenerative joint disease of aging (Figure 9). Thus, PC-1 may represent a specific molecular target for prevention of chondrocalcinosis.

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