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Age-related changes in cartilage endogenous osteogenic protein-1 (OP-1)

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Abstract

Articular cartilage has a poor reparative capacity. This feature is exacerbated with aging and during degenerative joint conditions, contributing to loss of motion and impairment of quality of life. This study focused on osteogenic protein-1 (OP-1) and its ability to serve as a repair-stimulating factor in articular cartilage. The purpose of this work was to develop a quantitative method for the assessment of the content of OP-1 protein in extracts from human articular cartilage and to investigate the changes in OP-1 mRNA expression and protein levels with aging of normal adult cartilage. Full thickness cartilage was dissected from femoral condyles of donors with no history of joint disease within 24 h of death. Levels of OP-1 mRNA expression were measured by a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) method; concentration of OP-1 protein was detected by new sandwich enzyme-linked immunosorbent assay (ELISA); qualitative changes in OP-1 forms were evaluated by Western blots with various anti-OP-1 antibodies. The sensitivity of the ELISA method allowed the detection of picogram quantities of OP-1 in cartilage extracts. We found that (1) concentration of OP-1 in normal cartilage is within the range of biological activity of OP-1 in vitro; and (2) during aging of human adult, articular cartilage, levels of OP-1 protein and message are dramatically reduced (more than 4-fold; $p < 0.02$). The major qualitative changes affected primarily mature OP-1. The results of the current study suggest the possibility that OP-1 may be critical for chondrocytes to maintain their normal homeostasis and could also serve as a repair factor during joint disease or aging.

Keywords: Human articular cartilage; Aging; Bone morphogenetic protein; Osteogenic protein-1; ELISA; PCR

1. Introduction

Osteoarthritis (OA) is a debilitating joint disease affecting primarily the elderly. OA is rare in young adults, developing symptomatically and radiographically in adults by their late 40s and 50s, and then, rapidly increases in prevalence from age 60 to 70 [1]. However, the mechanisms that turn normal aging of articular cartilage into the pathological OA process are currently unknown. In general, as any biological tissue or organ ages, function gradually declines and susceptibility to disease and injury increases [2]. During the last decade, a number of age-related changes in cartilage have been documented: (1) an increased denaturation of collagen type II [3]; (2) a decline in synthesis of DNA [4], proteoglycans (PG) and link protein [5–8]; (3) an increased sulfation of chondroitin sulfate [9]; (4) accumulation of hyaluronan [10] and cartilage intermediate layer protein [11]; (5) structural changes in fibromodulin [12]; (6) a decreased capability of assembling large molecular size aggregates [7]; (7) elevated levels of transglutaminase activity that promotes a pathologic matrix mineralization and cartilage degeneration [13]; and (8) increased apoptosis [14]. Importantly, with aging, the responsiveness of articular cartilage to different growth factors, such as transforming growth factor-β (TGF-β) [6,15], insulin-like growth factor-1 (IGF-1) [16], epidermal growth factor (EGF) [4], osteogenic protein-1 (OP-1) [17] and others, was also changed.

While the underlying causes of articular cartilage degeneration seen with age have not been identified, there is increasing evidence that cytokines and growth factors,

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especially those expressed endogenously in cartilage, play a critical mediatory role. Since growth factors, and particularly members of the bone morphogenetic protein family (BMP), are important regulators of matrix production that can also inhibit certain degradative processes, it is not difficult to envision how a loss in the biologically active endogenous growth factor could play a role in the development of matrix damage.

BMPs were originally identified as proteins capable of inducing ectopic endochondral bone formation in subcutaneous implants [18,19]. Subsequent molecular cloning revealed that the BMP family consists of a large number of related molecules that belong to the TGF-β superfamily. Although BMPs were initially found in the bone matrix, it is now clear that they are expressed in a variety of tissues. OP-1 is the seventh member of this family (BMP-7). It is synthesized as a large precursor, approximately three times larger than a mature protein, and is ultimately processed proteolytically at the C-terminal region to yield a mature disulfide-linked dimer. OP-1 is the most closely related to BMP-6 and BMP-5 (88% and 87% homology), and to a lesser extent to BMP-2 and BMP-4 (60% and 58%), with some homology to BMP-3 (42%) and TGF-β (about 30%) [20,21]. Originally, OP-1 was purified from bovine demineralized bone [22] with its recombinant form being subsequently cloned from human cDNA libraries in Chinese hamster ovary (CHO) cells [23]. The critical importance of BMPs for cartilage and bone formation was demonstrated using the transgenic approach: lack of some BMP genes caused skeletal abnormalities and eventually the lethality of mouse embryos [24,25]. Recent studies from our department [17,26–28] have focused on the potential role of exogenous OP-1 in human and bovine cartilage homeostasis and repair. We showed that human recombinant OP-1 caused a significant anabolic response in articular cartilage. It induced the synthesis of major matrix components (aggrecan and collagen type II) in human chondrocytes of different age [17,26] with continued expression of the chondrocyte phenotype. In addition, OP-1 has been shown to induce the synthesis of hyaluronan, its receptor CD44 and hyaluronan synthase-2, to promote the formation and retention of the extracellular matrix [28], and to counteract catabolic events, such as interleukin-1 (IL-1), fibronectin and collagen fragment-induced human cartilage degeneration [26,27,29]. When the effect of OP-1 on the Fn-f-challenged cartilage was compared to that of TGF-β, it was found that TGF-β was not able to block Fn-f-mediated PG depletion, but by itself promoted a decrease in cartilage PG content [27]. Importantly, recombinant OP-1 did not lead to chondrocyte proliferation and differentiation in human and bovine adult articular cartilage during short-term culture [17,30].

Noteworthy, we previously showed [31] that OP-1 is endogenously expressed by human adult articular chondrocytes. Moreover, in human articular cartilage, OP-1 is present in two forms, unprocessed, pro-form, and processed, mature-form, that are distributed in the inverted manner. Mature OP-1 is immunolocalized primarily in the superficial layer of cartilage, while pro-OP-1 is detected in the deep layer. The endogenous expression of OP-1 by articular chondrocytes indicates that articular cartilage has the potential to repair and might suggest the unique role of this BMP in tissue protection and regeneration. This is supported by recent data [32] where overexpression of OP-1 in mice led to the increased synthesis of matrix macromolecules, collagen type II and PGs.

The purpose of the current study was to investigate changes in endogenous OP-1 (protein and mRNA) with aging of human articular cartilage. We hypothesized that with aging, there will be a decline in the levels of endogenous OP-1, primarily, mature form of the protein, that might contribute to the elevated susceptibility of cartilage to the degenerative processes. To assess quantitatively the concentration of total endogenous OP-1 protein in cartilage extracts, we developed a sandwich OP-1 enzyme-linked immunosorbent assay (ELISA). We present here a quantitative data on age-related changes in endogenous OP-1 mRNA and protein and provide a qualitative analysis of these changes.

2. Materials and methods

2.1. Reagents

Human recombinant pro- and mature-OP-1, BMP-6, anti-pro (R2854) and anti-mature (1B12) OP-1 antibodies were provided by Stryker Biotech (Hopkinton, MA). Two other, anti-mature OP-1 antibodies (sc-9305 and MAB 354) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN). Electrophoresis grade reagents were purchased from Bio-Rad (Hercules, CA). Chemicals, either reagent or molecular biology grade, were purchased from Sigma (St. Louis, MO) unless otherwise noted. Keratanase (Ker; *Pseudomonas* sp.; EC 3.2.1.103), keratanase II (Ker II; *Bacillus* sp. Ks 36) and chondroitinase ABC (CHase; *Proteus vulgaris*; EC 4.2.2.2) were obtained from Seikagaku, Japan. Hyaluronidase (bovine testicular) was purchased from Sigma and Collagenase P (*Clostridium histolyticus*) was purchased from Boehringer Mannheim.

2.2. Tissue acquisition

Full thickness normal human articular cartilage was dissected from load bearing regions of femoral condyles of donors with no history of joint disease within 24 h of death. Samples from men and women ranging from 20 to 80 years old were obtained, with institutional approval, through the Regional Organ Bank of Illinois according to their protocol. After opening the joint, the surface of the cartilage was grossly examined. Although all cartilages were obtained from normal donors, not all of them appeared to be normal. Some samples revealed degenerative morpho-

logical changes. All cartilages were processed for either messenger RNA or protein extraction.

2.3. OP-1 antibodies

We used four different antibodies for this study. Antibodies obtained from Stryker Biotech (R2854 and 1B12) were previously described and specificity toward OP-1 was tested [31,33–35]. The polyclonal antibody R2854 was raised in rabbits against the monomeric pro-domain of the OP-1 molecule. For the detection of the mature domain of OP-1, we utilized three antibodies that were available to us either from commercial sources or from Stryker Biotech. Two monoclonal antibodies, 1B12 and MAB354, were raised against the monomeric mature domain of OP-1. A third, polyclonal antibody sc-9305 was raised against a 15-amino-acid synthetic peptide within the N-terminus of the mature OP-1 domain. The specificity of all antibodies was tested by the companies that produced these antibodies. However, taking into consideration the high homology between OP-1 and BMP-6 and the fact that the recombinant BMP-6 was cloned after the anti-OP-1 antibodies were produced, all antibodies used for the current study were tested for cross-reactivity with BMP-6 (Fig. 1).

2.4. Cartilage extraction

Five hundred milligrams of fresh donor cartilage was lyophilized overnight and the dry weight of the tissue was determined. Samples were pulverized in liquid nitrogen and 150 mg (dry weight) of cartilage tissue was extracted with 3.5 ml of ice-cold 1 M GuHCl buffer, pH 7.5, containing 10 mM $CaCl_2$, 50 mM Tris, and protease inhibitors (Protease inhibitor cocktail tablets, one tablet per 10 ml of ice-cold 1 M GuHCl buffer; Roche Diagnostics, Indianapolis, IN). The extraction was performed at 4 °C for 4 h with rotation. Supernatants were centrifuged at 2500 rpm for 10 min at 4 °C, dialyzed for 2 days in water (12,000–14,000 MW cutoff) and stored at 4 °C. To prove the efficiency of the extraction, the cartilage tissue underwent a second extraction after the supernatants were removed. These extracts were also analyzed by Western blotting and ELISA.

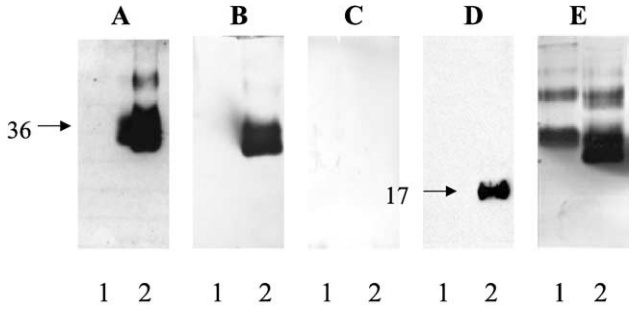

Fig. 1. Specificity of different anti-OP-1 antibodies as detected by Western blotting. (A) Anti-mature OP-1 antibody sc-9305 (Santa Cruz Biotechnology); (B) anti-mature OP-1 antibody 1B12 (Stryker Biotech); (C) anti-pro-OP-1 antibody R2854 (Stryker Biotech); (D) anti-mature OP-1 antibody MAB354 (R&D Systems); (E) antibody that recognizes both BMP-6 and BMP-7. Lane 1, recombinant BMP-6; Lane 2, recombinant BMP-7. A, B, C, and E—proteins were loaded under non-reduced conditions; D—proteins were reduced with 10% β-mercaptoethanol. Immunoreactive band at 36 kDa corresponds to mature OP-1 dimer; band at 17 kDa corresponds to mature OP-1 monomer; bands above 36 kDa correspond to the aggregates of the recombinant proteins. These data prove the specificity of the antibodies toward OP-1 protein.

2.5. SDS-PAGE and Western blot analysis

2.5 mg dry weight of each sample (lyophilized) was solubilized in a buffer containing 10 mM Tris, pH 6.5, 1% SDS, 10% glycerol, 0.016% bromphenol blue. Samples were loaded onto SDS-PAGE gel under reduced (with 2-mercaptoethanol) or non-reduced conditions. Western blots were performed following 12% SDS-PAGE gels. To reduce non-specific binding, blots were incubated with blocking solution containing 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.05% Tween 20 (TBS/Tween) (Bio-Rad) for 1 h at room temperature. The blots were incubated with primary antibody at suggested dilutions in TBS/Tween: 1:250 for R2854 and MAB354 anti-OP-1 antibodies, and 1:100 for sc-9305 antibody. As a secondary antibody, either ImmunoPure goat anti-mouse IgG (Pierce, Rockford, IL) or donkey anti-rabbit IgG (Pierce) conjugated with horseradish peroxidase at 1:10,000 dilutions in TBS/Tween was used. The blots were developed with the Enhanced Chemiluminescent (ECL-Plus) kit for Western blotting (Amersham Pharmacia Biotech, England). Specificity of the binding was compared with the binding of the antibodies to recombinant pro- or mature OP-1. Secondary antibodies were also tested for non-specific binding. The densities of specific immunoreactive bands was scanned by Fluor-S MultiImager (Bio-Rad) and quantified by Quantity One Software program (Bio-Rad).

2.6. Chemiluminescent sandwich ELISA

For sandwich ELISA, two anti-OP-1 antibodies, polyclonal sc-9305 and monoclonal 1B12, were used. Polyclonal anti-OP-1 antibody was utilized as a coating antibody. Plates were coated with 50 ng/well of this antibody in TBS, pH 7.5, and incubated overnight at 4 °C. Non-specific binding was blocked with 200 μl/well blocking solution (5% non fat dry milk in TBS/Tween, pH 7.5) for 2 h at room temperature. To generate a standard curve, mature recombinant OP-1 was diluted in TBS/Tween at different concentrations ranging from 10 to 0.01 ng/ml. Either OP-1 standard (100 μl) or cartilage extract was added to the plate and incubated for 1 h at room temperature. Second anti-OP-1 antibody (1B12) was applied at 1:1000 dilution in TBS/Tween and incubated at room temperature for 1 h. ImmunoPure goat anti-mouse IgG peroxidase conjugated antibody (Pierce) was used at 1:10,000 dilution as a secondary antibody. The reaction was developed with Supersignal ELISA Femto Maximum Sensitivity Substrate (Pierce).

The data were obtained through chemiluminescent ELISA plate reader Victor2 (Wallac) as relative light units (RLU).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted directly from cartilage tissue with acid-guanidinium thiocyanate as previously described [36]. Specific primer pairs were constructed for OP-1 [31] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). These primer pairs were designed to yield PCR products of different sizes (319 base pairs [bp] for GAPDH and 313 bp for OP-1). OP-1 primers used for nested RT-PCR: (a) 21-mer, antisense, location 1810–1830, 5′-TTTTCCTTTCGCACAGACACC-3′; (b) 20-mer, sense, location 1328–1347, 5′-TGCCATCTCCGTCCTCTACT-3′; and (c) 23-mer, sense, location 1518–1540, 5′-TTCCCCTCCCTATCCCCAACTTT-3′. The specificity of the primers was previously proven [31]. GAPDH primers were: sense, 5′-GGTATCGTGGAAGGACTCAT-3′ and antisense, 5′-ACCACCTGGTGCTCAGTGTA-3′. Approximately 1 μg of total RNA was trans- cribed using reverse transcriptase as described by Cs-Szabo et al. [36]. Five microliters of the resulting cDNA was am-plified by PCR using *Taq* DNA polymerase (Promega, Madi-son, WI) in the presence of specific upstream and down-stream primers [15 pM each; primers (a) and (b) for OP-1 gene]. 0.5 microliters of the first amplification product and a second sense primer [primer (c); nested primer] was used for a subsequent amplification step. To perform RT-PCR at the optimal conditions and to stay within the logarithmically linear product formation, 30 cycles were chosen (45 s at 95 °C, 30 s at 57 °C of annealing temperature and 45 s at 72 °C for the primers used), followed by the final extension for 5 min at 72 °C. PCR products were separated in 3% Metaphor agarose gels (FMC BioProducts, Rockland, ME) and visualized by ethidium bromide staining. Densities of the bands were measured using Fluor S MultiImager (Bio-Rad) with attached Quantity one software program (Bio-Rad). The densities of the OP-1 bands were normalized to the densities of the GAPDH bands to control variability among samples.

2.8. Statistical analysis

All results shown are mean ± S.D. of at least three separate experiments, with triplicate determination for each point. Covariation significance was determined by Pearson correlation of normal data.

3. Results

3.1. Antibody specificity

For the current study, we have used either commercially available antibodies or antibodies produced by Stryker Biotech. Although the specificity of these antibodies was previously proven, we have tested their cross-reactivity to the closely related member of the BMP family, BMP-6, of which the recombinant form became recently available. As a positive control, human recombinant processed (mature) OP-1 dimer was used (molecular weight is 36 kDa for dimer and 17 kDa for the reduced monomer). It is important to mention that with storage, recombinant OP-1 might form higher molecular weight aggregates that could be detected by the antibodies as a higher molecular weight band. In Fig. 1A–C, and E, BMP-6 and -7 were loaded onto the gels under non-reduced conditions; while in Fig. 1D, these proteins were reduced with 10% β-mercaptoethanol. As shown in Fig. 1, anti-mature OP-1 antibodies sc-9305 (Fig. 1A), 1B12 (Fig. 1B) and MAB354 (Fig. 1D) clearly recognized mature form of OP-1, both a dimer (36 kDa) and a monomer (17 kDa); none of the antibodies cross-reacted to BMP-6. Anti-pro-OP-1 antibody R2854 (Fig. 1C) showed no specific binding neither to BMP-6, nor to mature OP-1. As we showed previously [31], this antibody reacts only with pro-domain of OP-1. As a control, antibody that cross-reacts with both BMPs, BMP-6 and BMP-7, is shown in Fig. 1E.

3.2. OP-1 ELISA method

To quantify antigenic endogenous OP-1 present in tissue extracts and culture media, a sandwich ELISA method has been developed. Several parameters have been tested: different combinations of anti-OP-1 antibodies, effect of pH, various extraction buffers, enzyme digestions, normalization to the protein content or dry weight, etc. We found that the highest sensitivity of the method was achieved when the combination of polyclonal peptide antibody sc-9305 (coating antibody) and monoclonal 1B12 antibody was used. Standard curves were originated by utilizing human recombinant mature OP-1 at different dilutions ranging from 0.01 to 10 ng/ml (Fig. 2). The conformation of OP-1 epitope and therefore the sensitivity of the method depend also on the pH of the buffer used for ELISA. A wide range of pH values for TBS/Tween buffer was tested (Fig. 3). Since the extraction buffer has a neutral pH and at neutral pH the ELISA readings were in the middle range, this pH was chosen as a standard for OP-1 ELISA. To address the question of whether cartilage extracts have to be dialyzed before the assessment by ELISA, we have tested the effect of two extraction buffers (1 M GuHCl and lysis buffer) on ELISA readings. As shown in Fig. 4A, the presence of 1 M GuHCl buffer inhibited the binding of OP-1 by 50% or more, while the lysis buffer did not affect the ELISA results (Fig. 4B). However, when the same cartilage specimens were extracted with both buffers and then analyzed by ELISA, we found that more antigenic OP-1 could be extracted from cartilage tissue with GuHCl buffer than with lysis buffer (Table 1). To overcome this problem, GuHCl buffer was selected as an extraction buffer, and all samples had to be dialyzed before further analysis.

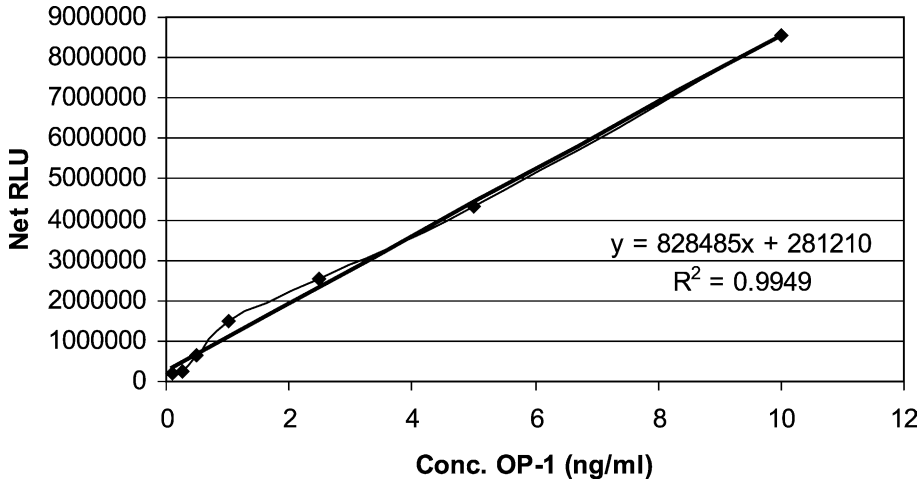

Fig. 2. Typical standard curve with human recombinant OP-1 generated by chemiluminescent sandwich ELISA method.

To standardize the ELISA method and to address the possible decline in cell numbers and depletion of matrix molecules that influence cartilage extractability with aging, before extraction, 500 mg of tissue (wet weight) was always lyophilized (to avoid the influence of matrix), pulverized in liquid nitrogen and extracted with 1 M GuHCl in a ratio of 150 mg (dry weight) of tissue per 3.5 ml of buffer. A repeated extraction of the remaining tissue with 4 M GuHCl buffer showed no extractable OP-1 left as detected by ELISA. To confirm quantitatively that 1 M GuHCl buffer extracts the most of the antigenic OP-1, aliquots of the same cartilage sample were extracted with a variety of extraction buffers including: (1) 1 M GuHCl, 0.005 M EDTA, 0.05 M NaCl; (2) 50 mM Tris, 1 M GuHCl; (3) 50 mM Tris, 4 M GuHCl; (4) 50 mM Tris, 20 mM Na_2HPO_4; (5) 50 mM Tris, 1% SDS; (6) 50 mM Tris, 0.15 M β-mercaptoethanol; (7) 50 mM Tris, 0.1 M NaCl, 8 M urea; and (8) 50 mM Tris, 1 M NaCl, 8 M urea. The choice of the dissociative/extraction agents was based on the specifics of the extraction of BMPs and the ability of BMPs to bind the components of cartilage extracellular matrix [19]. All these extracts were quantitatively analyzed by ELISA. 1 M GuHCl buffer was found to be the most appropriate for OP-1 extraction from human adult articular cartilage.

While seeking the most sensitive ELISA conditions, different enzymatic treatments of cartilage extracts were performed. Cartilage extracts were treated for 1 h at 37 °C with hyaluronidase (1 mg/ml), collagenase (5 mg/ml), chondroitinase ABC (0.1 U/ml), keratanase (0.1 U/ml), keratanase II (0.001 U/ml) and combinations of these enzymes and analyzed by ELISA. The values in the treatment groups were compared to control groups (no prior digestion) (Table 1). We found no significant differences between the groups in ELISA readings. Therefore, enzymatic digestion is not included in the standard protocol for ELISA. The high sensitivity of this method was achieved by utilizing Chemiluminescent Supersignal ELISA Femto Maximum Sensitivity Substrate (Pierce).

3.3. Cartilage ELISA results

Extracts from all cartilage samples were analyzed with the sandwich ELISA method described above. We found that with increasing age, the content of endogenous OP-1 protein significantly declined ($p < 0.02$; Fig. 5). Age of the donors and the levels of OP-1 protein showed a significant covariation (Pearson correlation, $p < 0.02$). Although cartilage samples were obtained from organ donors with no history of joint disease, not all of them appeared to be normal. It is noteworthy, that a decrease in the levels of OP-1 protein with age was found not only in the group that

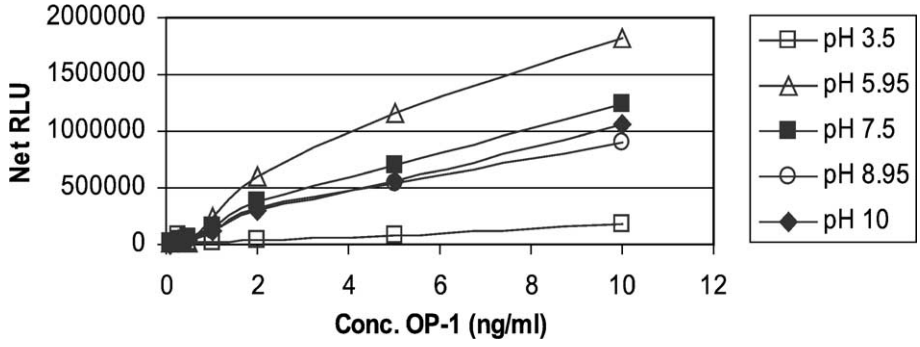

Fig. 3. Effect of pH of the TBS/Tween buffer on a standard curve generated by OP-1 sandwich ELISA.

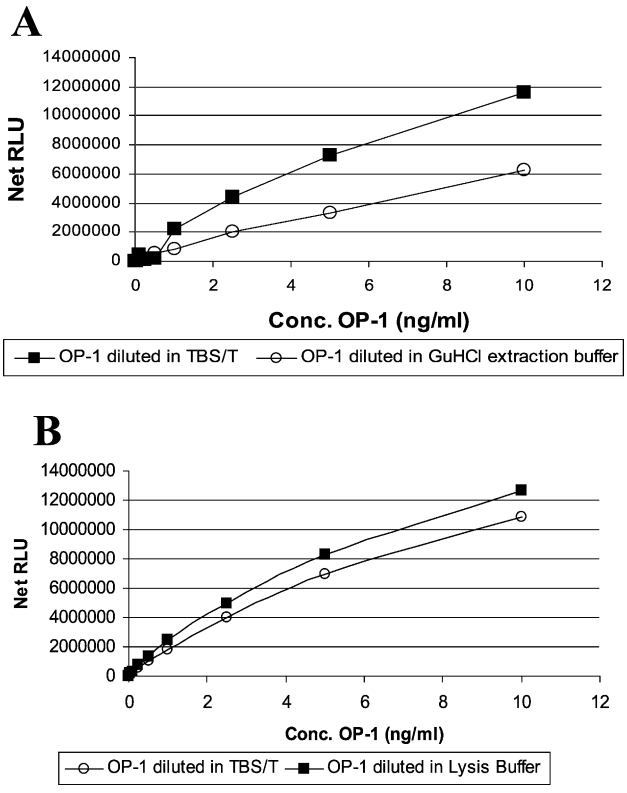

Fig. 4. Effect of 1 M GuHCl extraction buffer (A) and lysis extraction buffer (B) on a standard curve generated by OP-1 ELISA. Closed squares, human recombinant OP-1 diluted in TBS/Tween buffer; opened circles, human recombinant OP-1 diluted in lysis buffer.

consisted of normal tissues and tissues exhibiting early degenerative changes, but also in the subgroup of normal cartilages only. When specimens with normal histomorphological appearance were secluded in a separate subgroup and analyzed for the content of OP-1 protein, the same statistical differences were detected (open circles for normal cartilages only, Fig. 5).

3.4. Western Blot analysis

For Western blotting, representative samples were taken from each age decade and analyzed with anti-mature OP-1 antibody. The gels were scanned and the densities of the immunoreactive bands were measured. Two major OP-1

Table 1
Effect of enzymatic digestion on the content of endogenous OP-1 in extracts from the same cartilage specimen as detected by ELISA (mean ± S.D.)

Type of treatment	ELISA (ng/ml)
1 M GuHCl buffer only	0.03 ± 0.0027
1 M GuHCl buffer ± CHase, Ker, Ker II	0.027 ± 0.0019
1 M GuHCl buffer ± CHase, Collagenase	0.022 ± 0.0024
1 M GuHCl buffer ± Hyaluronidase	0.025 ± 0.0027
1 M GuHCl buffer ± Collagenase	0.0181 ± 0.0011
Lysis buffer	0.017 ± 0.0013
Lysis buffer ± CHase, Collagenase	0.017 ± 0.0009

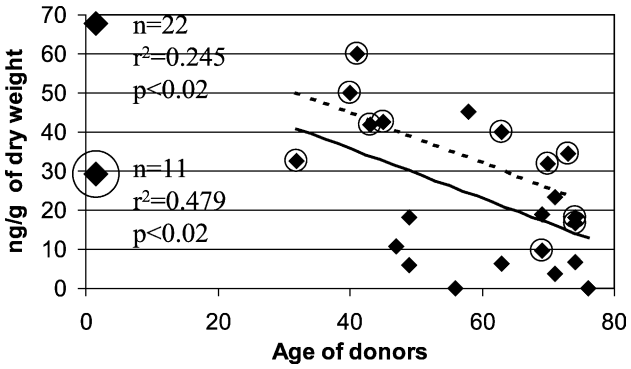

Fig. 5. Concentration of endogenous OP-1 protein detected by sandwich ELISA method in extracts from all tested human adult articular cartilages (solid squares) or in extracts from normal cartilages only (open circles).

bands were present in all tested cartilages regardless of the age of donors (Fig. 6A). These bands represented fully processed mature OP-1 form (molecular weight is 36 kDa) and the partially processed intermediate form of the OP-1 protein (molecular weight is 75 kDa). Semi-quantitative densitometric analysis of each of the bands demonstrated a decrease in the intensity of these bands with age, which

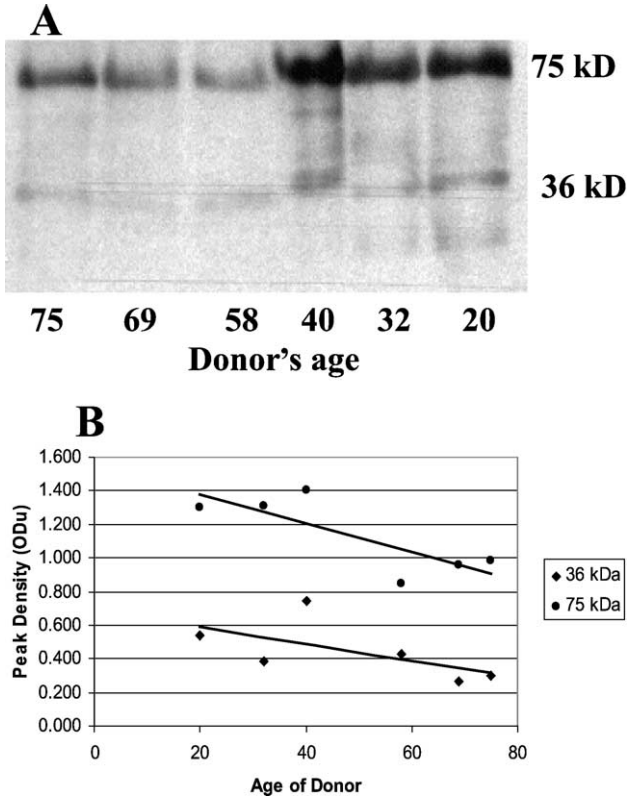

Fig. 6. Changes in OP-1 protein with age detected by Western blotting. (A) Representative Western blot with anti-mature OP-1 antibody of cartilage extracts from each age decade. Band at 75 kDa indicates a hemidimer form of OP-1 that consists of OP-1 mature dimer and one attached pro-domain [35]; band at 36 kDa corresponds to the mature OP-1 dimer. (B) Semi-quantitative image analyses of the Western blot described in A. Trendlines indicate the changes in the intensity of the major OP-1 immunoreactive bands.

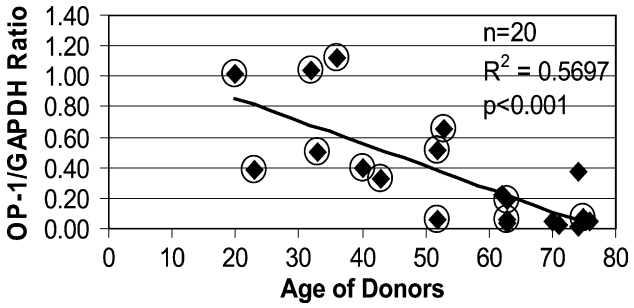

Fig. 7. Graphical illustration of the changes in OP-1 mRNA expression with aging of human adult articular cartilage. Semi-quantitative analysis of the PCR bands. The levels of OP-1 message normalized to the levels of GAPDH message. Solid squares represent all tested cartilage samples; open circles represent a subgroup of normal cartilages.

correlated to ELISA findings (Fig. 6B). Western blot results were normalized to tissue dry weight, as were ELISA data.

3.5. RT-PCR results

For PCR analysis, total RNA was extracted directly from cartilage tissue without previous chondrocyte isolation or culture. Aliquots of the same mRNA were used for nested RT-PCR with OP-1 and GAPDH specific primer sets. Both PCR products were amplified with the same number of cycles. GAPDH was chosen as the normalization factor, since it is a housekeeping gene. We found that with aging levels of GAPDH mRNA expression in normal cartilage did not vary for more than 10%. As it was identified on the protein level, OP-1 mRNA expression in articular cartilage decreased with the increasing age of donors (Fig. 7; $p < 0.001$).

4. Discussion

This study confirms an age-related decrease in the anabolic activity of human adult articular cartilage. The anabolic activity of cartilage was evaluated via the assessment of changes in endogenous OP-1, a BMP that is considered to be one of the most potent inducers of cartilage regeneration and repair. We observed greater than a 4-fold decline in OP-1 protein content between ages of 35 and 75 years. The data showed that there was a greater decline in OP-1 mRNA expression, where in some cartilages from older individuals, OP-1 message was barely detectable or was below the detection limit. Another important result of our study is that the concentration of endogenous OP-1 protein in normal articular cartilage is within the range of biological activity of OP-1 (50–100 ng/ml) in vitro, as we have shown previously [17,26–28].

One of the main difficulties in studying human tissue has been to distinguish between non-pathologically (normal) and pathologically progressive (degenerative) aging of articular cartilage. The uniqueness of the present study lies in the examination of age-related changes in human adult articular cartilage obtained from normal individuals through the collaboration with the Regional Organ Bank of Illinois in Chicago. For this study, cartilage from organ donors with no documented history of joint disease was selected. Although tissue was collected from normal individuals (not from patients), not all of the cartilages appeared normal. Some of them displayed degenerative changes described in detail in Chubinskaya et al. [37]. Therefore, tissue selected for the current study was divided into two subgroups. One group included the entire spectrum of cartilages, and one group consisted only of normal cartilages (normal histomorphological appearance). Critically, statistical changes in endogenous OP-1 with age were observed in both groups, suggesting that the decline in anabolic activity of human chondrocytes is associated not only with degenerative changes, but is truly an age-related process.

The decrease in endogenous OP-1 with aging of human articular cartilage is consistent with previous findings on changes in the levels of other growth factors and their receptors, for example, a decrease in mRNA expression of TGF-β1, 2 and 3 in aging equine articular cartilage [6] and increase in EGF synthesis and a decrease in the EGF-receptor mRNA by rat articular chondrocytes [4]. However, our study is the first to show an age-related decrease in endogenous OP-1 in human adult articular cartilage. Also, in the current study, we evaluated changes in both levels, message and protein, and found that there was a decrease in OP-1 mRNA and protein expression with age.

Even though it is apparent that elderly people have decreased levels of OP-1, it still remains unclear whether this phenomenon is a result of aging per se or is due to other age-related variables. The decrease in OP-1 protein in part could be due to the suppression of mRNA expression (as we demonstrated in this study), but also due to many other processes including but not limiting a general slower metabolic activity of older cells, increased degradation of OP-1 due to an activation of catabolic events in cartilage, increase in OP-1 binding proteins, changes in OP-1 receptors or other members of the BMP signaling pathway, etc. Although degradation of OP-1 protein could be one of the reasons in the decline of OP-1, we have not noticed an accumulation of the degradation products in cartilage matrix. As a consequence of aging, catabolic events could be induced and different proteinases present in cartilage could be activated leading to a degradation of a number of matrix proteins including mature OP-1. Also mature OP-1 might undergo autodegradation and might be degraded by serine proteinases.

The results obtained by Western blot analysis suggest that the predominant form of OP-1 in cartilage is the intermediate form (75 kDa), although some OP-1 is also present in the mature (active) form (36 kDa). Since 75 kDa is a partially processed form of OP-1 that contains in its structure one bound pro-domain and one non-covalently associated pro-domain in addition to two mature domains [35], we do not

expect this form to be biologically active. By analogy to TGF-β, pro-domain could function as a latency-associated protein that prevents binding of mature BMP molecule to its receptors [39]. However, the function of pro-domain is well described only for TGF-β, and it remains unclear whether pro-domain in other BMPs plays a similar role in preventing the biological activity of these molecules.

The effect of binding proteins on endogenous OP-1 pathway in cartilage is largely unknown. If there is an increase in BMP binding proteins with age as it is documented for IGF-1 binding proteins [38], the decline in OP-1 with age could be attributed by this phenomenon. The exploration of OP-1 binding proteins in human adult articular cartilage and their effect on different cartilage matrix molecules might shed the light on the entire OP-1/BMP pathway role in aging and disease. As it was shown by Martin et al. [38], an elevation of IGF binding proteins in rat articular chondrocytes was one of the reasons for the suppression of the synthetic activity of cells with age in response to IGF-1. Also our preliminary experiments indicate that BMP binding proteins added to cultures of human articular chondrocytes suppress OP-1 and lead to a decline in PG synthesis (unpublished data).

The results obtained in the present study confirm an age-related decline in endogenous OP-1, one of the most potent naturally occurring agents produced by chondrocytes that could counteract cartilage destruction and/or facilitate its repair. These data prompted us to develop a hypothesis that a decrease in endogenous OP-1 metabolism with aging may lead to an elevated susceptibility of cells to catabolic processes thus contributing/promoting cartilage degeneration. Further work is needed to confirm this hypothesis and to understand the function of endogenous OP-1 in cartilage. The availability of quantitative and qualitative methods described here will provide necessary tools for answering these and other questions and could be useful in monitoring therapies and/or disease prognosis. The results of this study suggest future means of using OP-1 to stimulate normal phenotypic activity in articular chondrocytes and maintain/restore function in affected joints.

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