Novel Fluorescent Compound (DDP) in Calf, Rabbit, and Human Articular Cartilage and Synovial Fluid

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ABSTRACT. ***Objective.*** To investigate the presence of and quantify 2,6-dimethyldifuro-8-pyrone (DDP), a novel fluorescent compound identified as in various calf, rabbit, and human tissue/fluid samples, to determine the DDP level in articular cartilage (AC) laminae, and to investigate the changes in cartilage DDP content with cartilage maturation.

Methods. Samples were obtained from calf (< 2 years), rabbit (< 2 weeks to 2 years) or human AC and synovial fluid (SF) as well as other non-cartilaginous tissues. SF and tissue samples were hydrolyzed with 6 M HCl (24 hours at 110º C), lyophilized, and dissolved in HPLC mobile phase. DDP and collagen crosslink peaks were measured using a fluorescence detector at excitation and emission wavelengths of 295 and 395 nm, respectively.

Results. DDP was detected from calf metacarpophalangeal joint AC (362 ± 48 pmol/mg dry weight), SF (4.5 ± 0.3 pmol/µl SF), and intervertebral disc (24 ± 4 pmol/mg). DDP was not detected in calf ligament, tendon, bone, ocular lens, cornea, or elastic cartilage. The DDP amount was greater in mid-deep cartilage lamina (448 ± 63 pmol/mg) than superficial-mid lamina (129 ± 52 pmol/mg) ($p = 0.008$). DDP level decreased with maturation in rabbit knee joint AC from 185 ± 40 (< 2 weeks) to 27 ± 3 (2 years) pmol/mg dry weight. DDP was not detected in adult rabbit ligament, tendon, meniscus, or bone. DDP was detected in human knee joint AC and SF. The DDP level in osteoarthritic lesions was present in lower concentrations (range: 0 to 96 pmol/mg dried AC) compared to intact AC (range: 63 to 236 pmol/mg) of the same knee.

Conclusion. DDP is a hyaline cartilage specific compound present in all articular cartilage samples from various articulating joints/animal species. DDP level increases with AC depth and decreases with cartilage maturation. DDP is a potential indicator of cartilage metabolism during normal growth, ageing, and cartilage disease. (J Rheumatol 2002;29:154-60)

Key Indexing Terms:
ARTICULAR CARTILAGE
SYNOVIAL FLUID
BIOCHEMICAL MARKER
FLUORESCENT COMPOUND

Tissue autofluorescence, the fluorescence of endogenous molecules, is dependent upon the tissue biochemical composition and morphological architecture, both of which undergo changes during maturation, ageing[1-4], and manifestation or progression of disease(s)[5-9]. Fluorophores are produced in biological tissues either by enzymatic (e.g., collagen pyridinium crosslinks) or non-enzymatic spontaneous chemical modifications of proteins (e.g., products of glycation or oxidation)[10-12].

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There are 2 known fluorescent molecules in articular cartilage (AC), namely collagen pyridinium crosslinks (pyridinoline and deoxypyridinoline) and pentosidine. Pyridinoline (Pyd), derived from 2 hydroxylysine aldehydes and a lysine residue[13,14], was first isolated from rat tail tendon and characterized in the mid 1970s by Fujimoto, *et al*[15,16]. Pyd is also present in other collagen-containing tissues such as synovium, meniscus, bone, and ligament. It is far more abundant in AC than in bone[17] and its concentration remains relatively constant in AC with age[18-20]. Besides Pyd, deoxypyridinoline (Dpyd) is abundant in bone and dentine. Dpyd is also detected in AC however, at levels much lower than Pyd. Pyd and Dpyd have been used extensively as reliable biochemical markers for the degradation of collagens. Both clinical and animal studies have shown urinary Pyd to be a marker of bone and cartilage breakdown in joints and Dpyd as a specific marker of bone loss[21].

Pentosidine, a condensation product of arginine, lysine, and ribose, is an end product of advanced glycation[22]. Pentosidine was isolated from human dura mater and characterized by Sell and Monnier in 1989[23]. Using fluorometry and high pressure liquid chromatography (HPLC) of hydrolysates, quantitation of pentosidine in various collagen-rich tissues

such as dura mater, skin, and ocular lens has shown that the amount of pentosidine increases exponentially with age[24-27]. Recently, pentosidine was detected in AC, tracheal cartilage, and body fluids of patients with bone and joint disorders[28-41]. Extremely high levels of pentosidine have been detected in the skin and ocular lens as well as in the plasma and urine of patients with diabetes and uremia[32].

The original focus of this research was to investigate the changes in collagen architecture and biochemical content of cartilage, in particular the collagen Pyd and Dpyd levels, with respect to maturation, ageing, and the progression of osteoarthritis (OA). While establishing the conventional procedure[20] to quantify collagen crosslinks in our laboratory, the HPLC chromatograph of AC also revealed a fluorescent peak in addition to the crosslinks. This peak eluted approximately 12 to 14 minutes after the known pyridinium peaks (elution time at 8 to 10 minutes). Interestingly, this peak was absent from non-cartilaginous human and animal tissues examined such as ligament, bone, tendon, meniscus, skin, stomach, liver, kidney, lung, heart, muscle, etc. An extensive literature search did not reveal any documentation of this peak. We therefore investigated the chemical composition and atomic connectivity of this novel compound[30]. Nuclear magnetic resonance spectroscopy, mass spectrometry, and single crystal x-ray diffraction determined the chemical structure of this peak now termed DDP after its IUPAC nomenclature, 2,6-dimethyldifuro-8-pyrone. DDP is a 204 Da, symmetrical aromatic compound with molecular formula $C_{11}H_8O_4$. In view of its articular cartilage specific nature, DDP could be a useful indication of AC metabolism associated with normal growth and ageing, and in diseases involving AC matrix degradation such as OA, rheumatoid arthritis (RA), chondromalacia patella, or osteochondritis. Although several markers are currently used to evaluate the presence of articular cartilage disease, only a few markers specify the stage (severity level) of the disease.

The purpose of this study was to determine the validity of DDP as an AC specific molecule by investigating the presence of and quantifying DDP in various tissues obtained from calf, rabbit, and humans. Further, we aimed to investigate the anatomic abundance of DDP in AC zones (superficial versus deep) and the changes in cartilage DDP content with maturation in rabbit knee joint.

MATERIALS AND METHODS

Tissue samples. Calf (age < 2 years) tissue/fluid samples were obtained from the abattoir (Ryding Regency, Toronto, Canada). Fully encapsulated metacarpophalangeal (MCP) joints were dissected within 24 hours of death; from each joint the synovial fluid (SF) was aspirated and AC was excised. In some joints, AC was excised separately from superficial-middle or middle-deep zone. This was achieved by manually dissecting very thin superficial slices and extending up to the middle lamina (i.e., from articulating surface approximately 35 to 40% of the entire cartilage thickness). The remaining exposed AC was harvested up to but not including the calcified cartilage and kept separately as cartilage from middle and deep laminae. White New Zealand male rabbit (age < 2 weeks to 2 years) tissue samples were obtained from the animals sacrificed for other studies. The right and left rabbit knee joints provided the source for AC. Human cartilage and tissue/SF samples were obtained from autopsy or knee joint transplants. Human SF samples were also collected from knee joint effusion of patients with knee joint arthropathy. Human plasma, serum, and urine samples were collected from volunteers.

The tissue/fluid samples from various sources were stored at –70°C until the day of experiment. Sea lamprey cartilage was also examined to determine whether DDP is present in primitive cartilage.

Reagents. The Hewlett Packard HPLC system consisted of 2 pumps (model 1050) and an automatic injector (series 1100). The fluorescence peak was examined using a Perkin Elmer fluorescent detector (model LC 240). Chromatographic separations were made on a C18 column (Phenomenex Spherisorb) with dimensions 250 × 4.6 mm, pore size 5 µm, and optical density 2. The following solvents were used: hydrochloric acid (Sigma, St. Louis, MO, USA), HPLC grade water (Omnisolve, Fisher Scientific, Ottawa, ON, Canada), and HPLC grade acetonitrile (Caledon Laboratories, Georgetown, ON, Canada). The ion-pairing agent used was heptafluorobutyric acid (HFBA) (Sigma). The Pyd and Dpyd standards were purchased from Metra Biosystems.

DDP calibrator. All sample peaks were quantified using the DDP calibrator prepared in house. A DDP calibrator was prepared from 16.62 gm dried calf MCP AC using the isolation and purification protocol outlined by Gahunia, *et al*[33]. For analytical purposes, the Metra Biosystems (Mountain View, CA, USA) Pyd and Dpyd crosslink calibrator and in-house DDP calibrator were combined to give one external calibrator. The values of DDP are expressed as pmol per mg dried tissue sample.

Measurements of DDP in tissue/fluid samples. The tissue samples were lyophilized, weighed, and then hydrolyzed with 1000 µl of 6 M HCl for 24 hours at 110°C. To process the SF, plasma, urine, serum, and vitreous humor samples, 1000 µl of tissue fluid was hydrolyzed with equal volume of 6 M HCl for 24 hours at 110°C. The hydrolyzed tissue or fluid samples were lyophilized, dissolved in 1000 µl of mobile phase (24% acetonitrile, 1% HFBA and 75% deionized water), briefly vortexed, and then filtered with 0.45 µm-pore filter (Gelman, Montreal, PQ, Canada). The DDP content was determined by injecting an aliquot of 30 µl hydrolysate onto the analytical column using reverse phase HPLC system that was protected by a guard cartridge. The flow rate was 1.0 ml/minute. DDP and pyridinium fluorescence peaks were monitored using a fluorescence detector at excitation λ = 295 nm and emission λ = 395 nm.

Statistical analysis. The results are presented as average ± standard error of mean (SEM). The statistical significance of the difference was determined using paired t-test between the groups. Values of p less than 0.05 were considered significant.

RESULTS

DDP calibrator. A total of 0.3 mg of DDP was purified, giving a yield of 18 µg DDP/gm dried weight AC. The molar extinction coefficient of DDP in 50% methanol was 4700 $Au \cdot M^{-1} \cdot cm^{-1}$, compared to 5700 $Au \cdot M^{-1} \cdot cm^{-1}$ for Pyd and 5000 $Au \cdot M^{-1} \cdot cm^{-1}$ for Dpyd. The peak elution times of Pyd, Dpyd, and DDP were 7.5, 8.5, and 20 min, respectively (Figure 1).

Bovine tissue. DDP was detected in calf MCP joint AC (362 ± 48 pmol/mg dry weight) and intervertebral disk (24 ± 4 pmol/mg dry weight tissue). A sample from the nasal cartilage that is rich with proteoglycan showed a high amount of DDP (1035 pmol/mg dry weight). DDP was not detected in calf ligament, tendon, bone, ocular lens, cornea, and elastic cartilage (Table 1). A significant difference (paired t-test, p = 0.008) in DDP level was noted in the AC superficial-middle zone (129 ± 52 pmol/mg dry weight) and middle-deep zone (448 ± 63

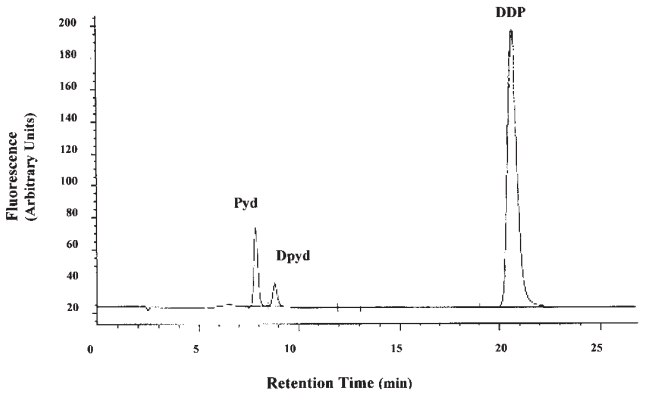

Figure 1. A typical chromatograph of the calibrator used for the quantitative analysis of pyridinoline (Pyd), deoxypyridinoline (Dpyd) and 2,6-dimethyld-ifuro-8-pyrone (DDP). The Pyd, Dpyd, and DDP peaks eluted at 7.5, 8.5, and 20 minutes, respectively. The DDP was isolated and purified from calf metacarpophalangeal articular cartilage.

Table 1. DDP detection in calf tissue samples.

Tissues Examined (n)	DDP/Tissue pmol/mg (± SEM)	Range pmol/ mg
Cartilage entire thickness (3)	362 (± 48)	300 to 456
Cartilage mid-deep zone (4)	448 (± 63)	331 to 626
Cartilage sup-mid zone (4)	129 (± 52)	0 to 247
Intravertebral disk (4)	24 (± 4)	16 to 29
Cornea (10)	ND	—
Ocular Lens (3)	ND	—
Ligament (10)	ND	—
Bone (5)	ND	—
Tendon (10)	ND	—
Nasal cartilage-bridge (1)	1035	—
Nasal cartilage periphery (3)	ND	—
Elastic cartilage (ear) (3)	ND	—

Data are expressed as means ± standard error of mean (SEM).
ND = Not detected.

pmol/mg dry weight) as well as superficial-middle zone and entire cartilage thickness (362 ± 48 pmol/mg dry weight) ($p = 0.02$). There was no significant difference ($p = 0.36$) between middle-deep zone and entire cartilage thickness.

DDP was detected in 11 samples of SF (4.5 ± 0.3 pmol/µl SF; range 0.86 to 8.6 pmol/µl SF). A typical chromatograph of calf SF is shown in Figure 2. DDP was not detected in vitreous humor fluid (n = 9).

Rabbit samples. Representative chromatographs of young (< 2 weeks) and adult (2 years) White New Zealand rabbit knee joint AC are shown in Figures 3A and 3B, respectively. Typically, 6 to 17-fold decrease in the DDP content of young versus adult rabbit AC was observed. In comparison, the Pyd content increased 3 to 6-fold between young and adult AC. The amount of DDP decreased with increasing age in rabbit AC from an average of 185 ± 40 pmol/mg dry weight AC (< 2 weeks) to 27 ± 3 (2 years) pmol/mg dry weight AC (Figure 4). DDP was not detected in adult rabbit ligament, tendon,

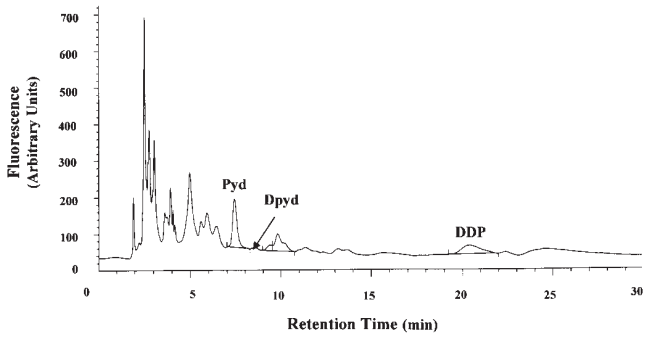

Figure 2. A typical chromatograph of the hydrolyzed synovial fluid obtained from calf metacarpophalangeal joint. The DDP peak eluted at 19.5 minutes. The Pyd and Dpyd peaks eluted at 7.3 and 8.4 minutes, respectively.

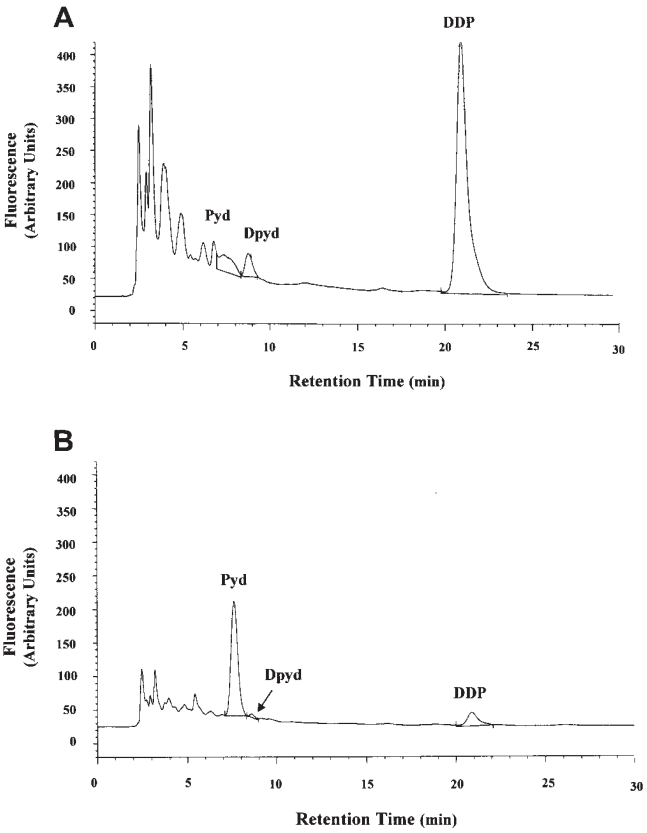

Figure 3. A chromatograph of the hydrolyzed articular cartilage (femoral condyle) of (A) a 7-day-old rabbit knee joint and (B) a 2-year-old rabbit knee joint. The DDP peak eluted at 20 minutes. The Pyd and Dpyd peaks eluted at 7.3 and 8.5 minutes, respectively.

meniscus, or bone (Table 2). However, a small amount of DDP was detected from the same tissues in young rabbits (≤ 1 kg).

Human samples. In adult human tissue samples, DDP was detected only in AC (Table 3). The amount of DDP present in adult AC (> 53 years old) ranged from 182 to 238 pmol of DDP/mg dried AC. DDP was also detected in the vertebral disk and trachea. DDP was not detected from adult non-cartilaginous tissue samples (ligament, tendon, meniscus, bone,

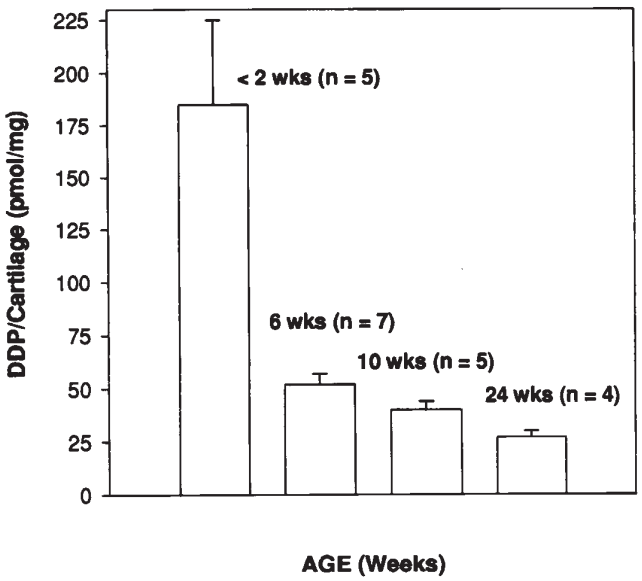

Figure 4. Bar graph showing the DDP level of rabbit knee joint articular cartilage at various ages. The data are given in pmol/mg and are expressed as means ± standard error of mean (SEM). Note the decrease in DDP level with age.

Table 2. DDP detection in rabbit tissue samples.

Rabbit tissue (Weight, kg)	Sample (n)	DDP/Tissue pmol/mg (± SEM)
Ligament (≤ 1)	2	6.9 (± 0.3)
Meniscus (≤ 1)	4	15.9 (± 1.8)
Bone (≤ 1)	1	3.65
Ligament (~4)	4	ND
Meniscus (~4)	3	ND
Bone (~4)	8	ND
Tendon (~4)	7	ND

Data are expressed as means ± standard error of mean (SEM).
ND = not detected.

esophagus, stomach, intestine, brain, heart, aorta, lungs, muscle, adipose tissue, liver, and kidney) as shown in Table 3. Investigation of OA knee joint showed lower DDP levels in cartilage with lesions compared to the intact cartilage of the same joint (Table 4).

DDP was not detected in serum (n = 10), plasma (n = 10), or urine (n = 10) samples. A summary of DDP content in the SF samples of patients with knee joint arthropathy is shown in Table 5. Out of 15 SF samples obtained from knee joint arthropathy patients or autopsy, DDP was detected in 10 SF samples and was either absent or below the detection limit in the remaining 5 SF samples. Preliminary data showed a higher level of DDP in SF of patients with knee joint disease (9 ± 2 pmol/µl of SF) compared to normal (< 3 pmol/µl of SF, n = 2). Further, investigation of tissue samples from a 3-day-old infant (autopsy) showed high concentrations of DDP in AC (525 pmol/mg dry weight AC) and sternum (295 pmol/mg dry weight AC).

Table 3. DDP detection in adult human tissue samples obtained from autopsy.

Tissue examined	Sample Size (n)	DDP/Tissue pmol/mg (± SEM)	Range pmol/mg
Adult articular cartilage	10	200 (± 16)	182 to 238
Vertebral disk	4	250 (± 87)	28 to 442
Trachea	5	75 (± 23)	13 to 131
Sternum	3	ND	—
Bone	10	ND	—
Tendon	3	ND	—
Meniscus	8	ND	—
Ligament	3	ND	—
Liver	5	ND	—
Pancreas	3	ND	—
Oesophagus	3	ND	—
Stomach	2	ND	—
Spleen	3	ND	—
Gall Bladder	2	ND	—
Brain	5	ND	—
Intestine	3	ND	—
Kidney	3	ND	—
Rectum	2	ND	—
Aorta	4	ND	—
Muscle	7	ND	—
Fat	4	ND	—
Prostate Gland	2	ND	—
Pituitary	1	ND	—
Lungs	3	ND	—
Skin	2	ND	—

Data are expressed as means ± standard error of mean (SEM).
ND = Not detected.

Table 4. DDP content of intact and lesioned femoral condyle articular cartilage. The samples were obtained from osteoarthritic knee joints (transplant or autopsy).

Case No.	Sex/Age (Years)	DDP/ AC Dry Weight (pmol/mg)	
		Intact Cartilage	Lesion Cartilage
1	F/54	236	17 (Sample 1), 43 (Sample 2)
2	M/71	157	122
3	F/55	68	15
4	M/71	154	0
5	F/45	231	96 (Sample1), 70 (Sample 2)
6	F/55	132	15

Lamprey samples. DDP was absent from the lamprey cartilage (n = 5), indicating that DDP is not detected in cartilage that lacks proteoglycans.

DISCUSSION

Successful isolation and purification of DDP has enabled the preparation of a calibrator for tissue quantification of DDP[33]. Investigators using a similar protocol (as outlined in this man-

Table 5. DDP content of synovial fluid samples from patients with knee joint arthropathy.

Case No.	Joint Disease	Sex/Age (Years)	CM-1/SF** (pmol/µl)
1	Rheumatoid arthritis	M/52	10.4
2	CPPD*	F/81	2.8
3	OA*** and CPPD*	F/81	10.3
4	Urate crystal	M/87	30.2
5	Urate crystal	M/75	4.7
6	Urate crystal	M/79	16.9
7	Cutaneous vasculitis	F/49	7.1
8	Rheumatoid arthritis	F/82	5.61

* Calcium pyrophosphate dihydrate crystals; ** Synovial Fluid; *** Osteoarthritis.

uscript) to determine collagen crosslinks in AC or SF did not observe the DDP peak because the conventional HPLC runs for crosslink analysis were 15 minutes; whereas the elution time of DDP is almost twice that of collagen crosslinks. Uchiyama, *et al*[34] studied fluorescent materials in AC, and despite characterizing 2 peaks of fluorescence excitation, they concluded from their (short) chromatographic analysis that there are only 2 types of molecules, pyridinoline, and pentosidine, contributing to the AC fluorescence.

DDP was detected in all AC and cartilage-containing tissues from different species as well as in small amounts in SF samples of bovine MCP and human knee joint. The absence of DDP from non-cartilagenous tissues (ligament, bone, tendon, meniscus etc.) examined in mature animals and humans indicates that DDP is an AC specific molecule. Examination of DDP concentration in cartilage zones showed that the amount of DDP per mg of dry weight AC is significantly higher in the middle-deep zone compared to the superficial-middle zone. Rabbit data showed that DDP level decreases in AC with maturation. The presence of DDP in tissues examined from young rabbits (≤ 6 weeks) suggests its potential as a marker for growth, development, and/or tissue differentiation. We have observed similar results in young versus old human AC as well as the relative abundance of DDP in deep versus superficial zone. Further, osteoarthritic knee joints revealed lower levels of DDP in lesioned AC compared to intact AC of the same joint.

The sea lamprey is a lower vertebrate with an entirely cartilaginous skeleton. The major connective tissue component of lamprey annular cartilage is lamprin, an insoluble non-collagen and non-elastin protein[35]. Cartilage structure of the sea lamprey is similar to elastic cartilage of higher vertebrates including auricle, epiglottic, and laryngeal cartilage[36]. The absence of DDP in lamprey cartilage and bovine elastic cartilage indicates that DDP is not associated with an elastic component of cartilage.

In the intervertebral disk, the bovine nucleus pulposus has galactosaminoglycan-rich and keratan-rich proteoglycan (PG) monomers and its nasal cartilage has chondroitin-sulphate-rich PG monomers. Stockwell[37] reported that the percentage of dry weight of PG and collagen in cartilage-containing tissues varies. For example, bovine nasal cartilage has 43% PG and only 35% collagen, whereas bovine articular cartilage has 14% PG and 72% collagen. Human epiphyseal cartilage has 15% PG and 37% collagen whereas human articular cartilage has 18% PG and 66% collagen. Further, Stockwell (1979) also reported that PG content increases with cartilage depth. Both observations (i.e., variation in macromolecular content of tissue source and increased proteoglycan content with articular cartilage depth) could explain the difference in DDP levels noted from bovine nasal cartilage versus AC as well as higher DDP level in the deep laminae compared to superficial laminae. In addition, the observation of lack of DDP in lamprey cartilage, which is devoid of proteoglycan, supports our finding that DDP is a product of acid hydrolysis of the carbohydrate moiety of AC.

Based on its chromatographic behavior, DDP (excitation at 306 nm and emission at 395 nm) is different from the fluorophores reported by Hormel, *et al*[38]. The collagen CNBr-peptide was measured at fluorescent profiles of 370 nm excitation and 440 nm emission. DDP is certainly different from glycated products such as pentosidine, which has fluorescence excitation and emission wavelengths optimal at 335 and 395 nm, respectively[3,34,39]. Furthermore, DDP occurs principally in AC, which makes it unlikely that it reflects the presence of pentosidine or conventional amino acids because these compounds also exist in non-cartilaginous tissues.

Biochemical markers are valuable tools in clinical medicine to study growth and development, detect latent disease, or monitor pre-existing disease and its treatment[40-44]. Identification of appropriate biological markers for disease activity in OA or other types of arthritis is a challenging and complex endeavor. A biochemical marker that reflects cartilage metabolism would be useful for assessing the stages of OA and in evaluating new therapeutic regimens. To date, several markers have been used in clinical and animal studies. However, only a few studies specify the stage (severity level) of the disease.

Pyd and Dpyd are products of collagen turnover from bone, cartilage, tendon, and ligament. Although the Pyd/Dpyd ratio is used as a marker to distinguish between destruction of cartilage and bone collagen, a recent report by Hein, *et al*[45] questioned the usefulness of Pyd/Dpyd ratio in urine. Using reverse phase HPLC they examined the levels of both Pyd and Dpyd in the urine and serum levels of 38 patients with RA. Since bone metabolizes at a higher rate than cartilage, crosslink levels from urine or serum samples reflect bone rather than cartilage metabolism. On the other hand, Odetti, *et al*[46] have shown that the total pentosidine concentration increased significantly with age. However, it remained constant in OA cartilage.

Two fluorophores have been previously characterized in

cartilage, pyridinoline, and pentosidine[18,19,47,48]. Although these compounds are accepted as candidate biomarkers for bone and connective tissue degradation, they are not unique to AC; therefore their measurement is not helpful in OA or RA. Interestingly, this study showed a cartilage specific molecule (DDP), which decreases in level with age and is detected in SF. Hence, DDP could reflect changes in cartilage metabolism during diseases involving cartilage degradation. Further, as an extention of this study, our preliminary investigations in cadaver knee joint cartilage show a decreased DDP level in OA cartilage with lesions compared to intact cartilage of OA joints. DDP may be useful in the evaluation of AC injury and repair in patients with OA. It is not yet known whether DDP or its *in vivo* precursor is necessary to maintain cartilage integrity. Nevertheless, due to its stability and specificity, DDP has potential as a biochemical marker in arthritic conditions.

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