

Age-related Changes in Human Bone Proteoglycan Structure

IMPACT OF OSTEOGENESIS IMPERFECTA*

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Proteoglycans (PGs) are a family of molecules that undergo extensive post-translational modifications that include addition of glycosaminoglycan (GAG) chains as well as *N*- and *O*-linked oligosaccharides to the protein core. PG composition and structure have been reported to alter with age. To test whether the post-translational modifications to PGs can serve as *in vitro* surrogate end point markers for chronological age, the extent of GAG modifications was determined for PGs derived from normal human bone cells of 14 donors (age range, fetal to 60 years). Isolated cells were steady state radiolabeled with ³⁵SO₄²⁻ and [³H]GlcN. For biglycan and decorin, iduronate content was linearly correlated with age (increased 1.5× between fetal and age 60 years). For the syndecan-like heparan sulfate PG, the *N*-sulfation of post-natal cells increased over 3.5-fold until reaching a plateau during the 4th decade of life. The amount of *O*-linked oligosaccharides was also found to decrease as a function of increasing normal donor age, whereas the specific activity of the metabolic precursor pool remained constant regardless of donor age. These age-related changes in post-translational modifications were then used to demonstrate that osteoblasts derived from patients with osteogenesis imperfecta did not exhibit facets of a pre-mature aging, but rather were arrested in a fetal-like phenotypic state. A growth matrix rich in thrombospondin altered PG metabolism in osteoblastic cells, resulting in the production and secretion of the fetal-like (rich in *O*-linked oligosaccharides) forms of decorin and biglycan. This effect was qualitatively different from the effect of transforming growth factor-β, which predominantly altered GAGs rather than *O*-linked oligosaccharides. No other Arg-Gly-Asp protein (fibronectin, vitronectin, type I collagen, osteopontin, and bone sialoprotein) showed any detectable effect on PG metabolism in bone cells. These results indicate that a proper matrix stoichiometry is critical for metabolism of PGs.

Osteogenesis imperfecta (OI)¹ is a heritable disease of the connective tissue characterized by bone fragility and skeletal

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¹ The abbreviations used are: OI, osteogenesis imperfecta; PG, pro-

teoglycan; BGN, biglycan; DCN, decorin; BSA, bovine serum albumin; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; TGF-β, transforming growth factor β; SAX, strong anion exchange; HPLC, high performance liquid chromatography; GAG, glycosaminoglycan; RGD, arginine-glycine-aspartate.

teoglycans (PGs) are a family of molecules that possess a core protein to which one or more glycosaminoglycan (GAG) chains are covalently attached (7). In addition, PG core proteins may also possess *N*- and *O*-linked oligosaccharides. Human bone cells in culture produce a large versican-like PG, a heparan sulfate syndecan-like PG, as well as biglycan (BGN) and decorin (DCN) (8). Osteoblast cells derived from OI patients were found to have reduced levels of collagen, osteonectin, large chondroitin sulfate PG, BGN, and DCN when compared with levels found in age-matched control bone cell cultures (9). The failure of OI osteoblast-like cells to parallel the normal donor age-dependent expression pattern of matrix genes was found to occur throughout long term culture (10). Osteoblast-like cell strains derived from OI patients had significantly reduced growth parameters (11). The reduction in maximal cell growth rates and levels of extracellular matrix components observed in OI-derived osteoblast-like cells, although abnormally low compared with age-matched controls, are equivalent to values observed in cells derived from elderly donors >60 years of age (12). Thus, OI could be exhibiting facets of normal aging and the observed phenotype of osteopenia and brittle bones would then share certain common features with age-related osteoporosis.

The fine structure of PGs have been reported to change as a function of donor age in other organ systems (13). The current study was undertaken to determine whether post-translational modifications to PGs can serve as surrogate biochemical markers to define donor chronological age. These markers were then

teoglycan; BGN, biglycan; DCN, decorin; BSA, bovine serum albumin; DMEM, Dulbecco's modified essential medium; FBS, fetal bovine serum; TGF-β, transforming growth factor β; SAX, strong anion exchange; HPLC, high performance liquid chromatography; GAG, glycosaminoglycan; RGD, arginine-glycine-aspartate.

TABLE I
Patient data

	NL	OI type I	OI type II	OI type III	OI type IV
<i>n</i>	14	6	2	6	8
No. fractures/year		0–2	^a	10–30	2–15
Mean age (years)	20	35	0.4	7	16
Range	0.25–60	8–60	0.25–0.45	2–13	3–45

^a Both type II patients died shortly after birth from respiratory insufficiency.

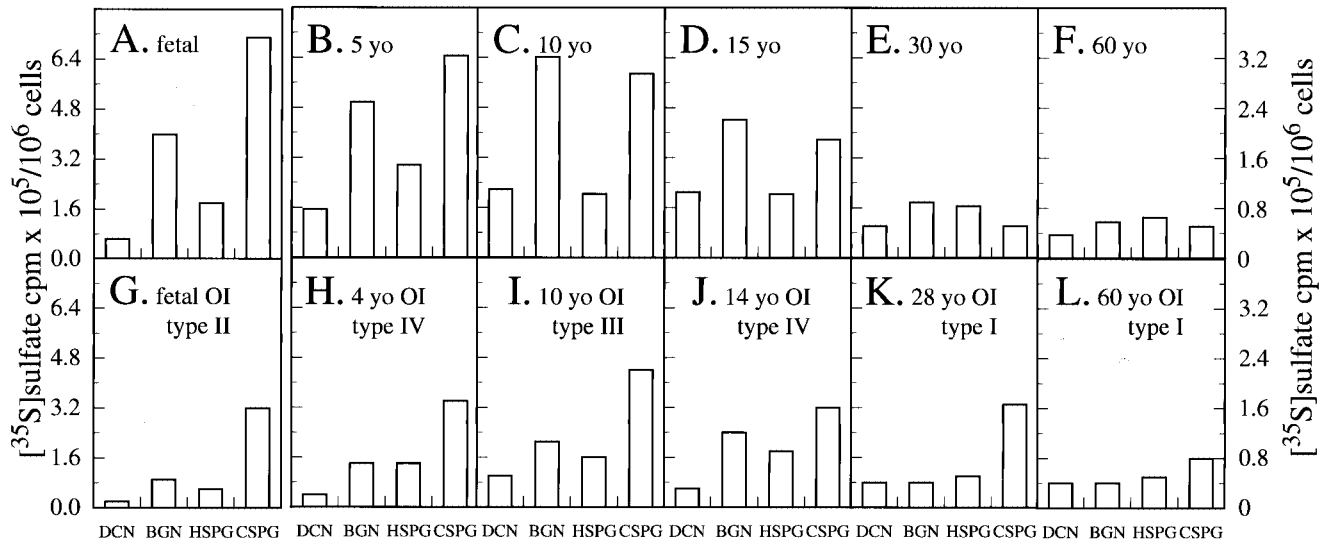


FIG. 1. **Proteoglycan distribution in normal and OI osteoblasts.** Osteoblasts derived from normal donors (A–F) or from patients with OI (G–L) were steady-state radiolabeled with $[^{35}\text{S}]\text{SO}_4^{2-}$. The levels of ^{35}S -labeled PGs in the medium and cell layer pools produced by osteoblasts in primary culture were determined as described under "Experimental Procedures." For comparative purposes, the total (medium + cell layer) levels normalized on a per 10^6 cell basis were graphed as a function of phenotype and donor age. HSPG, heparan sulfate syndecan-like PG; CSPG, versican-like chondroitin sulfate PG.

used to evaluate whether the expression pattern observed in bone cells derived from OI patients were consistent with an accelerated aging where the physiological age of the bone is well advanced beyond chronological age. The biological signals associated with these altered post-translational modifications were investigated.

EXPERIMENTAL PROCEDURES

Reagents—Ultrapure guanidinium HCl, glycine, and Tris-HCl were obtained from Invitrogen. Dulbecco's modified essential medium (DMEM) and heat-inactivated fetal bovine serum (FBS) were obtained from BioFluids, Inc. (Rockville, MD). Formamide, barium nitrite, tissue culture grade type IV collagenase, glutamine, penicillin, streptomycin, ascorbate, and bovine serum albumin (BSA) were purchased from Sigma. $[^{35}\text{S}]\text{SO}_4^{2-}$ and $[6\text{-}^3\text{H}]\text{GlcN}$ were obtained from PerkinElmer Life Sciences and Tran ^{35}S -label ($[^{35}\text{S}]\text{methionine/cysteine}$) was purchased from ICN, Inc. (Cosa Mesa, CA). Size exclusion chromatography resins Toyopearl HW 40(S), 55(S), and 75(F) were purchased from TosoHaas, Inc. (Montgomeryville, PA). Analytical HPLC SAX columns and Partisil PAC 10 columns were obtained from Phenomenex Inc. (Torrance, CA). Chondroitinase ABC (*Proteus vulgaris*) and ACII (*Arthrobacter aurescens*) were purchased from Seikagaku America (Falmouth, MA). Thrombospondin was isolated from human platelets (14); bone sialoprotein was isolated from a rat osteosarcoma variant, UMR-106-BSP (15); osteopontin (also called uropontin), isolated from human urine, was a gift from Dr. J. Hoyer, University of Pennsylvania (16). Serum-free conditioned medium (SFCM) was obtained from normal, primary human trabecular bone cells incubated in serum-free medium supplemented with 0.5% ITS+ (2.5 $\mu\text{g}/\text{ml}$ insulin/transferrin/selenium, 0.5 mg/ml BSA, and 0.5 $\mu\text{g}/\text{ml}$ linoleic acid) for 24 h prior to collection. ITS+, vitronectin, and fibronectin were purchased from Collaborative Research, Inc. (Bedford, MA). Bovine collagen type I was purchased from Worthington. TGF- β was purchased from R & D Systems, Inc. (Minneapolis, MN). Agarose was purchased from FMC Bioproducts, Inc. (Rockland, ME).

Human Bone Cell Culture—Recruitment of subjects donating osteoblasts was in accordance with approved procedures of the Institutional Review Board, The Johns Hopkins Bayview Medical Center. Normal

bone samples from 22 different donors were taken during surgery for fracture non-union and grafting with benign bone tumors. In some cases, samples were obtained during autopsy by sterile biopsy within 2–6 h of death. A diagnosis of OI was made based upon patient history of fractures, family history, presence of blue sclerae, and radiographic evidence of osteopenia. Bone was obtained from the following: 6 type I OI patients who exhibited limited skeletal deformity and were –1 to –3 S.D. below normal age-matched height; 2 type II OI patients that died shortly after birth from respiratory insufficiency; 6 type III OI patients that presented with severe skeletal deformities and dwarfism; and 8 type IV OI patients that showed skeletal deformities as well as significantly short stature. The mean age and range as well as number of fractures per year are give in Table I. The bone was cultured as described previously (9, 17). Briefly, bone was stripped of adherent periosteum, cut into small pieces, and treated with 250 units/ml type IV collagenase in DMEM for 2 h. The collagenase-treated bone chips were rinsed and then cultured in low calcium medium (0.2 mM $\text{CaCl}_2/\text{DMEM}$) containing 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 50 $\mu\text{g}/\text{ml}$ ascorbate. After about 5–7 days, outgrowth of bone cells started from the bone chips and confluency in 15-cm 2 dishes was usually reached after 4–6 weeks. For these studies, only first passage cells were used.

The first passage cells were seeded at a density of 20,000 cells per cm 2 in DMEM supplemented with 10% heat-inactivated FBS, glutamine, penicillin/streptomycin, and ascorbate. After 48 h this was changed to media from which the antibiotics had been omitted. After 24 h the cells were labeled with 50 $\mu\text{Ci}/\text{ml}$ of $[^{35}\text{S}]\text{SO}_4^{2-}$ and 10 $\mu\text{Ci}/\text{ml}$ $[6\text{-}^3\text{H}]\text{GlcN}$ in low sulfate-containing DMEM supplemented with 2% dialyzed FBS, ascorbate, and glutamine for 24 h. For harvesting, the cell layer was extracted at 4 $^\circ\text{C}$ in 0.05 M Tris buffer, pH 7.4, containing 4.0 M guanidine HCl, 0.5 M EDTA, and 2% Triton X-100 (extraction buffer) overnight. The medium was diluted 1:1 with extraction buffer and also incubated overnight. All labeling was done in triplicate. Cell number was determined in parallel cultures in triplicate at the end of the labeling period after trypsinization by use of a Coulter counter (Beckman Instruments, Hialeah, FL) in order to assess the proliferative capacity of osteoblasts.

Proteoglycan Isolation—Radiolabeled pools were desalted by chroma-

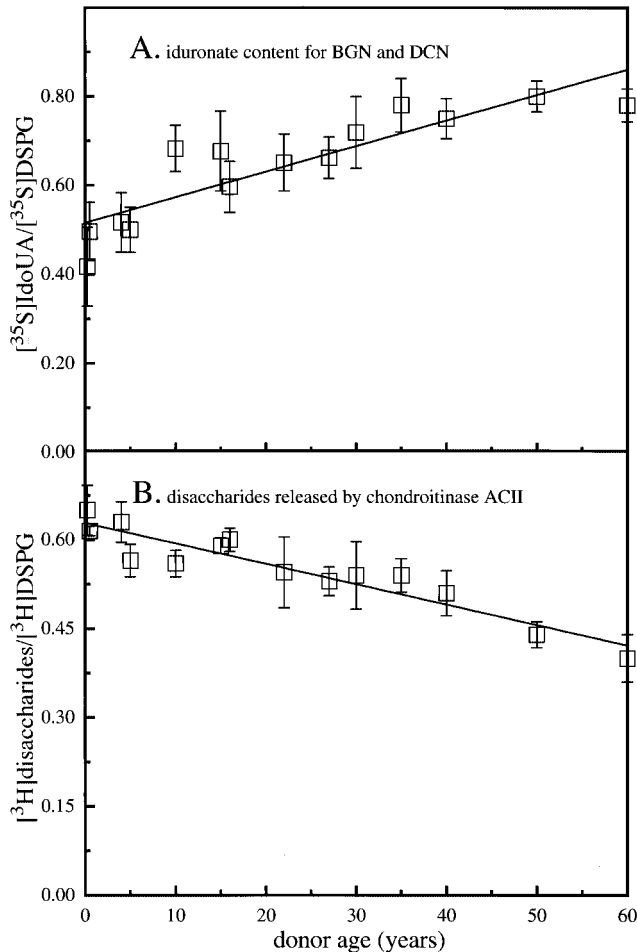


FIG. 2. Normal dermatan sulfate proteoglycan maturity. The iduronate content of BGN and DCN derived from normal donors was analyzed. $[^3\text{H}]$ Iduronate levels were determined by differential digestion with chondroitinase ABC and ACII as described under "Experimental Procedures." The resultant iduronate content was plotted as a function of donor age (A). The amount of $[^3\text{H}]$ disaccharides released by chondroitinase ACII digestion was determined by HPLC analysis and plotted as a function of donor age (B). Analyses were done in triplicate, and the error bars represent the means \pm S.D. *IdoUA*, iduronic acid; *DSPG*, dermatan sulfate, PG.

topography over a 1.0×25 -cm column packed with TSK-GEL Toyopearl HW 40(S) and equilibrated in 50% formamide, 40 mM KH_2PO_4 , 0.1 M KCl, and 0.5% Triton X-100 (18). After fractionation by charge density on a Nucleogen DEAE 4000-7 column, PGs were further separated by size distribution using a TSK-GEL ToyoPearl HW 75(F) column as described (18).

Proteoglycan Analysis—GAG chains were liberated by alkaline borohydride treatment (0.5 M NaBH_4 in 0.4 N NaOH for 24 h at 25 °C) and isolated by ToyoPearl TSK-GEL HW 40(S) chromatography (18). This chromatographic step enabled the resolution of GAG chains from O-linked oligosaccharides released by alkaline borohydride treatment, thus enabling the determination of the relative incorporation of $[^3\text{H}]\text{GlcN}/[^3\text{H}]\text{GalN}$ into both GAG chains and O-linked oligosaccharides. The size of the GAG chains was profiled by gel permeation chromatography using a 1.0×30 -cm ToyoPearl TSK-GEL HW 55(S) column equilibrated in 4 M guanidine HCl (19).

Glycosaminoglycan Structural Analysis—Structural analysis was done by digesting an aliquot of the isolated GAG with chondroitinase ACII and another aliquot with chondroitinase ABC. Digestion with 40 milliunits/ml chondroitinase ABC was carried out in a buffer containing 5 mM calcium acetate, 1 mM phenylmethylsulfonyl fluoride, 0.36 mM pepstatin, 0.1% Triton X-100, and 250 $\mu\text{g}/\text{ml}$ BSA in 100 mM Tris-HCl, pH 7.2. Digestion with 40 milliunits/ml chondroitinase ACII occurred in a buffer containing 100 mM sodium acetate, 0.1% Triton X-100, and 250 $\mu\text{g}/\text{ml}$ BSA in 100 mM Tris-HCl, pH 7.3. Digested products were analyzed by Centricon 3 microconcentrators and gel permeation chromatography as described (8). The percentage that is ACII-resistant reflects iduronic

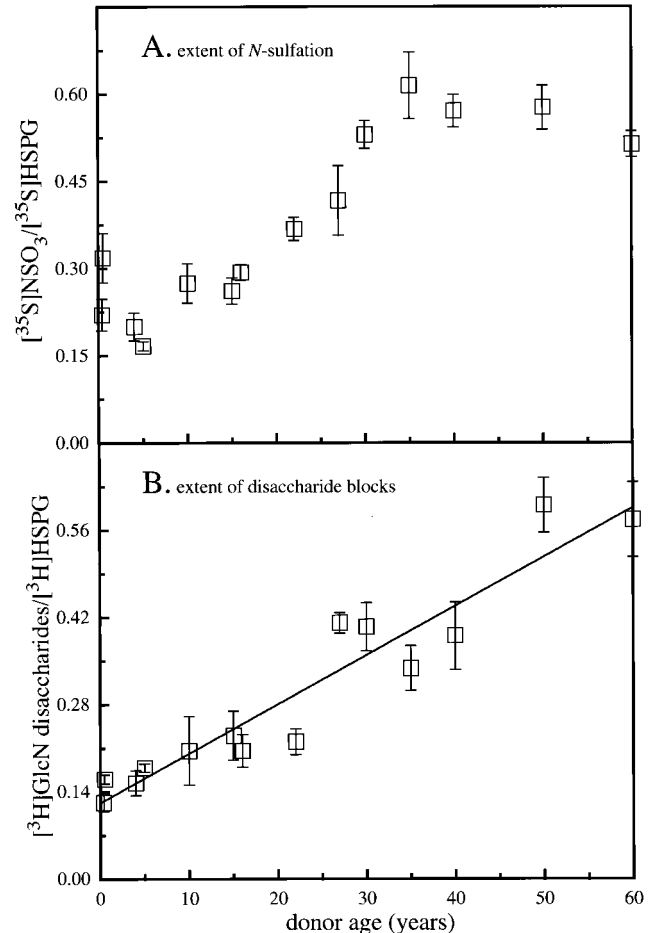


FIG. 3. Normal syndecan-like proteoglycan maturity. The degree of N-sulfation of heparan sulfate derived from normal osteoblasts was determined as described under "Experimental Procedures." The degree of N-sulfation (A) and the extent of N-sulfated disaccharide blocks (B) were plotted as a function of donor age. Analyses were done in triplicate, and the error bars represent the means \pm S.D. *HSPG*, heparan sulfate syndecan-like PG.

acid content. Radiolabeled disaccharides were analyzed by HPLC using a SAX column exactly as described (20).

Similarly, heparan sulfate can be characterized in terms of "maturity" by the degree to which its GlcN residues are N-sulfated. Heparan sulfate GAGs were depolymerized with pH 1.5 nitrous acid for 10 min at room temperature and quenched by the addition of 1.0 M Na_2CO_3 (21). Depolymerized samples were then reduced with NaBH_4 (0.25 M NaBH_4 in 0.1 N NaOH at 50 °C for 30 min). Free $[^35\text{S}]\text{SO}_4^{2-}$ was quantified by descending paper chromatography as reported previously (19). Disaccharide analysis was done by SAX column chromatography as described (22).

Determination of Specific Activity of $[^3\text{H}]\text{Glucosamine Labeling Pool}$ —The specific activity of the $[^3\text{H}]\text{GlcN}$ radiolabeling pool in bone cells from the age span under study was determined essentially as described (23–25). Briefly, the large chondroitin sulfate PG was isolated and digested with chondroitinase ABC (40 milliunits/ml). The resultant unsaturated disaccharides separated by HPLC and the amount of $[^35\text{S}]\text{SO}_4^{2-}$ and $[^3\text{H}]\text{GalN}$ associated with each disaccharide were quantitated by chromatography on a Partisil PAC 10 column (26).

Determination of Structural Effects of Different Matrix Growth Substrates—The entire surface of a 60-mm bacteriological Petri dish was coated with 0.5 ml of protein solution of each examined Arg-Gly-Asp (RGD)-containing protein solution in phosphate-buffered saline containing 1 mM CaCl_2 . After a 16-h incubation at 4 °C, the fluid was aspirated, and 0.5 ml of 60% methanol was added to each well (or the entire dish was covered with a thin layer of methanol) followed by 2 h of incubation at 4 °C. The methanol fixation was found to improve the stability and durability of the protein-coated area (15, 28) without significant changes of the biological activity of the substratum (as evaluated by attachment properties). Methanol was then aspirated, and

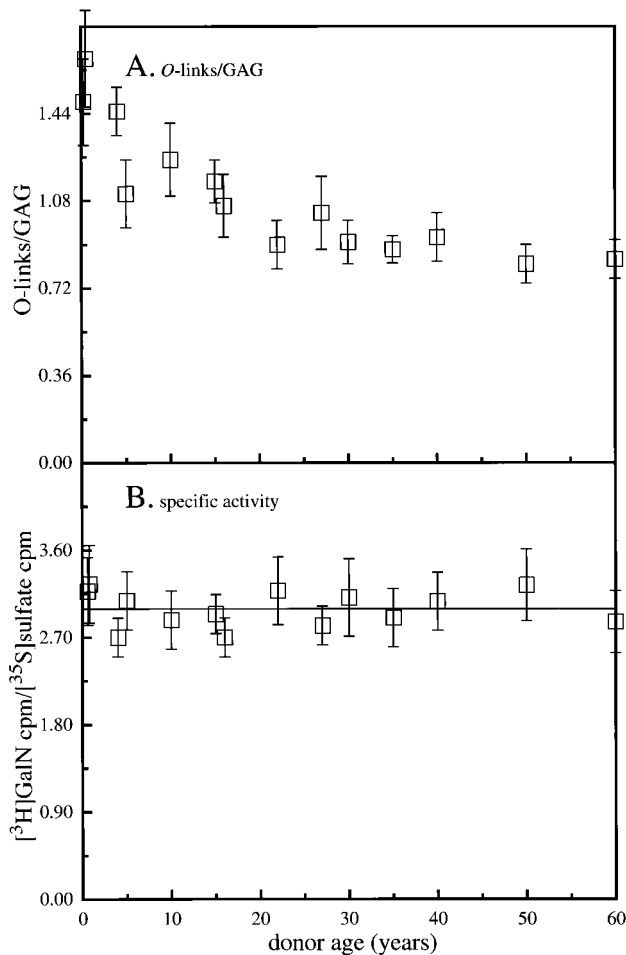


FIG. 4. **Normal O-linked oligosaccharide modifications.** The ratio of radiolabel incorporation into O-linked oligosaccharides to GAG chains was determined as described under "Experimental Procedures" (A). The specific activity of the metabolic precursor pool for the versican-like PG was determined by following the ratio of $[^3\text{H}]\text{GalN}$ to $[^{35}\text{S}]\text{SO}_4^{2-}$ in the unsaturated disaccharides produced by chondroitinase ABC digestion (B). For both O-linked modifications and specific activity, the values were plotted as a function of donor age; analyses were done in triplicate, and the error bars represent means \pm S.D.

the plates were washed for 30 min at 4 °C with washing buffer (50 mM Tris-HCl, pH 7.8, 110 mM NaCl, 5 mM CaCl_2 , 0.1 mM phenylmethylsulfonyl fluoride, 1% BSA, and 0.01% sodium azide). This was followed by washing three times with serum-free medium containing DMEM/Ham's F-12 (1:1) supplemented with 2 mM glutamine, 100 units/ml penicillin/streptomycin, 0.5% ITS+, and 50 $\mu\text{g}/\text{ml}$ ascorbate (serum-free medium).

Cells in third passage from normal donors over the age of 60 years, after reaching confluence, were released from culture dishes by trypsin-EDTA treatment and, following sedimentation, were resuspended in serum-free medium. Following a 30-min incubation at 37 °C, cells were plated onto plastic precoated with RGD-containing proteins at a density of 40,000 cells/ cm^2 , and 36 h after plating, medium was aspirated and replaced with serum-free medium containing 50 $\mu\text{Ci}/\text{ml}$ $[^{35}\text{S}]\text{SO}_4^{2-}$. After 12 h of labeling, media were collected, and cell layers were either extracted using 4 M guanidinium HCl or dissolved in gel sample buffer (GSB, consisting of 30% glycerol, 4% SDS, 2 M urea in 0.625 M Tris-HCl buffer). Aliquots of both medium and cell layer fractions were subjected to 3–15% bisacrylamide gradient gel electrophoresis and fluorography (8). Borohydrate-treated specimens (β -elimination) were analyzed by HPLC.

In order to characterize the GAG chains and O-linked oligosaccharides, medium fractions obtained from cells plated on RGD-containing proteins and labeled with $[6\text{-}^3\text{H}]\text{GlcN}$ were separated on 3–15% gradient SDS-PAGE. By using an Erickman gel slicer, 1-cm wide gel lanes were cut into 1-mm long slices and, after dissolving the gel slices in H_2O_2 at 60 °C overnight, the amount of incorporated $[^3\text{H}]\text{GlcN}$ + $[^3\text{H}]\text{GalN}$ was counted within each slice. A band containing the peak

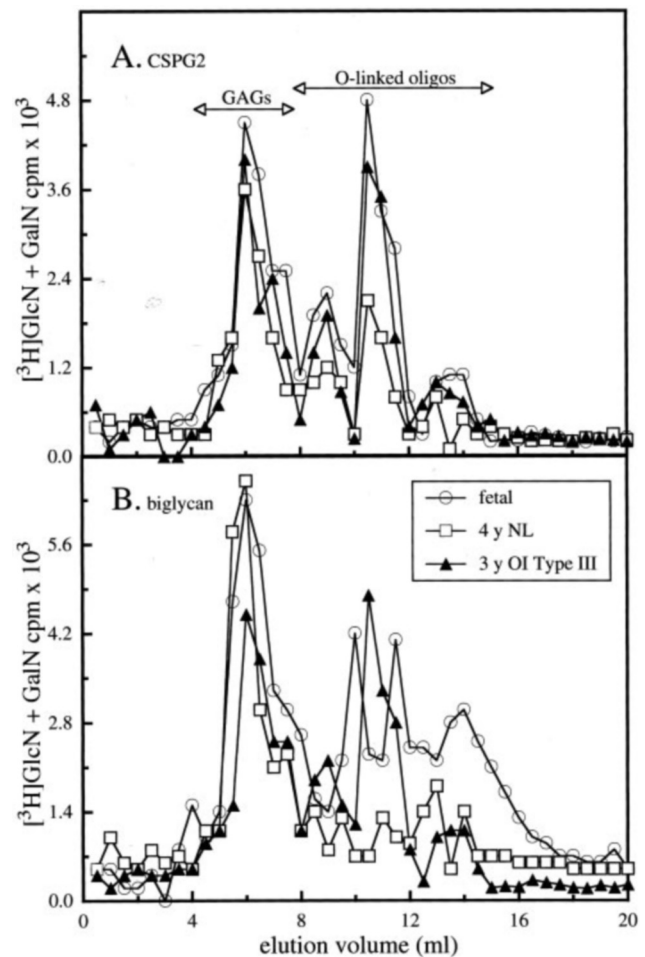


FIG. 5. **OI proteoglycan structural analysis.** $[^3\text{H}]\text{GlcN}$ - and $[^{35}\text{S}]\text{sulfate}$ -labeled PGs were isolated, purified, and subjected to alkaline β -elimination with NaBH_4 to cleave O-linked glycosidic-amino acid linkages. Label incorporation into intact versican-like PG (A) or BGN (B), GAG chains, and O-linked oligosaccharides was determined by chromatography on a Toyopearl TSK HW 40(S) column. Representative profiles derived from a fetal donor, a 4-year-old normal donor, and a 3-year-old OI type III donor are shown. CSPG2, versican-like chondroitin sulfate PG; NL, normal.

corresponding to BGN and DCN was excised from the remaining part of the gel, cut into small ($\sim 2/2$ -mm) fragments, washed with water, and lyophilized. Dried gel fragments were incubated with 0.3 M NaBH_4 in 0.4 M NaOH for 24 h in order to cleave off GAG chains and O-linked oligosaccharides (12). The reaction was stopped by bringing the pH to 1 with acetic acid for 1 h, and then samples were neutralized with 2 M NaOH. The samples were lyophilized, resuspended in water, and subjected to gel filtration analysis using the TSK-GEL HW 40(S) column equilibrated with ammonium acetate buffer (0.5 M ammonium acetate, 10% methanol, pH 6.0). The samples (0.2–0.5 ml volume) were run at the flow rate of 0.5 ml/min; 0.5-ml fractions were collected, and the amount of radioisotope incorporated into every fraction was counted in a scintillation counter.

RESULTS

Proteoglycan Distribution—In skin and cartilage, the distribution and structure of PGs change with age (13, 29–36). To test for such changes in osteoblasts, fingerprint profiles of the four PGs produced by bone cells derived from normal and OI donors were analyzed by steady-state radiolabeling and quantifying label incorporation into specific PGs (Fig. 1). For osteoblasts derived from normal donors, the versican-like chondroitin sulfate PG (CSPG) was initially the major PG. With increasing age, CSPG levels decreased, and other PGs increased in abundance, although absolute levels were reduced (Fig. 1, A–F). OI osteoblasts derived from patients of any age

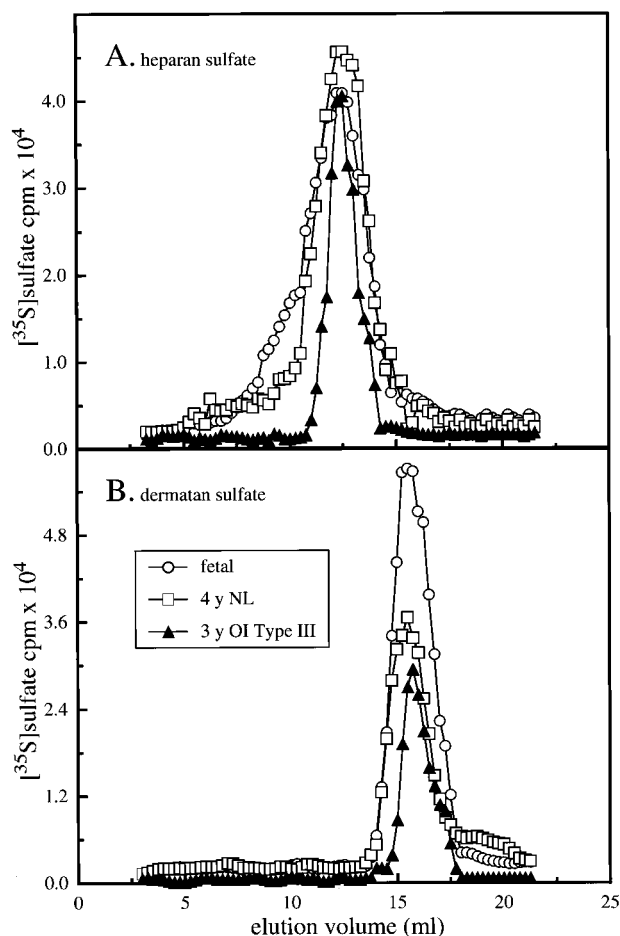


FIG. 6. OI glycosaminoglycan size. The relative size of the liberated free heparan sulfate (A) or dermatan sulfate (B) GAG chains derived from OI donors was analyzed by chromatography on a $50 \times 1\text{-cm}$ ToyoPearl TSK HW 55(S) column. Representative profiles derived from a fetal donor, a 4-year-old OI type III donor, and a 4-year-old normal donor. NL, normal.

exhibited a "fetal-like" PG fingerprint, with the CSPG staying the major component at all ages. The absolute levels of all four PGs were consistently low at all OI donor ages (Fig. 1, G–L) and similar to the absolute levels observed in cells derived from much older normal donors. Thus, OI-derived PGs exhibited a fetal distribution pattern, but levels produced were similar to those from aged normal donors. To distinguish whether the observed alterations in OI bone cell extracellular matrix production arose from a type I collagen mutation causing an arrest of the cells in a fetal-like state or whether a consequence on the mutation was a premature aging of the cells, the fine structure of the PGs was investigated.

Normal Proteoglycan Structure—The iduronate content of the dermatan sulfate containing PGs BGN and DCN derived from 14 different normal donors was determined by radiolabeling normal osteoblasts with [³⁵S]SO₄²⁻ and [³H]GlcN, isolating the GAGs, and carrying out a differential digestion with chondroitinase ABC versus ACII. The amount of radiolabeled material digested by ACII (which will not degrade iduronate-containing oligosaccharides) was compared with the amount digested by chondroitinase ABC (which degrades the GAG completely to disaccharides). The iduronate content was determined as ((ABC digested cpm – ACII digested cpm)/ABC digested cpm). A positive linear correlation between iduronate content and donor age was observed. Iduronate content increased 1.5-fold between fetal and 60 years of age (Fig. 2A). A complementary measure of the maturity of dermatan sulfate

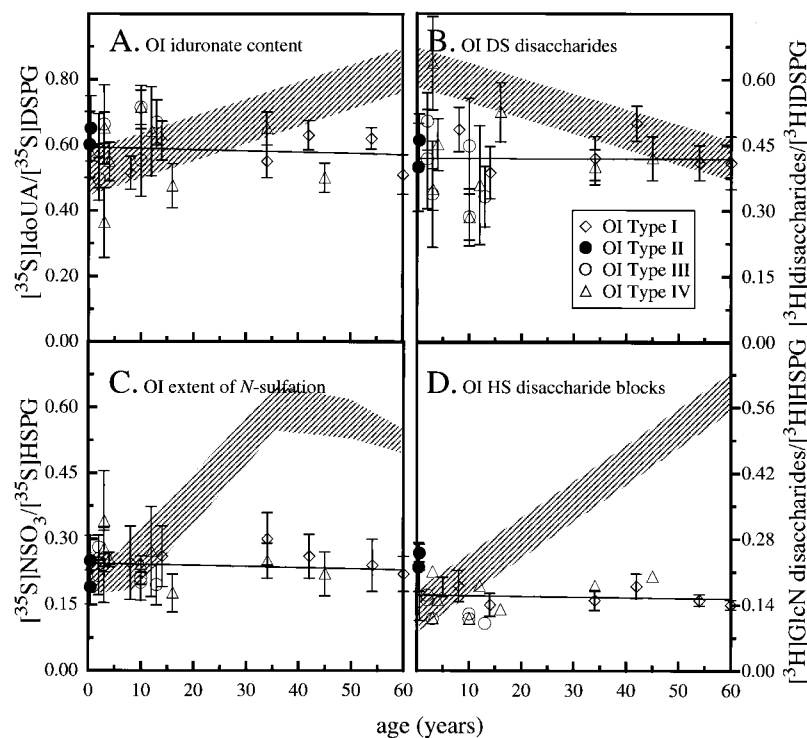
PGs was obtained by quantifying the levels of [³H]GlcN-labeled disaccharides released from chondroitinase ACII treatment by SAX column HPLC. An increase in iduronate content would be reflected in fewer disaccharides being released (as chondroitinase ACII does not cleave at iduronate moieties). The number of [³H]disaccharides released by chondroitinase ACII treatment decreased linearly with increasing donor age for chains derived from normal donors (Fig. 2B).

Similarly, heparan sulfate can be characterized in terms of "maturity" by the degree to which its GlcN residues were N-sulfated. Sulfation of GlcN residues is a key enzymatic step required before further enzymatic modifications, such as glucuronate epimerization and O-sulfation can occur (37). The extent of N-sulfation can easily be assessed with [³⁵S]GAGs by low pH nitrous acid deaminative treatment that will cleave heparan sulfate at GlcNSO₃ residues, liberating free [³⁵S]SO₄²⁻, disaccharides, tetrasaccharides, and larger oligosaccharides. The amount of free ³⁵SO₄²⁻ directly reflects the extent of N-sulfation. This was done for all normal donor-derived heparan sulfate chains. The results (Fig. 3A) indicated that chains derived from normal donors exhibited an age-dependent increase in extent of N-sulfation. The N-sulfation of post-natal heparan sulfate increased over 3.5-fold until reaching a plateau during the 4th decade of life. Analysis of the [³H]GlcN-labeled disaccharides generated following nitrous acid cleavage of N-sulfated disaccharide blocks by SAX column HPLC demonstrated a parallel measure of heparan sulfate maturity. Again, heparan sulfate chains derived from normal cells showed an age-related pattern with increasing disaccharide blocks with increasing age (Fig. 3B).

Normal Proteoglycan O-Linked Modifications—Previous work (12) has shown that the degree of O-linked oligosaccharide modification to the core proteins of bone PGs change significantly in samples derived from either fetal or newborn donors. A more extensive analysis of the influence of donor age on core protein post-translational modifications was undertaken. Radiolabeled CSPG and BGN derived from the osteoblast-like cells of 14 normal donors were isolated, and the amount of label incorporation into O-linked oligosaccharides and free GAG chains was analyzed by HPLC resolution following alkaline borohydride treatment. The levels of ³H-labeled O-linked oligosaccharides were expressed as the ratio of radiolabel incorporation into O-links versus radiolabel incorporation into GAG chains. The degree of O-linked post-translational modification decreased linearly with increasing age from fetal to age 30 for both the CSPG and BGN (Fig. 4A). After age 30, the ratio was constant.

Labeling Pool Specific Activity—When extracellular matrix component stoichiometry was determined *in vitro*, the resultant levels of radiolabel incorporated into specific matrix components is a function of the specific activity of the precursor pool. The possibility exists that age-related alterations in PG levels were caused by metabolic changes in the specific activity of precursor pools (that may be a function of patient age, bone cell metabolic state, etc.). The specific activity of precursor pools involved in PG synthesis was analyzed by determining the [³H]GlcN radiolabeling precursor pool activity that, because GlcN rapidly equilibrates with GalN, is the same as [³H]GalN. This was tested by determining the ratio of [³H]GalN to [³⁵S]SO₄²⁻ counts/min incorporation in the unsaturated disaccharides liberated from the CSPG by chondroitinase ABC digestion. This PG has a GAG composition of almost entirely chondroitin 4-sulfate (38). Thus, each unsaturated disaccharide produced by enzymatic digestion should possess one [³⁵S]SO₄²⁻ per [³H]GalN residue. When the ratio of [³H]GalN to [³⁵S]SO₄²⁻ counts/min in the unsaturated disaccharide was an-

FIG. 7. OI proteoglycan maturity. The maturity of BGN and DCN derived from OI donors was analyzed. [^3H]Iduronate levels (A) as well as the amount of [^3H]disaccharides released by chondroitinase ACII digestion (B) were determined and plotted as a function of donor age. The degree of *N*-sulfation of heparan sulfate derived from OI osteoblasts was determined. The degree of *N*-sulfation (C) and the extent of *N*-sulfated disaccharide blocks (D) were plotted as a function of donor age. Analyses were done in triplicate, and the error bars indicate means \pm S.D. The shaded area represents the range of the data observed for normal donor-derived PGs. *IdoUA*, iduronic acid; *DSPG*, dermatan sulfate PG; *DS*, dermatan sulfate; *HSPG*, heparan sulfate syndecan-like PG; *HS*, heparan sulfate.



alyzed, the ratio did not change significantly for normal samples (Fig. 4B).

OI Proteoglycan Structure—The observed age-related changes in normal PG structure suggest that these post-translational modifications could be used to test whether OI donor-derived osteoblasts exhibit a phenotype consistent with advanced aging or with an arrest in a fetal-like state. When the component structure of isolated PGs derived from OI donors was analyzed by size exclusion chromatography following β -elimination and borohydride reduction, distinct profiles were evident. A representative profile is shown in Fig. 5, where the levels of *O*-linked oligosaccharides from fetal, 4-year-old normal, and 4-year-old OI type III bone cell-derived samples of purified versican-like PG and BGN were determined. By this method of profiling, the GAG chains eluted in the void volume, so no conclusion was made on chain size variation. However, *O*-linked oligosaccharides were eluted in the included column volume, and comparisons were made. The OI-derived osteoblast strains exhibited increased levels of *O*-linked oligosaccharides similar to that seen in normal fetal-derived *O*-links. The size of the GAG chains between normal and OI-derived PGs was compared by HPLC analysis using a size exclusion chromatography column where the chains eluted in the included volume (Fig. 6). Intact GAGs from all OI bone cultures were similar in size as they co-eluted on gel permeation chromatography, consistent with the observed absence of changes in iduronate or *N*-sulfation content. Comparison of normal to OI-derived GAG chains did not yield a significant difference in the elution position. However, the polydispersity of the peaks reflecting carbohydrate structural heterogeneity was consistently greater in the normal donor-derived samples.

The GAG chains derived from OI donors were then analyzed for extent of post-translational modifications. This was done by analyzing the iduronate content in dermatan sulfate GAGs and *N*-sulfation in heparan sulfate GAGs isolated from osteoblast-like cells derived from 26 different OI donors. When the extent of iduronate epimerization in dermatan sulfate chains from BGN and DCN derived from OI osteoblasts was determined, the percentage of iduronate remained relatively constant with

increasing donor age (Fig. 7A). In contrast to normal cells, the number of disaccharides released from samples derived from OI osteoblasts remained relatively constant at all ages (Fig. 7B). Similarly, heparan sulfate chains derived from OI cells failed to express the normal age-dependent increase in *N*-sulfation and *N*-sulfated disaccharide blocks (Fig. 7, C and D).

OI donor-derived PG core protein *O*-linked modifications were profiled by comparing the ratio of *O*-linked oligosaccharides to GAGs (Fig. 8A). Milder OI forms (types I and IV) exhibited a ratio that was similar to the normal age-matched pattern. PGs derived from OI patients with a more severe phenotype (OI types II and III) exhibited higher *O*-linked glycosylation. To rule out the possibility that the observed differences in OI PG levels and structure were due to changes in the specific activity of the metabolic precursor pool, the specific activity of the [^3H]GalN and [^{35}S]SO $_4^{2-}$ precursor pools was investigated in OI-derived samples. The ratio of [^3H]GalN to [^{35}S]SO $_4^{2-}$ in the unsaturated chondroitin 4-sulfate disaccharide did not change significantly for all OI samples analyzed (Fig. 8B). The major differences between normal and OI-derived PGs did not arise from differences in the specific activity of the precursor pool. All PGs derived from OI donors failed to parallel the age-related increase in dermatan sulfate iduronate content or heparan sulfate *N*-sulfation seen in PGs derived from normal donors. The structural pattern observed in OI-derived PGs was similar to the structural features seen in normal PGs derived from fetal donors. By using the post-translational modifications of PGs as surrogate biochemical markers of physiologic age suggests that, in the presence of a mutation in type I collagen, osteoblast cells exhibit a phenotype arrested in a fetal-like state.

Extracellular Matrix Effects on Post-translational Modifications—The potential biological significance of these observations was investigated by testing whether the altered PG post-translational modification seen in cells from OI donors might be associated with the altered extracellular matrix composition, *i.e.* whether common constituents of extracellular matrix that are known to interact with cells via specific receptors are capable of inducing the fetal-like post-translational modifica-

tions of PGs in normal adult-derived osteoblasts. The extent of post-translational modifications to PGs can be grossly compared by migration position on SDS-PAGE. Adult-derived bone cells were plated on different matrix glycoproteins, and the cells were radiolabeled to follow PG levels and structure. Cells exposed to thrombospondin secreted PGs that migrate on the

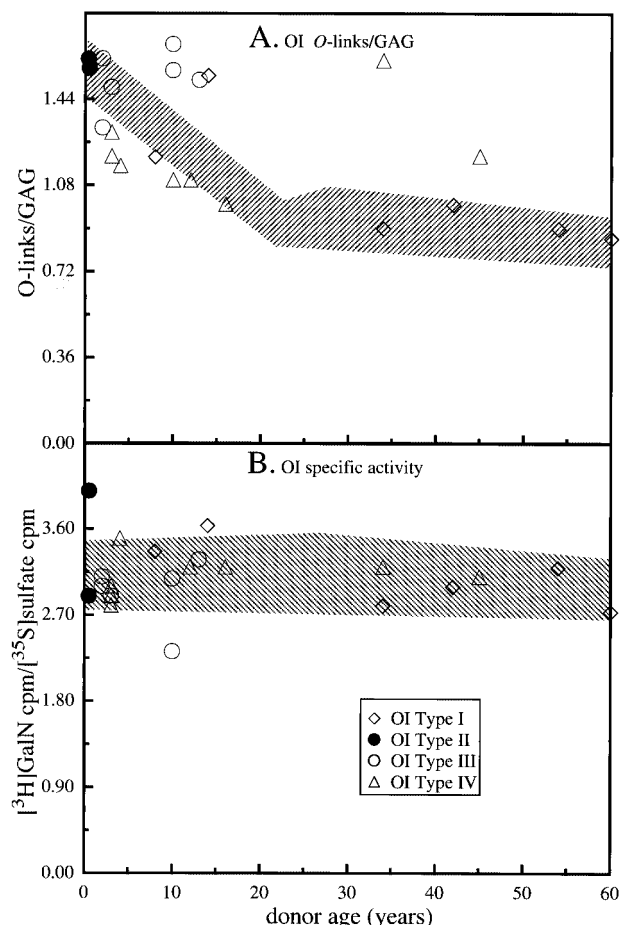


FIG. 8. OI O-linked oligosaccharide modifications and specific activity. The ratio of radiolabel incorporation into O-linked oligosaccharides to GAG chains was determined for BGN derived from OI donors (A). The specific activity of the metabolic precursor pool for the versican-like PG was determined in OI donor cells by following the ratio of $[^3\text{H}]\text{GalN}$ to $[^{35}\text{S}]\text{SO}_4^{2-}$ in the unsaturated disaccharides produced by chondroitinase ABC digestion (B). For both O-linked modifications and specific activity, analyses were done in triplicate, and the values were plotted as a function of donor age. The shaded area represents the range of the data observed for normal donor-derived PGs.

gel as higher M_r species than those secreted by cells plated on fibronectin, vitronectin, collagen, osteopontin, and bone sialoprotein (Fig. 9). This pattern was reproducible and occurred in all experiments and over extended time courses (data not shown).

A typical profile of alkaline borohydride treatment of a representative BGN sample purified as described from cells attached to collagen and thrombospondin is shown in Fig. 10A. This type of analysis made it possible to quantitate the relative incorporation of radioisotope into GAG chains and oligosaccharides. The high M_r BGN (secreted by cells plated onto thrombospondin) showed isotope incorporation mostly into O-linked oligosaccharides and to a lesser extent into GAG chains, whereas in the lower M_r BGN (secreted by cells grown on other RGD-containing matrix components), the major peak appears to be composed of GAG chains. TGF- β is known to co-purify with thrombospondin (39), and TGF- β has been implicated in the regulation of small PG metabolism (40–42). The effect of TGF- β on PG post-translational modification was investigated by adding exogenous TGF- β 1 prior to radiolabeling of the cells. However, BGN secreted by cells in the presence of TGF- β 1 exhibited a profile showing greater radiolabel incorporation into GAG chains with little effect on O-linked oligosaccharides (Fig. 10A, inset).

The ratio of O-linked oligosaccharides to GAGs was examined as a function of OI type and compared with normal adult and fetal donor ratios. When the ratios were averaged within a given OI type, increasing disease severity was associated with increasing O-linked oligosaccharide modifications (Fig. 10B). Between the mildest OI type and most severe type, the ratio increased 1.6-fold. The changes in the ratio paralleled the changes in total thrombospondin levels produced by these same cells. The ratio of O-linked oligosaccharides to GAGs was next determined in osteoblasts derived from an adult donor (65 years of age) whose cells were cultured and radiolabeled on either a type I collagen substrate, a collagen + TGF- β 1, or a thrombospondin substrate. The addition of TGF- β 1 to a collagen substrate decreased the ratio, consistent with an effect on radiolabel incorporation into the GAG. The presence of thrombospondin led to a 2-fold increase in the ratio of O-linked oligosaccharides to GAGs (Fig. 10C).

DISCUSSION

PG synthesis begins in the rough endoplasmic reticulum where N-linked oligosaccharides are added co-translationally to the nascent core protein (43). Once in the Golgi, high mannose N-linked oligosaccharides are modified, and O-linked oligosaccharides are added. GAG chain addition begins in the lumen of the endoplasmic reticulum and proceeds as the core

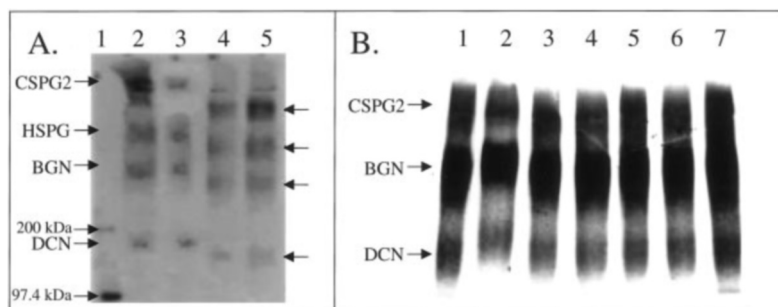


FIG. 9. Matrix RGD-protein effects on proteoglycan size. Osteoblasts derived from a 10-year-old type II OI donor and an age-matched normal were grown on tissue culture plastic, radiolabeled with $[^{35}\text{S}]\text{SO}_4^{2-}$, and the relative migration positions of the PGs determined by 3–15% SDS-PAGE analysis and fluorography (A). The lanes were loaded with molecular weight standards (lane 1), samples from the OI-derived medium pool (lane 2), OI-derived cell layer pool (lane 3), normal medium pool (lane 4), and normal cell layer pool (lane 5). Cells from a normal adult donor (age 65 years) were plated on either serum-free conditioned medium (lane 1), thrombospondin (lane 2), fibronectin (lane 3), vitronectin (lane 4), collagen type I (lane 5), osteopontin (lane 6), or bone sialoprotein (lane 7). The relative size of the PGs was profiled by 3–15% SDS-PAGE analysis (B). CSPG2, versican-like PG; HSPG, syndecan-like PG. Arrows mark the relative migration positions of the PGs.

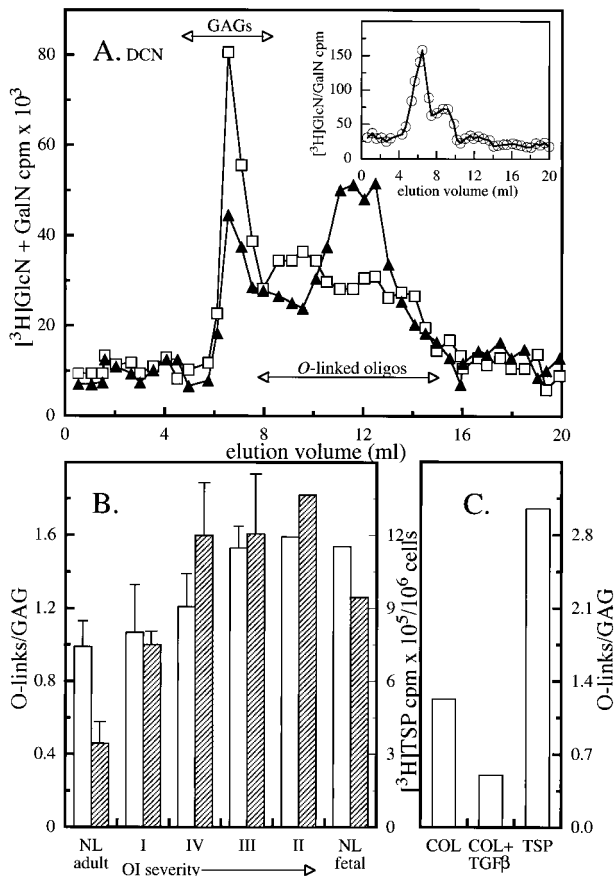


FIG. 10. Matrix RGD-protein effect on proteoglycan post-translational modifications. The products of alkaline borohydride treatment of BGN obtained from normal adult cells plated on collagen (open square) or thrombospondin (closed triangle) and labeled with $^3\text{H}[\text{GlcN}]$ were profiled by TSK-GEL HW 40(S) column HPLC (A). A representative profile of ^3H -labeled GAGs and O -linked oligosaccharides derived from BGN obtained from the same cells grown on collagen treated with $\text{TGF-}\beta_1$ is shown in the inset. The ratio of O -linked oligosaccharides to GAGs was averaged among all adult normal donors ($n = 12$), each OI type ($n = 6$ for type I, $n = 2$ for type II, $n = 6$ for type II, and $n = 8$ for type IV), and among normal fetal donors ($n = 2$) for comparison (B). For comparison, the levels of thrombospondin (cross-hatched bars) were averaged by phenotype from previously published values (17). Error bars represent means \pm S.D. The ratio of O -linked oligosaccharides to GAGs for BGN derived from normal adult cells grown and radiolabeled on collagen, collagen + $\text{TGF-}\beta_1$, or thrombospondin was determined (C).

protein traverses the intracellular secretory pathway (44). These GAG chains define the class of the PG by a general repeating disaccharide backbone. For heparin, heparan sulfate, chondroitin sulfate, and dermatan sulfate PGs, the backbone consists of (hexuronic acid- N -acetylhexosamine) $_n$ which can have sulfate ester substituents on either the uronic acid or hexosamine or both (7). Dermatan sulfate is distinguished from chondroitin sulfate by the epimerization of glucuronate residues to iduronate and by 2- O -sulfation of those iduronate residues. Heparin is distinguished from heparan sulfate by possessing a higher degree of N -sulfated glucosamine residues and more O -sulfation (of both the uronic acid and glucosamine residues). Dermatan sulfate containing PGs are defined as mature based on their iduronate content, whereas heparan sulfate containing PGs are defined by their extent on N -sulfation of glucosamine residues (37, 45).

The distribution and structure of PGs has been found to change with age in a number of different tissues/organs. The ratio of keratan sulfate to chondroitin sulfate chains was found

to increase with increasing age in lumbar intervertebral discs (29, 30). In intervertebral discs and hyaline cartilage, the size range of the PGs exhibited greater polydispersity with increasing age (33–35). The degree of N -sulfation of cell-associated heparan sulfate increased with age in gingival fibroblasts (31). In skin fibroblasts, the levels of versican were found to decrease and DCN increased with age (36). Older skin also exhibited a greater polydispersity in size. The current study was undertaken to determine whether similar age-related changes occurred in normal bone cell-derived PGs. Changes in the extent of post-translational modifications to both heparan and dermatan sulfate chains of bone PGs were observed in the current study. The correlation of donor age with changes in normal bone cell PG fine structure suggested that these specific alterations might be used as surrogate markers of a physiologic age of the tissue/cells synthesizing the PG. The utility of the PG fine structures as such markers was investigated by studying PGs derived from OI donors.

Previous work has shown that the stoichiometry of extracellular matrix components made by OI bone cells was different from that of age-matched controls (9, 17). In addition to type I collagen, the synthesis of the four bone PGs was reduced in OI cells (9). The levels of other matrix components, hyaluronan as well as fibronectin and thrombospondin, were elevated in the extracellular matrix pool compared with age-matched controls (17). The proliferative capacity and growth rate of OI cells were reduced relative to normal cells (11). Finally, the observed changes in OI bone matrix stoichiometry persisted in long term culture (10). The reduced levels of matrix expression in osteoblast-like cells derived from young donors with OI (compared with normal age-matched controls) were within the normal range seen for cells derived from healthy donors over the age of 60 years. One possible interpretation of the observed alterations in extracellular matrix stoichiometry was that OI osteoblasts fail to parallel the normal bone cell developmental expression pattern because they exhibit a prematurely aged expression pattern. If this were the case then biochemical markers of this aged state should be identifiable.

By using bone PG post-translational modification structure as surrogate markers for physiologic age, cells derived from OI donors are fetal-like in terms of PG distribution, GAG maturity, and O -linked modifications. The pattern of expression and fine structure of the PGs were consistent with OI-derived osteoblasts failing to follow a developmental differentiation pattern. This failure in differentiation may contribute to the pathophysiological consequences observed in the OI phenotype. Although the absolute levels are equivalent to cells derived from aged normal donors, both the distribution among the four PGs and the degree of O -linked oligosaccharide modifications were found to be similar to the fetal-like pattern. Thus the hypothesis that the reduction in extracellular matrix production as well as reduced proliferative rates of OI-derived osteoblast-like cells was a manifestation of advanced aging can be excluded. The observed lack of change in OI cell GAG modification (epimerization and sulfation pattern) was consistent with a failure of cells to progress from the fetal state of expression patterns. There was no clear correlation between severity of disease (OI type) and the type of PG fine structure. The observation of PGs possessing O -linked oligosaccharide structures of a fetal-like nature, which did not change with increasing age of the OI donor, point to a fundamental error in the normal differentiation pathway of osteoblasts as a consequence of a mutation in type I collagen.

When normal adult cells were cultured on thrombospondin (but not fibronectin, vitronectin, type I collagen, osteopontin, or bone sialoprotein) the post-translational pattern of small ma-

trix PGs closely resembles the pattern characteristic for osteoblastic cells obtained from OI donors as well as normal fetal donors, even though cells used for these set of experiments were obtained from a 65-year-old donor. Interestingly, both fetal bone cell cultures and cells derived from donors with OI synthesize and secrete significantly higher levels of thrombospondin than cultures obtained from normal adult donors (17). It may be that the over-glycosylation of the oligosaccharides observed for OI and fetal cells is a consequence of an "autocrine" effect of thrombospondin, synthesized in higher amounts by these cells. It is also possible that elevated thrombospondin and altered PG post-translational modifications reflect an underlying altered phenotypic expression pattern.

The major source of thrombospondin is the α -granules of platelets (46), from which it is released following platelet activation and degranulation. This mechanism is one of the first steps in the process of wound healing. It is possible that thrombospondin, apart from its involvement in promoting cell attachment via several different mechanisms described (47), also influences cell metabolism to make it more effective in the task of rebuilding damaged matrix. Thus, it is conceivable that under certain conditions (e.g. insufficient collagen and matrix production in OI as well as increased fractures) osteoblastic cells exist in a milieu that is essentially a damaged matrix, and part of the programmed response is expression of an "elevated thrombospondin/altered post-translational structure" phenotype with respect to their matrix metabolic activity.

The addition of TGF- β 1 to normal adult-derived bone cells did not produce the altered O-linked oligosaccharides associated with OI. OI bone cells do not exhibit a TGF- β -dependent increase in collagen synthesis and alkaline phosphatase activity seen with normal bone cells (48). OI osteoblasts had a greater reduction in TGF- β receptor I–III number than age-matched controls, and receptor affinity was unchanged, whereas ligand-induced modulation of receptor number was found to be impaired in the OI cells (49). Thus, the association of elevated thrombospondin levels with altered post-translational modifications in OI osteoblasts is unlikely to involve TGF- β .

Currently, over 250 distinct type I collagen mutations have been identified in OI patients, with point mutations resulting in substitutions for glycine residues being the most common (50, 51). Although there are a large number (>50) of exons in *COL1A1* and *COL1A2* genes, single exon deletions are a rare finding (52). Some mutations lead to the functional loss of the affected allele, which has been suggested to cause mild (type I) forms of OI (53). Mutations in the collagen genes of the severely affected patients are thought to lead both to a reduced type I collagen synthesis, secretion, and deposition of structurally abnormal collagen molecules into the matrix (39, 54, 55). Histomorphometric studies of human OI types III and IV bone have suggested that OI is associated with a high bone turnover rate (56, 57). For adult OI type IA and perhaps in children with mild disease, the turnover rate may not be as high (27, 58). The increased turnover is believed to arise from increased osteoclastic activity in the presence of inadequate matrix production by osteoblasts.

Based upon our *in vitro* observations of the effects of type I collagen mutations on osteoblast proliferation, extracellular matrix component expression, and PG fine structure, it is apparent that the mutations that give rise to OI cause pleiotropic effects far beyond the production of a structurally aberrant type I collagen molecule. We hypothesize that although the genotype for most cases of human OI is caused by mutations in type I collagen, the phenotype may be defined by an altered

bone matrix stoichiometry and concomitant altered osteoblast differentiation state, *i.e.* the pathophysiological consequences of mutations in OI arise not solely from structural aberrations in type I collagen but also from alterations in osteoblastic differentiation. Altered osteoblastic differentiation may arise from altered extracellular matrix feedback into cellular metabolism.

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