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Purification and characterization of iduronic acid-rich and glucuronic acid-rich proteoglycans implicated in human post-burn keloid scar*

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ABSTRACT

Small proteoglycans (PGs), extracted from human keloid scar tissue with 4M guanidinium chloride and fractionated by DEAE-cellulose chromatography, were separated by ethanol precipitation into one L-iduronic acid-rich and one D-glucuronic acid-rich fraction. The size of the L-iduronic acid-rich PG was 102 kDa with a 27 kDa glycosaminoglycan chain, that of the D-glucuronic acid-rich PG was 90 kDa with a 26 kDa glycosaminoglycan chain, and the protein core of both PGs was 14.5 kDa. The two PGs carried sulfate groups mostly attached at C-4 of the 2-amino-2-deoxy-D-galactose units. The N-terminal amino acid sequence of both was similar to human bone PGII (decorin), normal and hypertrophic scar, and human dermal tissue PG.

INTRODUCTION

The healing of a burn wound, especially one with a loss of dermal tissue, results in different types of scars, namely, normal, hypertrophic, or keloid[1]. The last-named are associated with excessive connective tissue deposition that extends beyond the confines of the original wound[2]. Histologically, it is difficult to distinguish between hypertrophic and keloid scars. Both show characteristic whorls and nodules of collagen, as opposed to normal scars in which the collagen fibers lie in bundles parallel to the surface of the skin. The whorls persist in keloid scars, but ultimately flatten in hypertrophic scars. Keloid scars contain broad, eosinophilic, refractive, and hyaline-like collagen fibers[3]. When keloid and hypertrophic scars are examined by transmission electron microscopy, the collagen fibrils are larger and more irregular in the keloid scars. This irregularity may reflect significant differences in terms of collagen fibrillogenesis, organization, and

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metabolism[4]. Various aspects of the biology of collagen behavior, both *in vitro* and *in vivo*, are related to other components of the extracellular matrix[5–8].

Proteoglycans (PGs) are widely distributed in the extracellular matrix of various connective tissues, and the bundles of collagen in various scar tissues are abundantly coated with them[9]. The compositions of PGs from bovine[10–12] and porcine skin[13] have been reported. Two different PGs have been found in newborn-calf skin[12] and fetal-calf skin[14]. In order to understand the role of PGs in different types of post-burn scars, we have isolated and characterized PGs from human skin[15], and human normal and hypertrophic scars[16]. The composition of the PGs from human skin differs from that of scar tissue, suggesting that they may have a role in scar formation. To understand this role we isolated PGs from keloid scar tissue and characterized them with respect to the core proteins and glycosaminoglycan (GAG) chains.

EXPERIMENTAL

Materials. — Keloid scar samples were obtained after surgery and were characterized by their raised, overhanging border and a nodular-growth pattern more exaggerated than that of hypertrophic scar[17]. The ages of the scar samples were 19 and 14 months after burn injury and were from a 4-year-old female patient and a 2-year-old male patient, respectively. Guanidinium chloride (Gdm·Cl) ultrapure quality was from Schwarz/Mann Biotech, benzamidine hydrochloride was from Aldrich; and phenylmethylsulfonyl fluoride, Δ-Di-OS [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose], Δ-Di-4S [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) -D-galactose 4-sulfate]; and Δ-Di-6S [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate] were from Sigma; chondroitinase ACII and ABC, chondroitin 4-sulfate, and chondroitin 6-sulfate were from Miles Research Lab. Glycosaminoglycan standards were supplied by Drs. M. Matthews and J. A. Cifonelli, University of Chicago (Contract No. 1-AM-5-2205 from NIH, Bethesda, MD). Sepharose Cl-4B and Sepharose CL-6B were from Pharmacia LKB Biotechnology Inc., and DEAE-cellulose ion-exchange resin was from Whatman Inc.

Analytical procedures. — The protein and uronic acid contents of various column fractions were determined by measuring the absorbancy at 280 nm and by a carbazole reaction[18], respectively. Amino acids were determined after hydrolysis with 6M HCl for 20 h at 110° and by treatment with phenyl isothiocyanate as described earlier[19]. The column used was an Altrex 3-μm ODS and the elution was as described in the Waters "Picotag" literature. Hexose content was determined by the anthrone reaction[20], protein content by the Lowry method[21], and sialic acid content as described by Jourdian *et al.*[22]. Hexosamines were determined with an amino acid analyzer after 8 h of hydrolysis with 4M HCl at 100°. The sulfate content of PG samples was measured by the method of Antonopoulos[23]. The N-terminal amino acid sequence of the PG samples was determined with an Applied Biosystems model 477A gas-phase sequencer with on line HPLC detection of Pth-amino acids.

Glycosaminoglycan oligomer analysis. — PG samples (1 mg) in 0.15M NaCl–0.1M sodium acetate, pH 5.0 (1 mL) were treated with testicular hyaluronidase (50 units in 1 mL of buffer) for 1 h at 37° to release oligomers by the method of Cowman *et al.*[24]. The digest was analyzed for oligomers by poly(acrylamide) gel electrophoresis.

Δ-Disaccharide analysis. — Samples of PG (100 μg) were treated[25] either with chondroitinase ABC or ACII in 0.05M Tris·HCl, pH 8.0 (0.025 unit in 20 μL) for 24 h at 37°. The digests were analyzed by cellulose acetate-plate electrophoresis as described by Miyamoto *et al.*[26].

Core protein analysis. — The PG sample (1 mg) in 30mM sodium acetate, 0.1M Tris, 5mM phenylmethylsulfonyl fluoride, 10mM *N*-ethylmaleimide, 10mM EDTA, 0.1M 6-aminohexanoic acid, and 5mM benzamidine·HCl, pH 8, was treated with chondroitinase ABC (0.5 unit) for 24 h at 37°. The reaction mixture was dialyzed, lyophilized, and analyzed by sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS–PAGE).

Cellulose acetate-plate electrophoresis. — PGs or their GAG chains were analyzed by electrophoresis on cellulose acetate plate (6 × 7.5 cm) as described by Capelleti *et al.*[27]. After electrophoresis, the cellulose acetate plates were stained with Alcian Blue.

Poly(acrylamide) gel electrophoresis. — PGs, GAG chains, and GAGs treated with testicular hyaluronidase were analyzed by poly(acrylamide) gel electrophoresis and Alcian Blue staining as described by Cowman *et al.*[28].

Sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS–PAGE). — SDS–PAGE of the protein cores of PG_{IdoA} and PG_{GlcA}, obtained after digestion with chondroitinase ABC as described above, was performed according to the method of Swann *et al.*[16] using a 1% SDS–7% poly(acrylamide) gel electrophoresis and staining with Coomassie Blue.

Viscosity measurements. — The viscosity of PGs was measured as previously described[29].

Proteoglycans (PGs) extraction and fractionation. — The epidermis was removed from the keloid scar tissue by scraping with a blade, and PGs were extracted by the procedure previously[30] described. The extract was fractionated to obtain various PGs by the modified, differential ethanol procedure[16]. The PG_{IdoA} (L-iduronic acid-rich PG) preparation was then fractionated on a Sepharose CL-4B column eluted with 3M Gdm·Cl. The PG_{GlcA} (D-glucuronic acid-rich PG) fraction contained hyaluronan (HA) and was separated from the latter by chromatography on a DE-52 column (18 × 1.5 cm) eluted with 0.2M NaCl (50 mL) in 7M urea–0.5M Tris, pH 6.5 (buffer *A*), and then a linear gradient of 0.2M NaCl in buffer *A* (60 mL) and M NaCl in buffer *A* (60 mL). The PG_{GlcA} fraction was dialyzed against water, lyophilized, rechromatographed on a Sepharose CL-6B column (136 × 1 cm), and eluted with a 3M Gdm·Cl buffer.

Isolation of glycosaminoglycan carbohydrate side-chains. — The PG sample (1 mg) was treated with 50mM NaOH–M $NaBH_4$ (500 μL) for 72 h at 37 ° according to the procedure described by Carlson[31] to release glycosaminoglycan chains.

RESULTS

Proteoglycans from human post-burn scar tissue were extracted under dissociative conditions and fractionated by DEAE-cellulose column chromatography as described previously[30]. The peak containing PGs (Fig. 1, peak B; ref. 30) was further fractionated by the differential ethanol–precipitation method[16,32]. The yields of PG and the composition of the GAG side-chains (analyzed by cellulose acetate electrophoresis, data not given) of the fractions precipitated with 20, 30, 40, 50, and 75% ethanol are given in Table I. The 30 and 40% PG_{IdoA} fractions contained a single PG having a single GAG chain and the same mobility as standard dermatan sulfate (DS) in cellulose acetate electrophoresis. The 75% PG fraction contained hyaluronan (HA) and another

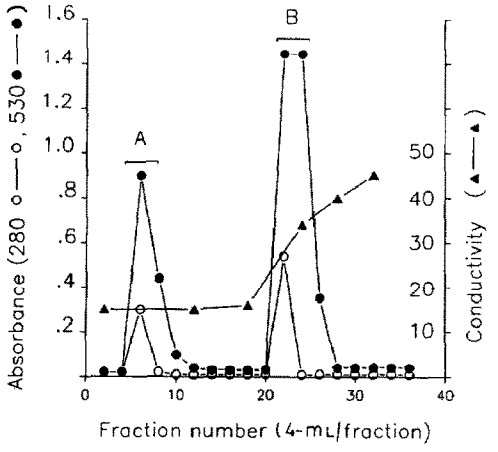

Fig. 1. DE-52 ion-exchange chromatography of the PGs obtained by a 75% ethanol precipitation. The column fractions were analyzed to determine the protein content (A_{280}), the uronic acid content (A_{530}), and the conductivity. The fractions were pooled to yield Fractions A and B.

TABLE I

Yield and composition of PG fractions from human keloid scar obtained by differential ethanol precipitation

Ethanol fraction (% v/v)	*Yield*		*Components detected*[a]				
	(mg)	*% recovered*	*HP*	*DS*	*HS*	*HA*	*CS*
20	2.2	3.8		+	[b]		
30	19.1	36.9		+			
40	18.9	32.5		+			
50	8.5	14.6		+	[b]	[c]	
75	9.4	16.2				+	+

[a] By the presence of an Alcian Blue-stained band having a similar mobility to reference GAG samples. [b] Presence of minor amount of the band. [c] Presence of trace of the band. Abbreviations: HP, Heparin; DS, dermatan sulfate; HS, Heparan sulfate; HA, hyaluronan; and CS, chondroitin sulfate.

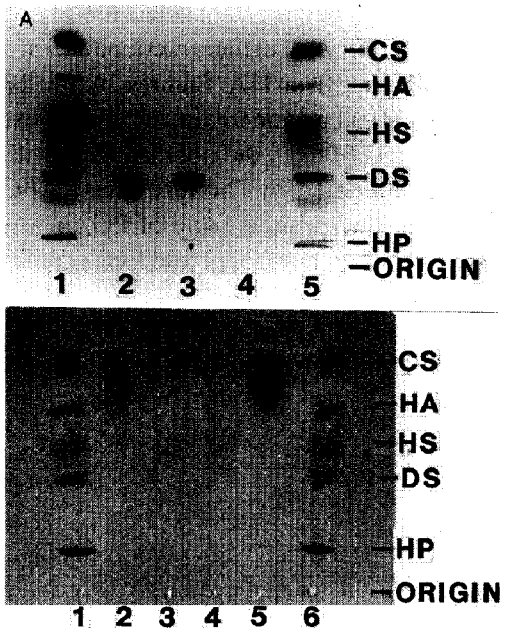

Fig. 2. Cellulose acetate electrophoresis of PG_{IdoA} (A) and PG_{GlcA} (B): (A) Lanes 1 and 5, reference GAGs (heparin, HP; dermatan sulfate, DS; heparan sulfate, HS; hyaluronan, HA; and chondroitin 4- and 6-sulfate, CS); lane 2, PG_{IdoA}; lane 3, PG_{IdoA} digested with chondroitinase ACII; and lane 4, PG_{IdoA} digested with chondroitinase ABC. (B) Lanes 1 and 6, reference GAGs; lanes 2 and 5, PG_{GlcA} : Lane 3, PG_{GlcA} digested with chondroitinase ABC; and Lane 4, PG_{GlcA} digested with chondroitinase ACII.

TABLE II

Amino acid compositions[a] of PG_{IdoA}, PG_{GlcA}, and pig skin CS.PG

Amino acid	*Keloid scar*		*Pig skin*
	PG_{IdoA}	PG_{GlcA}	*CS.PG*[b]
Aspartic acid	119	85	57
Threonine	53	85	51
Serine	81	106	136
Glutamic acid	140	143	150
Proline	84	93	97
Glycine	64	92	151
Alanine	51	70	58
Half-cystine	[c]	[c]	[c]
Valine	53	59	78
Methionine	14	14	[c]
Isoleucine	31	22	35
Leucine	140	46	86
Tyrosine	15	24	9
Phenylalanine	34	38	34
Lysine	53	63	32
Histidine	22	26	10
Arginine	46	36	16

[a] Residues/1000 residues. [b] Results taken from Damle *et al.*[33]. [c] Not determined.

type of PG of higher mobility than standard DS, but having the same mobility as standard chondroitin sulfate (CS). This latter PG fraction was further fractionated on a DEAE-cellulose column (Fig. 1) to give a PG (peak B) free from HA. Figures 2A and 2B show the mobilities of PG_{IdoA} and PG_{GlcA} before and after enzyme treatment in cellulose acetate electrophoresis. The Alcian Blue-positive band of PG_{IdoA} did not disappear with chondroitinase ACII treatment, but was totally eliminated by chondroitinase ABC treatment (Fig. 2A). On the other hand, the Alcian Blue-positive band of PG_{GlcA} was completely digested by both enzymes (Fig. 2B), suggesting that PG_{IdoA} and PG_{GlcA} differ in the structure of the GAG side-chains.

PG_{IdoA} was further purified by chromatography on a Sepharose CL-4B column (Fig. 3A) and eluted as a single peak. Its size was determined by use of a logarithmic plot

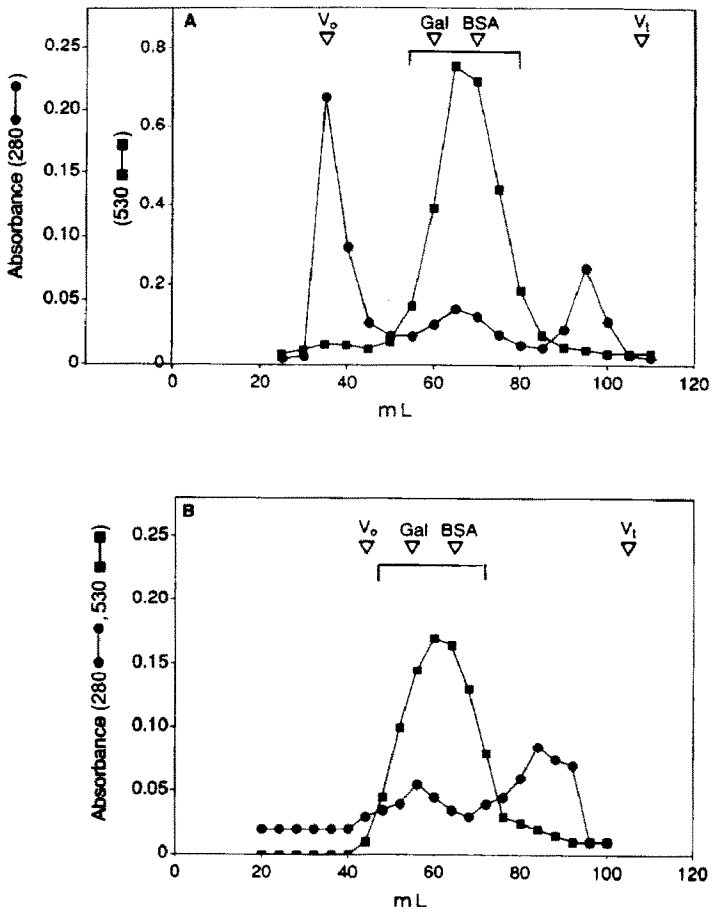

Fig. 3. Elution profiles of PG_{IdoA} from a column (138 × 1 cm) of Sepharose CL-4B and of PG_{GlcA} from a column (136 × 1 cm) of Sepharose CL-6B eluted with 3M Gdm·Cl. The fractions were analyzed for uronic acid content by the carbazole method (A_{530}) and for protein content by u.v. absorbance at 280 nm. The arrows indicate the elution positions of Dextran Blue 2000 (V_0), β-D-galactosidase (Gal, M_r 116 kDa), bovine serum albumin (BSA, M_r 66 kDa), and uronic acid (V_t). The fractions were pooled as indicated.

of K_{av} values (β-D-galactosidase, M_r ~116 kDa; bovine serum albumin M_r ~66 kDa; data not shown) and PG_{IdoA} was estimated as 102 kDa. Similarly, PG_{GlcA} was eluted as a single peak from a Sepharose CL-6B column (Fig. 3B) and its size estimated as 90 kDa. The sizes of both PGs were larger than the size of PG from human dermis and smaller than that of PG from human epidermis[15].

The amino acid compositions of PG_{IdoA}, PG_{GlcA}, and small CS.PG from pig skin[33] are listed in Table II. The proportion of leucine in PG_{GlcA} and CS.PG from pig skin was lower, and that of serine higher, in comparison to PG_{IdoA}. Similar variations were found for human, post-burn, hypertrophic scar copolymeric DS–CS.PG[34]. Although the amino acid composition of the large cartilage PG (PG-LA) is similar to that of PG_{GlcA}, it is different from that of PG_{GlcA} because the GAG chains of PG-LA are attached at the C-terminus[35,36]. The carbohydrate, sulfate, and protein content, and the content of Δ-disaccharides liberated by chondroitinase ABC and ACII are given in Table III. The proportion of L-iduronic acid in PG_{IdoA} and PG_{GlcA} was ~98 and 10%, respectively. Both PGs contained mainly disaccharide 4-sulfates.

The presence of glucosamine and sialic acid suggested that PG_{IdoA} and PG_{GlcA} contain *N*-linked oligosaccharides. Such oligosaccharides were found in calf skin DS–PG previously[11]. The two PGs were smaller than other cartilage PGs and did not aggregate with hyaluronan as determined by viscosity measurements[29].

Alkaline–borohydride treatment of PG_{IdoA} and PG_{GlcA} liberated GAG side-chains. Densitometric tracing of poly(acrylamide) gel electrophoresis (Fig. 4) showed that these GAGs were polydisperse and slightly larger than those from human, post-burn, normal or hypertrophic scar GAGs[16]. The M_r values of PG_{IdoA} and PG_{GlcA} GAG

TABLE III

Chemical compositions of PG_{IdoA} and PG_{GlcA} from human keloid scar

Component	PG_{IdoA}	PG_{GlcA}
IdoA–GalNAC(SO_4)[a]	~98	10
GlcA–GalNAC(SO_4)[a]	<1	90
Hexuronic acid[b]	12.6	11.0
Galactosamine[b]	14.2	12.6
Glucosamine[b]	<1	2.1
Hexose[b]	2.5	4.1
Sialic acid[b]	0.24	3.5
Sulfate[b]	14.0	13.7
Protein[b]	18.5	16.1
Δ-Disaccharides[c]		
Δ-Di-4S	95	96
Δ-Di-6S	<1	[d]
Δ-Di-0S	<1	[d]

[a] Percentage of the total glycosaminoglycan that is IdoA–GalNAC(SO_4) or GlcA–GalNAC(SO_4), as determined from the amounts of unsaturated disaccharides generated by digestion with chondroitinase ABCase and ACIIase. [b] Percentage of the dry weight of PG by procedures described in the Experimental section. [c] Percentage of the total Δ-disaccharides. [d] Not detected.

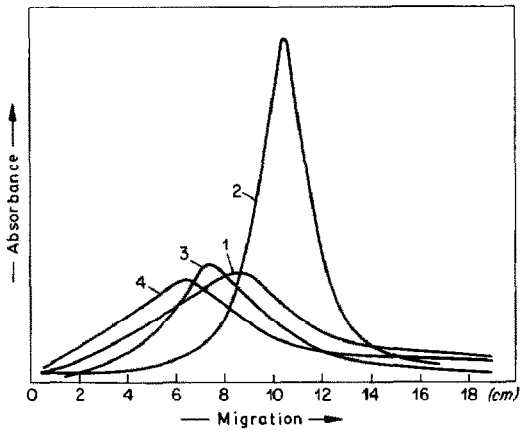

Fig. 4. Densitometric tracings of Alcian Blue-stained poly(acrylamide) gel electrophoresis (PAGE) of GAGs obtained after alkali–borohydride treatment of PG: (1) Reference hypertrophic scar DS–GAG (M_r 23.2 kDa); (2) reference normal scar DS–GAG (M_r 20 kDa); (3) keloid scar PG_{GlcA}-GAG; and (4) keloid scar PG_{IdoA}-GAG.

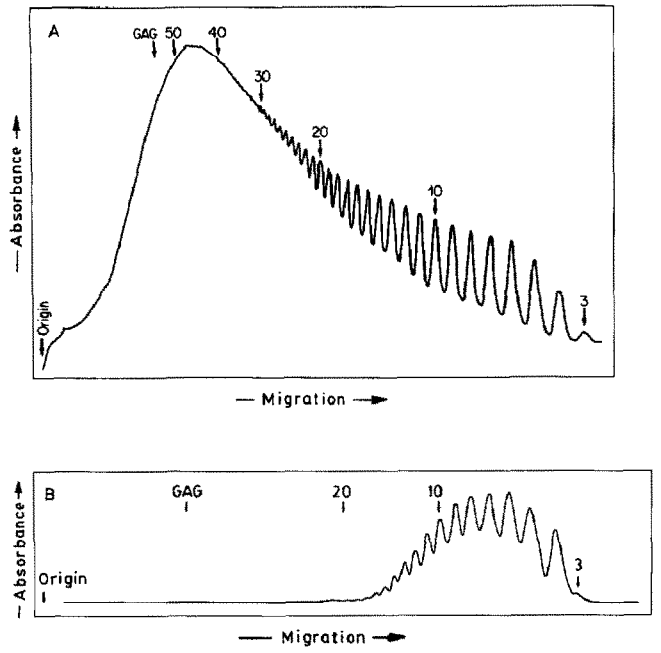

Fig. 5. Densitometric tracings of Alcian Blue-stained PAGE of oligosaccharides released after testicular hyaluronidase treatment of PG_{IdoA} (A) and PG_{GlcA} (B). The arrows marked 10, 20, 30, 40, and 50 indicate the mobility of oligosaccharides that contain 10, 20, 30, 40, and 50 disaccharide units. The arrows marked GAG show the mobility of PG_{IdoA} and PG_{GlcA}-GAG chains.

side-chains were 27 and 26 kDa, respectively, based upon the logarithmic plots of mobility and the sizes of standard GAGs from normal (M_r 20 kDa) and hypertrophic (M_r 23.2 kDa) scars (data not given). Testicular hyaluronidase digestion[24,28] of both PGs yielded different patterns of oligomers (Figs. 5A and 5B). The largest oligosaccharide obtained from PG_{IdoA} GAG chains, after hyaluronidase treatment, contained ~50 disaccharide units, whereas the largest oligosaccharide of GAG chains from PG_{GlcA} had only ~20 disaccharide units. These differences suggested variations in the structure of the GAG chains of the PGs.

PG_{IdoA} was treated with chondroitinase ABC in order to estimate the size of its protein core, and the released core was analyzed by SDS–PAGE. A comparison of the Coomassie Blue-stained bands of the enzyme chondroitinase ABC (Fig. 6B) and chondroitinase ABC-treated PG_{IdoA} (Fig. 6A) showed that the size of the PG_{IdoA} protein core is 14.5 kDa. The size of the PG_{GlcA} protein core obtained by chondroitinase ABC treatment was also 14.5 kDa (Fig. 6C). The ~14 kDa-band was broad owing to different proportions of residual carbohydrate residues linked to the core protein that is obtained after enzyme digestion. The size of the protein cores, although smaller than that of fetal calf skin[14] (56 kDa), is similar to that of human dermis[15] (14–21.5 kDa).

The N-terminal amino acid sequences of PG_{IdoA} and PG_{GlcA} are given in Table IV. Both PGs had the same N-terminal amino acid sequence as that reported for human skin[15] and bone PGII[37].

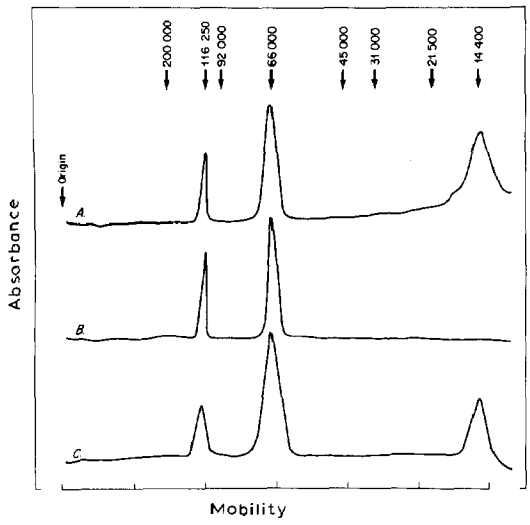

Fig. 6. Densitometric tracings of SDS-PAGE of PG_{IdoA} protein core obtained by digestion of PG_{IdoA} with chondroitinase ABC (A), of chondroitinase ABC (B), and of PG_{GlcA} protein core obtained by digestion of PG_{GlcA} with chondroitinase ABC (C). The arrows mark the peak mobilities of reference peptides: Myosin (200 kDa), β-D-galactosidase (116.25 kDa), phosphorylase B (92 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydride (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

TABLE IV

N-Terminal amino acid sequences of human small PG present in various tissues

Residue No	*Keloid scar*		*Normal skin*		*Hypertrophic scar*		
	PG_{IdoA}	PG_{GlcA}	Epidermis[a]	Dermis[a]	DS[b]	Copolymeric	DS–CS[c]
1	Asp(?)	Asp(?)	Asp	Asp	Asp	Asp	
2	(Glu)	Glu	Glu	Glu	Glu	Glu	
3	Ala	Ala	Ala	Ala	Ala	Ala	
4	[d]	[d]	[d]	[d]	[d]	[d]	
5	Gly	Gly	Gly	Gly	Gly	Gly	
6	Ile	Ile	Ile	Ile		Ile	
7	Gly	Gly	Gly	Gly		Gly	
8		Pro	Pro	Pro		Pro	
9		Glu	Glu	Glu		Glu	
10		Val	Val	Val		Val	
11		Pro	Pro	Pro			
12			Asp	Asp			
13			Asp	Asp			
14			Arg	Arg			
15			Asp(?)	Asp(?)			
16			Phe	Phe			
17			Glu	Glu			
18				Pro			
19				Ser(?)			
20				Leu			

[a] Ref. 15. [b] Ref. 16. [c] Ref. 34. [d] Not detected.

DISCUSSION

Small PGs, which are ubiquitous components of the extracellular matrix, are involved in regulation of collagen fibril diameter through a mechanism thought to involve the protein core[38,39]. Do the structural changes in the composition of small PGs that occur as a result of burn injury affect the remodelling of the tissue and result in different types of scars? The following structural changes may occur: (a) change in the size of the GAG side-chains, (b) degree and location of sulfation, (c) change in the size of core protein, and (d) degree of D-glucuronic acid epimerization at C-5. Earlier studies from this laboratory[16,30] demonstrated that alteration in the amount of PGs, as well as changes in their distribution take place, implying a function for these macromolecules in different types of scar formation. The results presented herein indicate that human, post-burn, keloid scar tissue contains two types of PGs, one rich in L-iduronic acid and the other rich in D-glucuronic acid. The protein cores of these two PGs are similar in size and have identical N-terminal amino acid sequences, but they differ in amino acid composition. The GAG chains of both PGs show some similarity in that they are both mainly 4-sulfated, but they differ in their content of L-iduronic and D-glucuronic acid, as well as in their size. Moreover, these PGs are larger in size than the PGs from normal human skin and from hypertrophic scars.

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