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Iduronic acid-rich proteoglycans (PG_{IdoA}) and human post-burn scar maturation: isolation and characterization ☆

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Abstract

Proteoglycans (PGs) were extracted from human hypertrophic and normal scar tissues from two different stages of maturation after burn injury, under dissociative conditions (4 M guanidinium chloride containing proteinase inhibitors). The extracts were fractionated by ion-exchange chromatography, followed by ethanol precipitation, to give PG-containing iduronic acid (PG_{IdoA}). The size of the PG_{IdoA} decreased with the maturation of scars. Glycosaminoglycan (GAG) chains from PG_{IdoA} were released by alkaline borohydride treatment, and their M_r values were evaluated by polyacrylamide gel electrophoresis. The M_r values for PG_{IdoA} protein cores of the hypertrophic scars (5+ years and 2–5 years) and normal scar (5+ years and 2–5 years) were 22.6, 25, 19 and 21 KDa, respectively. The iduronic acid content of PG_{IdoA} from both types of scar increased in their maturation phase. The M_r values of PG_{IdoA} decreased with maturation. PG_{IdoA} carried the sulfate group mainly attached at C-4 of the 2-amino-2-deoxy-D-galactose residue. The NH_2-terminal amino acid sequences of all the PG_{IdoA} were similar to those of normal human skin or bone PG II (decorin) (i.e., Asp-Glu-Ala-B-Gly-Ile-Gly-Pro-Glu-Val-Pro-Asp-Asp-Arg).

Keywords: Iduronic acid; Proteoglycan; Burn scar; Collagen formation

1. Introduction

Remodeling of human burn tissues involving dermis results in three different types of scar: normal, hypertrophic, or keloid [2]. Hypertrophic scars can be distinguished from

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Abbreviations: GAG, glycosaminoglycan; M_r, molecular mass; PG, proteoglycan; PG_{IdoA}, iduronic acid containing proteoglycan; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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normal scars in that they are elevated and hard to the touch, while normal scars are flat and soft [3,4]. Proteoglycans (PGs) are important macromolecules present in skin [5–10]. Changes in the amounts and distributions of proteoglycan containing iduronic acid PG_{IdoA} in different scar tissues, in comparison to normal skin, have recently been reported [11]. These alterations are reflected in the disorganization of collagen bundles formed during the repair process. PG_{IdoA} binds noncovalently to the surface of collagen fibrils [12–16] and may be involved in this process. There is evidence that PG_{IdoA} may play a role in collagen fibrillogenesis of type I and type II collagens, in vitro [17,18]. A recent study reported that the core protein of the leucine-rich PG (decorin) interacts with collagen fibrils [19]. These findings suggest that PG_{IdoA} plays an important role in regulation of collagen fiber formation. To understand the role of PG_{IdoA} in scarring, particularly after burn injury, we have isolated PG_{IdoA} from different scar tissues [20,21]. Clinically, scars progress towards stability (i.e., lack of change with time). Immature scars are raised, red, and firm. Normal scars become flat, white, and soft as they mature. In contrast, hypertrophic scars become raised, hard, red, stiff, flaky, and itchy as they mature [3,22]. The objective of this study, therefore, was to determine the changes in PG_{IdoA} at two stages of human scar maturation. PG_{IdoA}s from normal and hypertrophic scars at two stages of maturation (i.e., 2–5 years: changing, stable, and immature; and 5+ years: unchanging, stable, and mature) [22] after burn injury have been isolated and characterized. These two stages were chosen because of the difficulty of obtaining the human tissues at more frequent intervals.

2. Experimental

Materials.—Normal and hypertrophic scar samples were obtained after surgery from Shriners Burns Institute, Boston. The ages of the scars were 2–5 years and 5+ years after burn injury, and they were from ~11 year old female and male patients. The number of patients in each group was five. Guanidinium chloride (Gdm.Cl) ultrapure quality was from Schwartz/Mann Biotech; benzamidine hydrochloride and 6-aminohexanoic acid were from Aldrich Chemical Co.; and phenylmethanesulfonyl fluoride, *N*-ethylmaleimide, ethylenediaminetetracetic acid (EDTA), Δ-di-4*S*-[2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic) acid D-galactose 4-sulfate], and Δ-di-6*S*-[2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose-6-sulfate] were from Sigma Chemical Co.; chondroitinases ABC and AC and chondroitin 4- and 6-sulfate were from Miles Laboratories. Glycosaminoglycan (GAG) standards were supplied by Drs. M. Matthews and J.A. Cifonelli, University of Chicago (Contract No. 1-AM-5-22-5 from NIH, Bethesda, MD). DEAE-cellulose ion-exchange resin (DE-52) was from Whatman Inc., and Sepharose CL-6B was from Pharmacia LKB.

Extraction of proteoglycans (PGs).—The fat and epidermis from scar tissues were removed by scraping with a blade, and PGs were extracted as previously described [11]. The scar tissues are readily extractable by Gdm.Cl (~80%) in dissociative conditions [23]. Briefly, the tissue (10 mL/g) was extracted twice with 4 M Gdm.Cl buffer containing proteinase inhibitors [sodium azide (0.02%, w/v), 10 mM EDTA, 100 mM 6-amino hexanoic acid, 5 mM benzamidine hydrochloride, 0.5 mM *N*-ethylmaleimide, and 1 mM phenylmethanesulfonyl fluoride]. The tissue was extracted for 24 h at 4°C. The extract was

separated from the residual tissue by centrifugation and dialyzed against water until free from Cl^- ions, and equilibrated with 7 M urea, 0.5 M Tris, pH 6.5 buffer A. PGs were obtained by chromatography on a DE-52 (Whatman) column eluted with buffer *A*, 0.2 M NaCl in buffer *A*, and then a gradient of 0.2 M NaCl in buffer *A* and 1 M NaCl in buffer A. The fraction containing PG was dialyzed against water and lyophilized.

Fractionation of PGs.—The above fraction containing PG was fractionated by modified differential ethanol precipitation procedure [24] to give pure PG_{IdoA}.

Sepharose CL-6B gel filtration.—PG_{IdoA} fractions were chromatographed on a Sepharose CL-6B column (92×2.5 cm), and eluted with a 3 M Gdm.Cl buffer. The fractions were analyzed for uronic acid content.

Analytical procedures.—The protein and uronic acid contents were determined by measuring the absorbance at 280 nm, and by a carbazole reaction [25], respectively. The *N*-terminal amino acid sequence of the PG_{IdoA} samples were determined with an Applied Biosystems Model 477A gas-phase sequencer with online HPLC detection of PTH-amino acids. Protein content was determined by the Lowry method [26] and hexose content by anthrone reactions [27]. Hexosamines were analyzed colorimetrically by modified Morgan–Elson procedures [28]. The sulfate content of PG samples was measured by the method of Antonopoulos [29].

Isolation of glycosaminoglycan (GAG) carbohydrate side-chains in PG_{IdoA}.—The PG_{IdoA} sample (1 mg) was treated with 50 mM NaOH–1 M $NaBH_4$ (500 μL) for 72 h at 37°C to obtain GAG chains, according to the procedure of Carlson [30].

Δ-Disaccharide analysis.—PG_{IdoA} samples (100 ug) were treated [31] either with chondroitinase ABC or AC II in 0.05 M Tris·HCl, pH 8.0 (0.025 unit in 20 μL) 24 h at 37°C. The digests were analyzed by cellulose acetate plate electrophoresis as described by Miyamoto et al. [32].

Electrophoresis on cellulose acetate plates.—PG_{IdoA} or their GAG chains were analyzed by electrophoresis on a cellulose acetate plate (6×7.5 cm), as described by Capelleti and co-workers [33]. After electrophoresis, the PG_{IdoA}/GAGs were visualized after staining with Alcian Blue.

Molecular weight determination of GAG chains by polyacrylamide gel electrophoresis (PAGE).—The above isolated GAG chains were then analyzed by PAGE and the Alcian Blue staining method described earlier [21].

Isolation of PG_{IdoA} protein core.—PG_{IdoA} sample (1 mg) in buffer (30 mM sodium acetate, 0.1 M Tris, 5 mM phenylmethanesulfonyl chloride, 10 mM *N*-ethylmaleimide, 10 mM EDTA, 0.1 M 6-aminohexanoic acid, and 5 mM benzamidine hydrochloride, pH 8.0) (1 mL) was treated with chondroitinase ABC (0.5 unit) for 24 h at 37°C. The digest was dialyzed and lyophilized, and the protein core was analyzed by gel electrophoresis[34].

Sodium dodecyl sulfate–polyacrylmaide gel electrophoresis (SDS–PAGE).—SDS–PAGE on 1% SDS-7% poly(acrylamide) gel was performed as described earlier [34]. Gels of PG_{IdoA} protein cores obtained after digestion of PG_{IdoA} with chondroitinase ABC as described above were stained with Coomassie Blue [35].

3. Results

Studies in human skin [36] and scar tissues [24] have shown that there are differences in the distribution, type, and structure of PG_{IdoA}. However, at what stage of the repair process

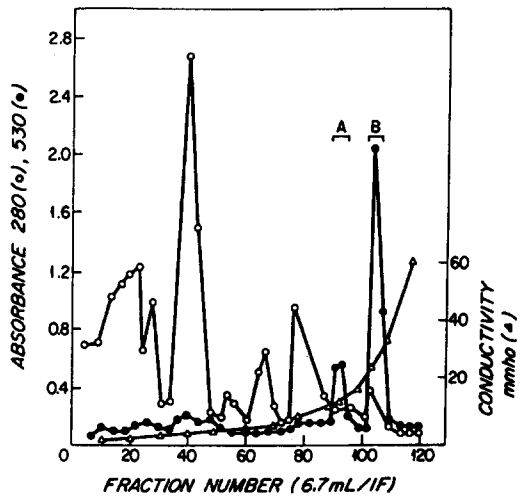

Fig. 1. DEAE-cellulose ion-exchange chromatography of constituents from hypertrophic scar, age 5 + years after burn injury and extraction with 4 M Gdm.Cl. The column fractions were analyzed to determine the uronic acid content (●), protein content A 280 nm (○), and the conductivity (Δ). The uronic acid positive fractions were pooled to yield fraction A (HA) and B (PGs).

these changes occur and how these changes influence collagen fibril assemply (i.e., scarring and its maturation) is unclear. To understand the role of PGs in scar maturation, we have now studied the changes in PG_{IdoA} structure and distribution from hypertrophic and normal scars at two intervals after burn injury: 2–5 years and 5 + years. PGs were isolated from human hypertrophic and normal scars by extraction with 4 M Gdm.Cl containing proteinase inhibitors, followed by their fractionation on a DEAE-cellulose column. The elution profile of hypertrophic scar (5 + years) is shown in Fig. 1. The two uronic acid-positive peaks (A and B) were pooled. Peak A contained hyaluronan (HA) and peak B contained PGs. The elution profile of the other scar tissues were similar (data not given). The distribution of HA, PGs, and protein is summarized in Table 1. The percent yields of the total constituents of PG fractions decreased as scars matured for both types; conversely, the percent yield of

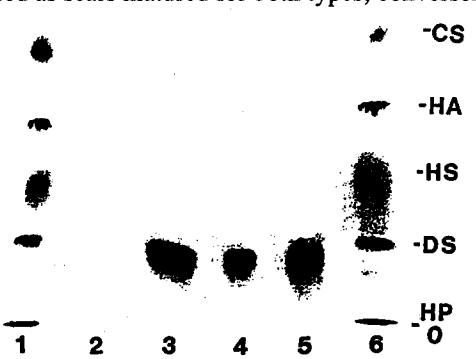

Fig. 2. Cellulose acetate electrophoresis of PG_{IdoA} from hypertrophic scar (5 + years) stained with Alcian Blue. Lanes 1 and 6, reference GAGs (heparin, HP; dermatan sulfate, DS; heparan sulfate, HS; hyaluronan, HA; and chondroitin 4- and 6-sulfate, CS); lane 2, PG_{IdoA} digested with chondroitinase ABC; lane 3, PG_{IdoA} digested with AC II and lanes 4 and 5 PG_{IdoA} from 30 and 40% ethanol precipitated fractions, respectively.

Table 1
Recovery of non-uronic acid constituents from normal and hypertrophic scar tissues at two different stages of maturation from pooled tissue samples

Type of scar tissue from patients of 11+ years of age	Age of the scar after burn injury (years)	Constituents (mg/g wet weight of the tissue)					
		Protein		Hylauronate		Proteoglycan	
		(mg)	(%)	(mg)	(%)	(mg)	(%)
Hypertrophic	2–5	10.42	81.34	1.03	8.04	1.36	10.62
Hypertrophic	5+	12.74	87.62	0.52	3.58	1.28	8.80
Normal	2–5	17.65	90.42	0.60	3.07	1.27	6.51
Normal	5+	15.59	89.50	0.81	4.70	1.01	5.8

protein content in hypertrophic scar increased. Overall, small differences between hypertrophic and normal scar tissue were found. PG fractions from different stages of maturation were fractionated by dissolving in 4 M Gdm.Cl and sequentially precipitating with 20, 30, 40, 50, and 75% (v/v) ethanol to obtain pure PG_{IdoA}. The recoveries of PGs from different fractions are given in Table 2. In the hypertrophic scar (both 2–5 and 5+ years groups), the 30 and 40% ethanol-precipitated fraction contained pure PG_{IdoA}. However, in the normal scar (5+ year group), the 30, 40, and 50% fractions contained pure PG_{IdoA}, and 30 and 40% for the 2–5 year group, as evaluated by cellulose acetate plate electrophoresis (data not shown)[33]. The 30 and 40% PG_{IdoA} obtained from hypertrophic scar (5+ years) contained pure PG_{IdoA}, with the same mobility as standard dermatan sulfate in cellulose acetate plate electrophoresis (Fig. 2). The purity of the PG_{IdoA} fractions was further established by treatment of these with chondroitinases AC and ABC under the conditions described earlier [34]. Chondroitinase AC treatment of PG_{IdoA} produced no change, whereas chondroitinase ABC treatment of PG_{IdoA} totally digested the Alcian Blue band. The pure PG_{IdoA} fractions used for subsequent analysis were representative of the same cuts from ethanol fraction (i.e., 30%).

Elution profiles of dissociative Sepharose CL-6B chromatography (data not given) of PG_{IdoA} from different scar tissues showed a single broad peak. The K_{av} values of these from normal scar (5+ years, and 2–5 years) were 0.47 and 0.50, and PG_{IdoA} from hypertrophic scar (5+ years and 2–5 years) were 0.48 and 0.49, respectively. This data indicates that PG_{IdoA} in the mature stage of scar formation (i.e., 5+ years after burn injury) had very minor differences in comparison to PG_{IdoA} from immature scars (i.e., 2–5 years after burn injury).

A comparison of the chemical composition of the pure PG_{IdoA} (Table 3) shows that PG_{IdoA} from mature scar contained more iduronic acid. The presence of *N*-acetylneuraminic acid and neutral sugar suggests that all the PG_{IdoA} contained oligosaccharides. Such oligosaccharides were previously found in calf skin[10] and scar tissues [24]. The results of Δ-disaccharide analysis, obtained after PG_{IdoA} treatment with chondroitinase ABC, indicate that all the PG_{IdoA} are mainly 4-sulfated. Minor differences were found in the protein content of PG_{IdoA} with increases in the maturation phase of the scar tissues after burn injury.

Following alkaline borohydride treatment, GAG chains were released from PG_{IdoA}. The M_r values of these GAG chains were determined by PAGE and plotting the logarithm of

Table 2
Yield and distribution of PGs in different ethanol precipitated fractions

Scar Tissue (ethanol fraction % v/v)	Yield (total recovery of PGs as ~90% in all the fractions) mg (% recovered)	Component detected [a]				
		HP	DS	HS	HA	CS
Hypertrophic (2–5 years)						
20	6.03 (9.97)	[c]	+	[c]		
30	20.67 (34.16)		+			
40	8.02 (13.25)		+			
50	10.69 (17.67)		+	[c]	[c]	
75	15.10 (24.95)			[c]	[b]	+
Hypertrophic (5+ years)						
20	0.94 (2.43)	[c]	+	[c]		
30	11.85 (30.64)		+			
40	8.25 (21.33)		+			
50	8.98 (23.72)		+	[c]	[c]	
75	8.65 (22.37)				[b]	+
Normal (2–5)						
20	26.13 (29.63)		+			
30	26.43 (29.97)		+			
40	9.72 (11.02)		+			
50	11.94 (13.54)		[b]	[b]	[b]	
75	13.96 (15.83)				[b]	+
Normal (5+ years)						
20	9.85 (5.03)	[c]	+			
30	65.93 (33.69)		+			
40	28.14 (14.38)		+			
50	52.79 (26.97)		+			
75	39.00 (19.93)			[c]	[c]	+

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

the M_r values of standards against the R_f values (Fig. 3). The data suggests that M_r values of GAG chains decreased as scars matured. All the PG_{IdoA} were treated with chondroitinase ABC to obtain their protein cores. Analysis of the protein cores was carried out using SDS–PAGE [34]. The molecular size of the major peptides from all PG_{IdoA} were similar (i.e., 21–22 kDa and a second component of M_r value 17–17.5 kDa). The size of the protein cores were the same as reported by us earlier [24] in pooled scar tissues from different stages of maturation. This suggests that the protein core of PG_{IdoA} does not change during maturation phase of the scar tissue.

The NH_2-terminal amino acid sequences of the PG_{IdoA} from different scar tissues were identical to those reported for decorin (PG-II containing one GAG chain) from human skin [36], scar [24], and bone [37], but differed from bovine skin after A9 [38]. The yield of different amino acid residues was reasonably good. Hypertrophic scar had a noticeable percent of biglycan (PG-I containing two GAG chains), whereas normal scar did not. Attempts to free PG-II from PG-I contamination were unsuccessful.

Table 3
Chemical composition of PG_{IdoA}

Component	Scar			
	Hypertrophic		Normal	
	5+ years	2–5 years	5+ years	2–5 years
IdoA-GalNAc (SO_4) [a]	~90	~80	~88	~79
GlcA-GalNAc (SO_4) [a]	<5	<12	<10	<12
Hexuronic acid [b]	15.24	17.16	15.24	16.3
Hexosamine [b]	16.31	16.75	15.01	16.07
Hexose [b]	2.51	2.22	2.13	2.25
Sialic acid [b]	0.24	0.45	0.25	0.22
Sulfate [b]	9.3	9.1	8.2	8.0
Protein [b]	20.2	22.8	19.6	21.4
Δ-Disaccharide [c]				
Δ-D14S	92	91	90	92
Δ-D16S	2	4	6	4
Δ-D1-0S	[d]	3	[d]	[d]
M_r, values of protein core released after CSABCase digestion [e]	21–22	21–22	21–22	21–22

[a] Percentage of the total GAG that is IdoA-GalNAc(SO_4) or GlcA-GalNAc(SO_4) as determined from the amounts of Δ-disaccharides obtained after digestion with chondroitinase ABCase and ACase.
[b] Percentage of the dry weight of PG.
[c] Percentage of the total Δ-disaccharides.
[d] Not detected.
[e] A second component of M_r value 17–17.5 kDa was present in all the PG_{IdoA} preparations.

4. General discussion

Proteoglycan (PGs), a diverse group of heterogeneous glycoconjugates that consist of a protein core to which different GAG chains and oligosaccharides are covalently attached [39–41], are abundant in the extracellular matrix of skin and scar tissues [20].

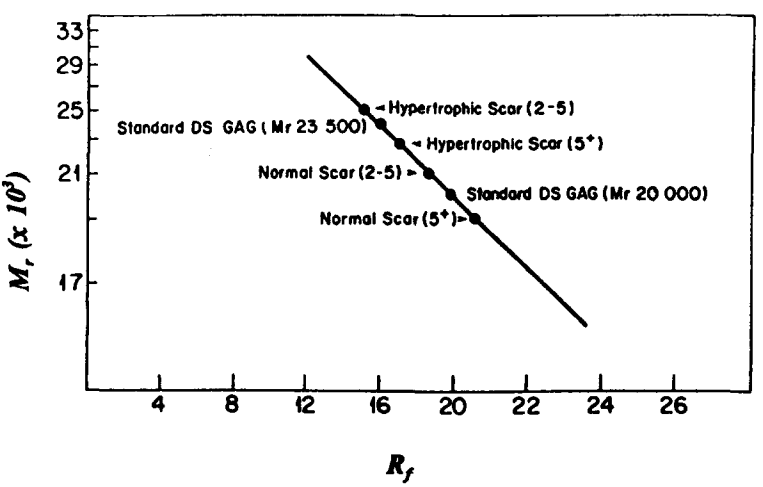

Fig. 3. Molecular weight determination of DS GAG chains obtained by alkaline borohydride cleavage from PG_{IdoA} of different scar tissues by SDS–PAGE.

Wounds produced by excessive heat are complex and their repair involves a series of constituents [42]. PGs and/or their GAG side chains are involved in the early stages of wound healing [43,44] and also in subsequent reorganization of the matrix constituents in scar formation and maturation [42]. In summary, PGs appear to act upon the wound healing process for a fairly long period. Hence, a minor modification in the structure ofPGs, either in the size of their protein core and/or GAG chains or degree of sulfation, may influence the type of scar formation and maturation (a process involving the formation of disorganized collagen bundles). Recently, evidence has been presented which demonstrates that a small PG (decorin) protein core interacts with collagen fibrils [19].

Several studies in this laboratory [11,24,34] have demonstrated that PG alterations occur in normal and hypertrophic scars. The objective of this study was to define the stages of scar maturation where changes in PG structure take place. The data reported here show that the following structural changes in PG_{IdoA} occur in the maturation phase of both normal and hypertrophic scars: (*a*) the size of the GAG chains decreases, (*b*) the degree of epimerization of the C-5 of D-gluuronic acid residue to L-iduronic acid increases on the basis of IdoA-GalNAc (SO_4) released after digestion with chondroitinase ABC, and (*c*) the amounts of PG decrease in mature tissue. The time scales, i.e., 2–5 and 5+ years, were chosen because: (*a*) tissues at different time intervals are hard to obtain and (*b*) Engrav [22] suggested that these time intervals are when the scar is maturing (2–5 years) and fully matured (5+ years).

5. Conclusions

In conclusion, the data presented in this study suggests that alteration in the structure of PG_{IdoA} occurs during the process of scar formation and maturation. Knowledge of these changes contributes towards our understanding of the biochemical processes involved in burn scarring and will hopefully aid our goal of reducing the scarring that generally follows burn injury.

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