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Age-related differences in human skin proteoglycans

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Previous work has shown that versican, decorin and a catabolic fragment of decorin, termed decorunt, are the most abundant proteoglycans in human skin. Further analysis of versican indicates that four major core protein species are present in human skin at all ages examined from fetal to adult. Two of these are identified as the V0 and V1 isoforms, with the latter predominating. The other two species are catabolic fragments of V0 and V1, which have the amino acid sequence DPEAAE as their carboxyl terminus. Although the core proteins of human skin versican show no major age-related differences, the glycosaminoglycans (GAGs) of adult skin versican are smaller in size and show differences in their sulfation pattern relative to those in fetal skin versican. In contrast to human skin versican, human skin decorin shows minimal age-related differences in its sulfation pattern, although, like versican, the GAGs of adult skin decorin are smaller than those of fetal skin decorin. Analysis of the catabolic fragments of decorin from adult skin reveals the presence of other fragments in addition to decorunt, although the core proteins of these additional decorin catabolic fragments have not been identified. Thus, versican and decorin of human skin show age-related differences, versican primarily in the size and the sulfation pattern of its GAGs and decorin in the size of its GAGs. The catabolic fragments of versican are detected at all ages examined, but appear to be in lower abundance in adult skin compared with fetal skin. In contrast, the catabolic fragments of decorin are present in adult skin, but are virtually absent from fetal skin. Taken together, these data suggest that there are age-related differences in the catabolism of proteoglycans in human skin. These age-related differences in proteoglycan patterns and catabolism may play a role in the age-related changes in the physical properties and injury response of human skin.

Keywords: decorin / glycosaminoglycans / proteoglycans / skin / versican

Introduction

The dermis is a tissue that contains an extensive extracellular matrix, and its physical properties are determined primarily by this matrix. Although collagen is the major extracellular matrix molecule of the dermis (Pinnell and Murad 1983), other molecular components are present and these components also contribute to the overall mechanical properties of skin. Among the non-collagenous components of the dermis are proteoglycans (Bianco et al. 1990; Meyer and Stern 1994; Zimmermann et al. 1994; Bernstein et al. 1995; du Cros et al. 1995; Bode-Lesniewska et al. 1996; Ågren et al. 1997; Danielson et al. 1997; Carrino et al. 2000; Bayer-Garner et al. 2002). Previous analysis has shown that the most abundant proteoglycans in extracts of postnatal human skin are decorin and versican (Carrino et al. 2000). In addition, extracts of postnatal human skin also contain a catabolic fragment of decorin, termed decorunt, whereas virtually no decorunt, if any, is detected in extracts of fetal human skin (Carrino et al. 2003). Another age-related difference observed with extracts of human skin is the presence of a larger proportion of versican in fetal compared with adult skin (Carrino et al. 2000). Immunohistochemical analysis has revealed that versican colocalizes with elastic fibers in skin (Zimmermann et al. 1994; Bernstein et al. 1995), whereas decorin is known to interact with collagen (Brown and Vogel 1989), to colocalize with collagen in skin (Fleischmajer et al. 1991) and to be crucial for robust tensile strength of skin through the establishment of a sound collagenous network (Danielson et al. 1997).

Because the mechanical properties of the dermis change as a function of age (Daly and Odland 1979; Takema et al. 1994), and because the physical properties of the dermis are determined principally by the extracellular matrix (Motokawa and Tsuchi 2003; Ruggiero et al. 2005), age-related changes in dermal extracellular matrix molecules are expected to be involved in age-related changes in the mechanical properties

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of skin. Age-related differences have been reported for dermal collagen (Bentley 1979; Lavker et al. 1987; Mays et al. 1988; Yamauchi et al. 1988; Moragas et al. 1998). In order to extend previous results regarding age-related differences in skin proteoglycans (Carrino et al. 2000), versican and decorin were isolated and analyzed from extracts of human skin obtained from various ages. The analyses indicate that there are differences between fetal and adult skin in the relative amounts of versican and decorin, in the size and sulfation patterns of the glycosaminoglycans (GAGs) of versican and in the size of the GAGs of decorin. These age-related differences in human skin versican and decorin have the potential to result in functional differences in these molecules and, hence, may be involved in the age-related changes in the biological properties of human skin.

Results

Core proteins

The antibodies and antisera that were used to analyze the core proteins of versican interact with defined regions of the core protein (Figure 1). Monoclonal antibody 12C5 recognizes an epitope in the G1 region of versican (Asher et al. 1995). The epitope for monoclonal antibody 2B1 (Isogai et al. 1996) is in the G3 region (K Kimata, personal communication). The antisera anti-GAG-α, anti-A and anti-D were raised against peptides from the chondroitin sulfate-attachment region of versican. Anti-GAG-α recognizes a peptide near the carboxyl-terminal end of the GAG-α region (Dours-Zimmermann and Zimmermann 1994), whereas anti-A and anti-D react with, respectively, peptides near the amino-terminal end and at the carboxyl-terminal end of the GAG-β region (Zimmermann et al. 1994). Anti-DPEAAE and anti-NIVSFE are neoepitope antisera (Sandy et al. 2001). As such, the peptides that they recognize, DPEAAE and NIVSFE, respectively, must occur at the carboxyl terminus in order for the antisera to react (Sandy et al. 2001). These sequences were selected for antisera production because of similarity to known ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs)-mediated cleavage sites in human aggrecan and rat brevican (Sandy et al. 2001). The NIVSFE sequence occurs near the amino-terminal end of the GAG-α region, and it is therefore present in versican V0 and V2, but not V1 (Dours-Zimmermann and Zimmermann 1994). Cleavage at this site by an ADAMTS generates a species of ~66 kDa from both V0 and V2, which reacts with the anti-NIVSFE neoepitope antiserum (Westling et al. 2004). The DPEAAE sequence occurs near the amino-terminal end of the GAG-β region, and it is therefore present in versican V0 and V1, but not V2 (Dours-Zimmermann and Zimmermann 1994). This sequence is within the peptide recognized by anti-A (Zimmermann et al. 1994). Cleavage at this site by an ADAMTS generates a species of ~66 kDa from V1 and a much larger product from V0, ~250 kDa in the results reported herein. Both of these species are detected by the anti-DPEAAE neoepitope antiserum (Sandy et al. 2001).

The results of the versican core protein analyses are summarized in Figure 1 as indicated by the reactivities with the various antibodies. The bands identified in the versican

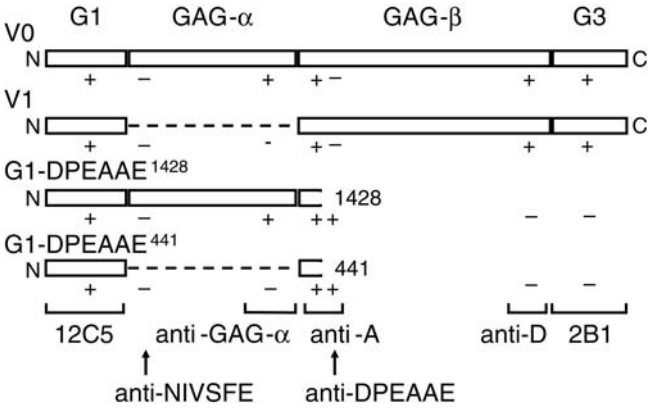

Fig. 1. Antibody binding to the core protein of human versican. Shown diagrammatically are the four major versican core protein species observed in human skin and the locations at which various antibodies bind. N and C at each end of the core protein diagram indicate the amino and carboxyl termini, respectively. The brackets for those antibodies whose exact epitope is not known (12C5 and 2B1) span the region in which the epitope is located. The brackets for the polyclonal antisera (anti-GAG-α, anti-A and anti-D) span the peptides used to raise the antisera. G1, G3, GAG-α and GAG-β refer to the different regions of the versican core protein. Also shown are the reactivities of the various antibody probes to the different human skin versican molecules (+, reactivity observed; −, no detectable reactivity).

immunoblots as bands 1–4 correspond, respectively, to the V0 isoform of versican, the V1 isoform, a catabolic fragment of V0 and a catabolic fragment of V1. Irrespective of the donor age, human skin samples show four major versican core protein bands on immunoblots probed with monoclonal antibody 12C5 (Carrino et al. 2000; Figures 2–4, and data not shown). Three of these, bands 1–3 (Figure 2), are recovered in diethylaminoethyl (DEAE) pool 2 (i.e. the high-salt eluate of the column), whereas one of these, band 4 (Figure 3), is recovered in DEAE pool 1 (i.e. the low-salt eluate of the column) and appears to lack chondroitin sulfate (Sorrell et al. 1999). In all the samples, fetal or adult, band 1 is much fainter than bands 2 and 3 (Figures 2 and 4).

Like 12C5, anti-A recognizes all three of the versican core protein isoforms recovered in DEAE pool 2 (bands 1–3, Figure 2A). In contrast, anti-D recognizes only bands 1 and 2, whereas anti-GAG-α recognizes only bands 1 and 3 (Figure 2A). The versican molecule recovered in DEAE pool 1 (i.e. band 4 of Figure 3) is not recognized by anti-D or anti-GAG-α, but is recognized by anti-A (data not shown). Another monoclonal antibody to the core protein of versican, 2B1, gives a pattern of reactivity like that of anti-D, that is, bands 1 and 2 are recognized whereas bands 3 and 4 are not recognized (Sorrell et al. 1999 and data not shown). Taken together, these data indicate that bands 1 and 2 contain both the amino- and the carboxyl-terminal regions of versican, whereas bands 3 and 4 lack the carboxyl-terminal regions (Figure 1).

The neoepitope antiserum anti-DPEAAE recognizes only bands 3 and 4 (Figures 2 and 3), which suggests that these bands are catabolic fragments of versican. Reactivity of bands 3 and 4 with anti-DPEAAE is consistent with these bands lacking the carboxyl-terminal regions of versican. A second

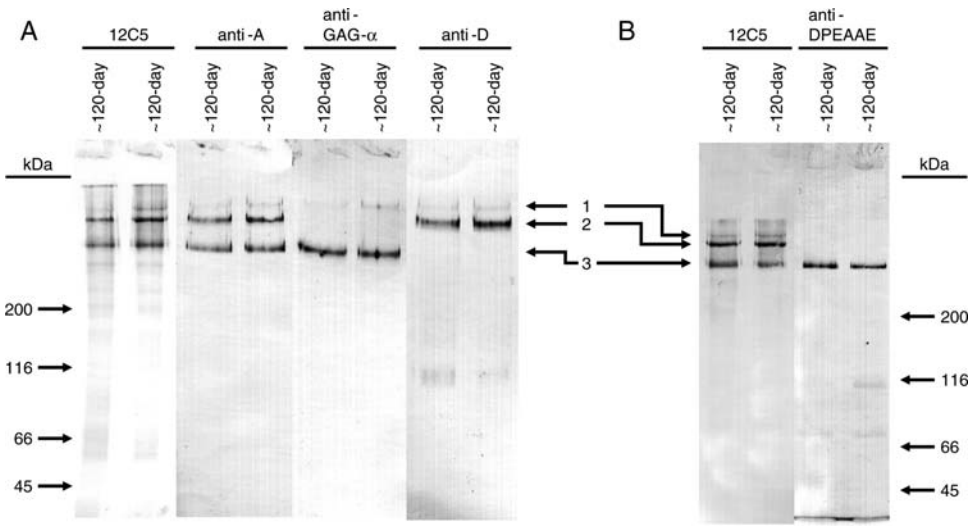

Fig. 2. Versican core protein immunoblot analysis: DEAE pool 2. Proteoglycans isolated from two different samples of human fetal skin of 120 days EGA were eluted from an anion exchange column with 1 M sodium chloride, treated with chondroitinase ABC, subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS–PAGE) on a 5% gel and electrotransferred to Immobilon-P. Separate blots were probed with the indicated antibody or antiserum. The blots in (**A**) are from a blot transferred from one gel, whereas the blots in (**B**) are from a different gel. The three major bands detected with 12C5 are labeled 1, 2 and 3.

versican neoepitope antiserum, anti-NIVSFE, shows no reactivity with samples of human skin proteoglycans, although a positive control from human hippocampus shows the expected 66-kDa band (data not shown). Importantly, the only major bands detected by 2B1 and anti-D are bands 1 and 2 (Figure 2 and data not shown); no other major bands are detected. The pattern of reactivity obtained with anti-D and 2B1, which recognize different areas in the carboxyl region of versican, suggests that the carboxyl portions of versican that are generated by cleavage at the DPEAAE site are not retained as such within the tissue, since such fragments should contain chondroitin sulfate and, hence, should be recovered in DEAE pool 2.

The results with samples of adult skin are qualitatively similar to those obtained for fetal skin in that bands 1–3 are detected on 12C5 immunoblots of DEAE pool 2 (Figure 4, left panel). Like fetal samples, only bands 1 and 2 of adult samples are recognized by 2B1 (Figure 4, middle panel), and only band 3 of adult samples is recognized by anti-DPEAAE (Figure 4, right panel). On 12C5 immunoblots, band 3 of adult samples has a lower intensity relative to bands 1 and 2 than is observed for fetal samples (Figure 4, left panel).

The core proteins of decorin were also examined in proteoglycan samples from skin of various ages (Figure 5). For the detection of human decorin core protein, immunoblots were probed with monoclonal antibody 6B6, which recognizes amino acids 57–65 of the core protein (Sawada et al. 2002). After chondroitinase treatment of DEAE pool 2, 6B6 immunoblots revealed somewhat different patterns of decorin core proteins for fetal and adult samples. The fetal samples that were examined show two major decorin core protein bands migrating at ~45 kDa (Figure 5). In contrast, the adult samples, which range in age from 19 to 82 years, show a third decorin core protein band that migrates ahead of the other two bands (Figure 5). For this study, it was possible to obtain only one postnatal sample, which is 5 weeks of age. This sample shows a pattern of decorin core proteins like that of fetal skin rather than adult skin (Figure 5). The 6B6-positive band migrating at ~14 kDa in the adult samples is the core protein of the decorin catabolic fragment, decorunt (Carrino et al. 2003).

Glycosaminoglycans

The chondroitin/dermatan sulfate of the major proteoglycans extracted from human skin (versican, decorin and decorunt) was analyzed after isolation of each of these proteoglycans. The initial step in fractionating the different proteoglycans for analysis of their GAGs involves separation of the large proteoglycans (i.e. versican) from the small proteoglycans (decorin, decorunt and biglycan) by Sepharose CL-2B chromatography. This fractionation indicates that fetal skin contains a larger proportion of versican than adult skin. As a proportion of the total GAGs measured by Safranin O, fetal skin contains $33.7 \pm 7.9\%$ of its GAGs as versican [$n = 3$, age range 85–103 days estimated gestational age (EGA)], whereas adult skin contains $3.3 \pm 1.6\%$ versican ($n = 14$, age range 20–56 years; data not shown). The larger proportion of versican GAGs in fetal skin compared with adult skin correlates with published results based on toluidine blue staining intensity on sodium dodecyl sulfate polyacrylamide electrophoresis (SDS–PAGE) and on composite agarose–polyacrylamide gels (Carrino et al. 2000). This difference is also observed in the staining intensity for versican core proteins on SDS–PAGE and composite gel immunoblots (Carrino et al. 2000).

There are several differences in the disaccharide patterns of the chondroitin/dermatan sulfate of versican from fetal skin compared with adult skin (Table I). One major difference is in the ratio of the two most abundant disaccharides, ΔDi6S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*

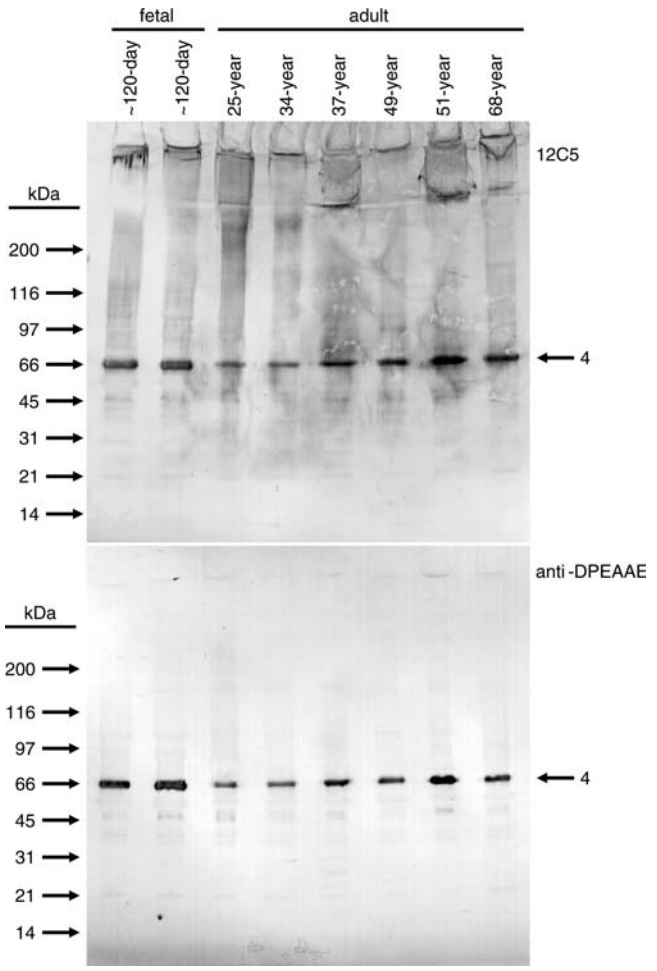

Fig. 3. Versican core protein immunoblot analysis: DEAE pool 1. Material isolated from eight different samples of human skin of the indicated ages and recovered in DEAE pool 1 was subjected to immunoblot analysis for versican core proteins. Each of the two different samples of fetal skin is 120 days EGA. Samples were subjected to SDS–PAGE on 5–17.5% gradient gels, and the gels were electrotransferred to Immobilon-P. Separate blots were probed with 12C5 (upper panel) or anti-DPEAAE (lower panel). The major band detected with 12C5 is labeled band 4.

-sulfo-D-galactose] and ΔDi4S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose]. Fetal skin versican contains ~60% ΔDi6S and ~20% ΔDi4S, whereas adult skin versican contains somewhat more ΔDi4S than ΔDi6S (Table I). Thus, the ratio of ΔDi6S to ΔDi4S is 3-fold higher for versican of fetal skin compared with the average for versican across all adult skin samples. In addition, the overall degree of sulfation is lower for fetal skin versican, as indicated by the higher percentage of ΔDi0S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose] and by the calculated average number of sulfates per disaccharide (Table I). The only oversulfated disaccharide that is consistently detected in human skin versican is ΔDi2,6S [2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose] (Table I). This disaccharide is present at 2–4% and shows an increase in relative amount in adult skin versican. There is also a general, though not systematic, decrease in the size of the GAGs of human skin versican in adult samples relative to fetal samples. It should be noted that the determination of GAG chain length depends heavily on the quantitative values obtained for the non-reducing termini. These chain length determinations are, therefore, greatly affected by even modest imprecision in the values obtained for the non-reducing termini, which is potentially problematic, because the non-reducing termini represent such a small percentage of the total digestion products. Interestingly, the non-reducing termini consistently show a higher level of 4-sulfation, even in fetal skin versican, where 6-sulfation predominates in the internal disaccharides. Over the adult age range examined (20–56 years), no significant differences were detected in the disaccharide composition of versican (Table I).

Human skin decorin shows little age-related difference in its GAGs (Table II). One major age-related difference is an overall smaller size for the GAGs, a difference which is also observed for versican. Unlike versican, the sulfation patterns of the GAGs of human skin decorin show no major differences for fetal and adult samples. Adult skin decorin contains consistently lower percentages of unsulfated and 6-sulfated disaccharides compared with fetal skin decorin (Table II), which mirrors differences in versican (Table I).

At each age examined, there are clear differences between the GAGs of human skin versican and human skin decorin. For example, the overall length of the GAGs of decorin is less than that for versican (Tables I and II). Also, the sulfation patterns of versican and decorin show several differences. While versican contains no more than 51% ΔDi4S and no less than 36% ΔDi6S at any age examined, ΔDi4S comprises a substantial percentage of the disaccharides in decorin, 83–88%, and ΔDi6S is found in very low amounts, 4% or less (Tables I and II). Human skin decorin differs further from versican in that it contains ΔDi2,4S [2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-d-galactose]/ΔDi2,4,6S [2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose] as its major oversulfated disaccharide, rather than ΔDi2,6S (Tables I and II). Unlike versican, which shows consistently detectable levels of only one oversulfated disaccharide, decorin displays significant amounts of a second oversulfated disaccharide, ΔDi2,6S, which is present in the GAGs of human skin decorin at levels of less than 2% (Tables I and II). At each age examined, decorin shows a higher level of sulfation than versican, as indicated by the percentage of ΔDi0S and by the calculated average number of sulfates per disaccharide, though the sulfation difference between versican and decorin is less pronounced in adult skin than in fetal skin (Tables I and II). Like versican, 4-sulfation predominates at the non-reducing termini of human skin decorin (Tables I and II). Not surprisingly, the disaccharide analysis of decorunt gives results that are similar to those obtained for decorin (Tables II and III). This similarity is expected, since there is evidence that decorunt is a catabolic fragment of decorin, which presumably arises by proteolytic cleavage of decorin (Carrino et al. 2003).

Decorin catabolic fragments

Decorunt consists of the amino-terminal 40% of decorin with the amino acid sequence VRKVTF^{140} as its carboxyl terminus

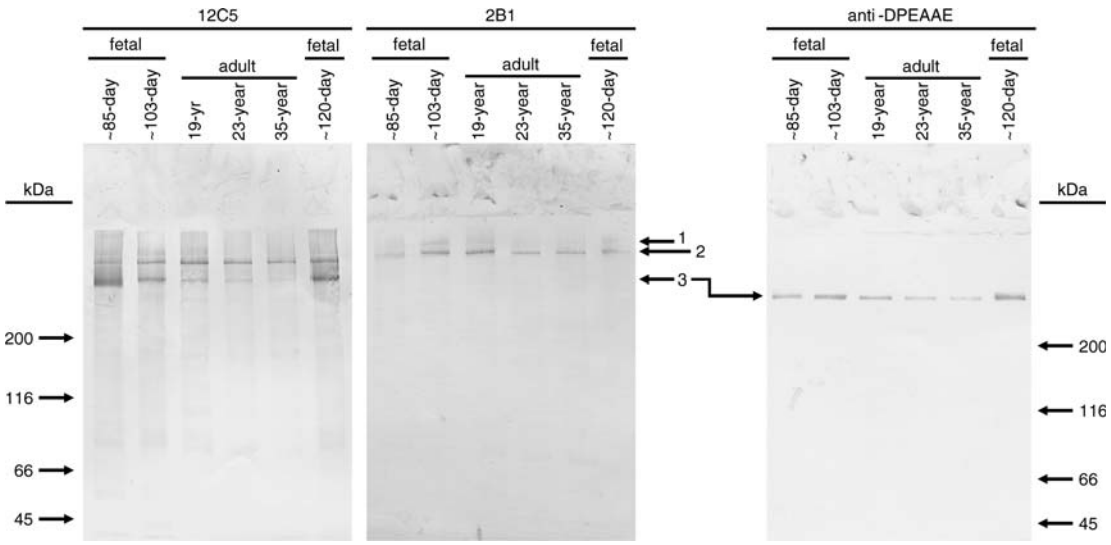

Fig. 4. Versican core protein analysis: Adult skin samples. Versican core protein analysis was done on proteoglycans recovered in DEAE pool 2 for three different samples of adult human skin of the indicated ages and three different samples of fetal human skin of the indicated EGA. Samples were treated with chondroitinase ABC, subjected to SDS–PAGE on a 5% gel and electrotransferred to Immobilon-P. Separate blots were probed with the indicated antibody or antiserum. The three major bands detected with 12C5 are labeled 1, 2 and 3 and correspond to the same bands indicated in Figure 2.

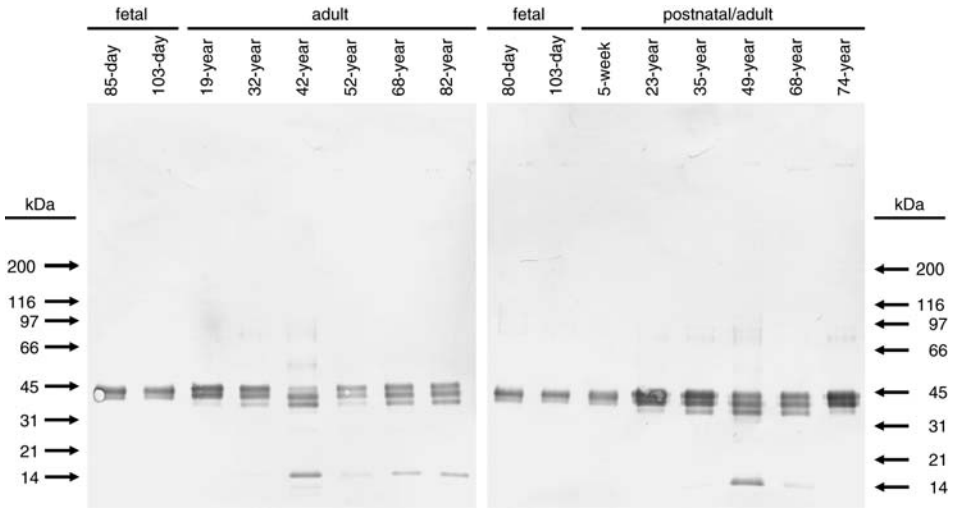

Fig. 5. Decorin core protein analysis. Decorin core protein analysis was done on proteoglycans recovered in DEAE pool 2 for 16 different samples of skin of the indicated ages. The two different 103 days EGA fetal samples are from the same individual, one trunk skin (left panel) and the other scalp skin (right panel). Samples were treated with chondroitinase ABC, subjected to SDS–PAGE on a 5–17.5% gradient gel and electrotransferred to Immunobilon-P. The blots were probed with 6B6.

(Carrino et al. 2003). It was previously found that decorunt can be separated from decorin by chromatography on octyl-Sepharose and that decorunt can be identified on immunoblots by its specific reactivity with a neoepitope antiserum, anti-VRKVTF, which was generated to recognize the carboxyl terminus of decorunt and which does not recognize decorin (Carrino et al. 2003).

Further analysis of decorunt revealed additional decorin catabolic fragments, which are termed non-decorunt catabolic fragments of decorin. The non-decorunt fragments were first observed when a new lot of octyl-Sepharose was purchased to replace the depleted lot that was used for the initial fractionations of decorunt from decorin. Both lots were procured from the same supplier under the same catalog number. With the original lot of octyl-Sepharose, decorunt eluted in the column unbound, whereas decorin bound to the column and was eluted during the guanidinium chloride gradient (Carrino et al. 2003). With the new lot, both decorin and decorunt bound to the column and eluted at a similar concentration of guanidinium chloride, whereas a distinct population of decorin catabolic fragments eluted in the column unbound. This is evident by immunoblot comparison of samples chromatographed on the old and new lots of octyl-Sepharose. On immunoblots probed with anti-VRKVTF, decorunt is detected

Table I. Human skin versican disaccharide analysis

Disaccharide	Fetal			Adult				
	85 days	103 days[a]	103 days[a]	20 years	24 years	37 years	52 years	56 years
ΔDi0S (%)	14.1	12.2	18.3	5.4	7.2	4.0	10.4	3.2
ΔDi6S (%)	61.7	62.7	54.2	43.3	38.2	38.1	35.9	44.1
ΔDi4S (%)	21.3	21.8	23.9	45.9	47.3	51.1	48.8	45.1
ΔDi2S	ND	ND	ND	ND	ND	ND	ND	ND
ΔDi4,6S (%)	ND	ND	ND	0.6	0.6	ND	ND	ND
ΔDi2,6S (%)	2.1	2.4	2.6	3.5	4.3	4.3	3.6	3.8
ΔDi2,4S/ΔDi2,4,6S (%)	ND	ND	ND	ND	0.8	ND	ND	ND
6S-GalNAc (%)	0.3	0.1	0.3	0.4	0.5	1.2	0.3	1.6
4S-GalNAc (%)	0.5	0.9	0.8	0.9	1.2	1.3	1.1	2.1
Chain length[b]	131	99	87	77	58	39	74	26
Sulfation[c]	0.87	0.89	0.83	0.97	0.95	0.98	0.92	0.97

ND, not detected; 6S-GalNAc, N-acetylgalactosamine-6-sulfate; 4S-GalNAc, *N*-acetylgalactosamine-4-sulfate; ΔDi2S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose.
[a]These proteoglycan samples are from the same individual (column 3, trunk skin; column 4, scalp skin).
[b]Number averaged disaccharides per GAG chain.
[c]Average sulfates per disaccharide.

Table II. Human skin decorin disaccharide analysis

Disaccharide	Fetal			Adult						
	85 days	103 days[a]	103 days[a]	20 years	24 years	37 years	42 years	49 years	52 years	56 years
ΔDi0S (%)	2.8	4.3	3.4	1.7	1.9	1.4	1.6	1.9	0.8	0.9
ΔDi6S (%)	6.3	7.3	6.4	3.6	2.3	2.8	3.4	0.8	1.5	2.5
ΔDi4S (%)	85.1	83.0	84.5	86.1	86.1	86.8	83.5	88.4	87.7	88.5
ΔDi2S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ΔDi4,6S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ΔDi2,6S (%)	0.7	1.3	0.9	1.0	0.9	0.8	1.1	1.2	1.6	0.3
ΔDi2,4S/ΔDi2,4,6S (%)	3.2	2.6	2.7	4.9	5.5	5.0	6.4	5.3	5.0	4.5
6S-GalNAc (%)	0.5	0.1	0.2	0.4	0.1	0.01	0.2	0.2	0.1	0.1
4S-GalNAc (%)	1.4	1.5	2.0	2.4	3.1	3.1	3.8	2.3	3.2	3.3
Chain length[b]	52	64	45	35	30	30	24	39	29	29
Sulfation[c]	1.0	1.0	1.0	1.1	1.1	1.1	1.1	1.1	1.1	1.1

ND, not detected; 6S-GalNAc, N-acetylgalactosamine-6-sulfate; 4S-GalNAc, *N*-acetylgalactosamine-4-sulfate; ΔDi2S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose.
[a]These proteoglycan samples are from the same individual (column 3, trunk skin; column 4, scalp skin).
[b]Number averaged disaccharides per GAG chain.
[c]Average sulfates per disaccharide.

Table III. Human skin decorunt disaccharide analysis

Disaccharide	Adult						
	20 years	24 years	37 years	42 years	49 years	52 years	56 years
ΔDi0S (%)	1.6	1.8	0.8	1.7	1.5	1.4	1.2
ΔDi6S (%)	4.1	2.7	1.9	3.6	2.2	4.7	4.0
ΔDi4S (%)	86.0	85.5	85.5	83.2	86.9	82.7	85.9
ΔDi2S	ND	ND	ND	ND	ND	ND	ND
ΔDi4,6S (%)	0.3	ND	ND	ND	ND	ND	ND
ΔDi2,6S (%)	1.0	0.9	ND	1.2	2.0	ND	0.4
ΔDi2,4S/ΔDi2,4,6S (%)	4.4	6.1	8.2	6.4	5.1	7.2	5.0
6S-GalNAc (%)	0.4	0.2	0.9	0.3	0.2	1.3	0.5
4S-GalNAc (%)	2.1	2.7	2.6	3.6	2.2	2.7	3.0
Chain length[a]	40	33	27	25	42	24	28
Sulfation[b]	1.1	1.1	1.1	1.1	1.1	1.1	1.1

ND, not detected; 6S-GalNAc, N-acetylgalactosamine-6-sulfate; 4S-GalNAc, *N*-acetylgalactosamine-4-sulfate; ΔDi2S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose.
[a]Number averaged disaccharides per GAG chain.
[b]Average sulfates per disaccharide.

in the unbound pool with the old lot and in pool 2 with the new lot (Figure 6, lower panels). On immunoblots probed with 6B6, which recognizes decorin and decorin catabolic fragments, a reactive band migrating between 45 and 31 kDa is observed in the column unbound for both lots, whereas a faint band migrating at a similar position is detected in pool 2 for the sample chromatographed on the new lot (Figure 6, upper panels). This latter band presumably represents decorunt. For both lots, decorin, the 6B6-positive band migrating between 80 and 100 kDa, elutes in pool 2 (Figure 6, upper panels). Similar results are obtained with proteoglycans from two other adult skin samples (data not shown). When aliquots from two samples were separately chromatographed on the two lots of octyl-Sepharose, the fractionation conformed to the specific lot (data not shown).

On the basis of the results with the new lot of octyl-Sepharose, the 6B6-reactive material that did not bind to the old lot of resin is now interpreted to contain both decorunt and the non-decorunt catabolic fragments of decorin. The

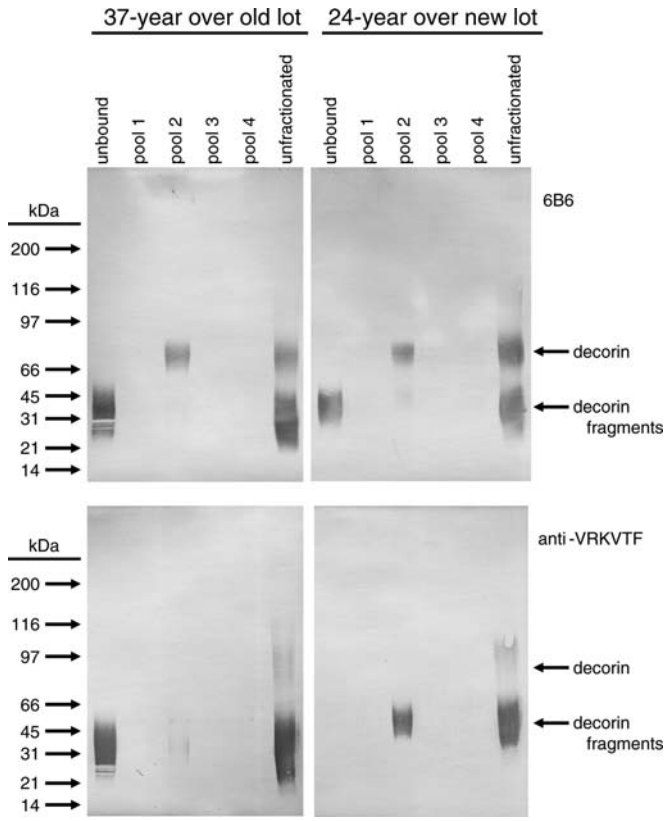

Fig. 6. Octyl-Sepharose chromatography of proteoglycans on different lots of octyl-Sepharose: column pools. Two different samples of proteoglycans recovered in DEAE pool 2 were chromatographed on Sepharose CL-2B to separate versican from the small proteoglycans, and the small proteoglycans were further fractionated on octyl-Sepharose. Each sample was fractionated on a different lot of octyl-Sepharose, designated the old lot or the new lot. Aliquots of each octyl-Sepharose pool were subjected to SDS–PAGE on a 5–17.5% gradient gel and electrotransferred to Immunobilon-P. Separate blots were probed with either 6B6 (upper panels) or anti-VRKVTF (lower panels). For comparison, a lane of unfractionated material was also included. The migration positions of decorin and decorin catabolic fragments are indicated.

samples that were used for GAG analysis of decorunt (Table III) were prepared with the old lot. Thus, both decorunt and the non-decorunt fragments were included in this analysis. A third lot of octyl-Sepharose, which was subsequently purchased, has elution characteristics like those of the new lot (data not shown). The basis for the difference in the elution properties of the old and the new lots of octyl-Sepharose is not clear but may result from differences in the number and/or distribution of the octyl groups on the resin, which may affect the binding properties of the resin.

Owing to their lack of reactivity with anti-VRKVTF, the 6B6-reactive bands recovered in the column unbound with the new lot differ from decorunt in their carboxyl termini. Their retention by anion exchange chromatography indicates that these molecules contain the GAG attached to serine-4 of mature decorin and, hence, have amino termini which are not truncated beyond this amino acid. Because of this and based on the known location of the 6B6 epitope within the core protein of decorin (Sawada et al. 2002), any protein components of the non-decorunt catabolic fragments of decorin should be at least 4.2 kDa. Chondroitinase ABC-treated aliquots of these fragments were analyzed on Coomassie blue-stained tris-tricine gels and on 6B6 immunoblots of tris-tricine gels. However, no protein bands could be identified as the core proteins of the non-decorunt fragments in spite of repeated attempts with several different skin samples (data not shown). It is possible that the non-decorunt fragments, in contrast to decorunt, do not have a defined carboxyl terminus, so that the protein components of these fragments are too heterogeneous in size to give rise to distinct bands on gel electrophoresis. Taken together, these results indicate that adult human skin contains additional catabolic fragments of decorin besides decorunt and also indicate that there can be differences in the elution characteristics of different lots of octyl-Sepharose.

Discussion

Previous work identified three major proteoglycans in human skin: versican, decorin and a catabolic fragment of decorin, termed decorunt (Carrino et al. 2000, 2003). Biglycan was also detected, but is present at lower amounts (Carrino et al. 2000). In these studies, detailed structural characterization of versican and decorin was not performed. With respect to versican, the data in this report indicate that four core protein species are most prominent: the V0 and V1 splice variants and the catabolic fragments of these molecules with DPEAAE as the carboxyl termini. This determination is based on the pattern of reactivity with antibodies to defined locations within the core protein of versican (Figure 1). As indicated by the relative intensities of the bands detected with 12C5, the V1 isoform is more abundant than the V0 isoform in human skin. This correlates with results obtained at the transcriptional level by reverse transcriptase–polymerase chain reaction (Cattaruzza et al. 2002) and at the translational level with cultured human dermal fibroblasts (Zimmermann et al. 1994). The results presented in this report indicate that these relative amounts of V0 and V1 also occur at the translational level *in vivo*.

In the present study, no evidence was found for the V2 and V3 isoforms in human skin. The V2 isoform has been detected only in the central nervous system (Schmaltfeldt et al. 1998; Zimmermann and Dours-Zimmermann 2008; Dours-Zimmermann et al. 2009), and its absence from skin is consistent with this observation. The V3 isoform lacks both of the GAG-attachment regions (GAG-α and GAG-β) and, hence, should not contain GAGs (Zako et al. 1995). As such, the expectation is that V3 does not bind to DEAE-Sephacel and would be excluded from the proteoglycan analysis described in this report. However, since V3 contains the G1 region, this isoform should bind to DEAE-Sephacel for the same reasons that the catabolic fragment of V1 does, as described later in the Discussion. Thus, if V3 is present in human skin, it should be detected on 12C5 immunoblots of DEAE pool 1 as a band migrating at ~75 kDa, and no such band is observed (Figure 3). This suggests that V3 is not present in skin at the protein level, although V3 has been reported in skin at the transcriptional level (Cattaruzza et al. 2002).

On the basis of their patterns of antibody reactivity (Figure 1), bands 3 and 4 of Figures 2–4 are identified as

catabolic fragments of V0 and V1, G1-DPEAAE^{1428} and G1-DPEAAE^{441}, respectively, according to the nomenclature of Sandy et al. (2001). The latter should lack chondroitin sulfate, as is observed for this molecule (Sorrell et al. 1999). A molecule similar in size to band 4 and recognized by 12C5 has been observed in extracts of central nervous system tissue (Asher et al. 1991, 1995). This molecule was found to have a low isoelectric point (Asher et al. 1991), which likely explains the binding of band 4 to the anion exchange columns. Band 4 is not recognized by the neoepitope antiserum anti-NIVSFE. Cleavage of the V0 isoform at this site would be expected to give rise to a fragment, G1-NIVSFE^{405}, which would have a size similar to that of the V1 fragment, G1-DPEAAE^{441}, and also be devoid of chondroitin sulfate. The lack of reactivity with anti-NIVSFE suggests that, in human skin, versican fragments terminating in DPEAAE are the major fragments that accumulate, and cleavage at the NIVSFE site may not occur. The G1-DPEAAE^{1428} and the G1-DPEAAE^{441} fragments are also present in human aortic intima (Sandy et al. 2001).

The relative intensities of bands detected with 12C5 in fetal and adult skin suggest that there is a lower level of versican catabolism in adult skin compared with fetal skin, although other interpretations are possible. The metalloproteinases ADAMTS1 and ADAMTS4 have been shown to cleave versican at DPEAAE (Sandy et al. 2001), and the former has been detected in skin (Krampert et al. 2005). It has previously been reported that versican, through its G3 domain, has the capacity to interact with fibrillin-1 and that this interaction is not abrogated by 4 M guanidinium chloride (Isogai et al. 2002). Thus, the possibility exists that the carboxyl regions removed from versican by cleavage at DPEAAE are not detected among the extracted molecules because these fragments are not efficiently extracted from the tissue, although in the present study, extraction was performed with not only 4 M guanidinium chloride, but with this chaotropic agent plus detergent.

Although versican shows no qualitative age-related differences in its pattern of core proteins, a small age-related difference is observed in the polydispersity of decorin core proteins. The fetal samples show a pattern of decorin core proteins similar to previous observations (Glössl et al. 1984; Scott and Dodd 1990; Schönherr et al. 1993; Ramamurthy et al. 1996), whereas adult samples show an additional band with higher mobility. It is not known if the differences in decorin core protein polydispersity result from differences in the number of asparagine-linked oligosaccharides or retention of the propeptide in some decorin molecules (Sawhney et al. 1991) or if the smallest of the three major decorin core protein bands in the adult samples results from catabolism. Because the core protein of decorin is involved in a number of molecular interactions (Brown and Vogel 1989; Schönherr et al. 1998; Santra et al. 2002), the possibility exists that there are age-related differences in fetal and adult skin with regard to the core protein-mediated interactive properties of decorin, such as its binding to collagen, which may affect the tensile properties of skin (Danielson et al. 1997).

Versican, but not decorin, shows age-related differences in the disaccharide pattern of its GAGs. For the ages examined, differences are found only between fetal skin versican and adult skin versican. The differences in disaccharide composition observed for versican from fetal and adult human skin represent, to our knowledge, the first such differences reported for this proteoglycan, although age-related disaccharide pattern differences have been reported for aggrecan from a number of species (De Luca et al. 1977; Roughley and White 1980; Thonar and Sweet 1981, Madsen et al. 1983; Dziewiatkowski et al. 1989). Age-related differences have also been reported for the disaccharide composition of heparan sulfate of human abdominal aorta (Feyzi et al. 1998). The GAGs of versican have been shown to be involved in interaction of this proteoglycan with other molecules, such as selectin, CD44 and chemokines (Kawashima et al. 2000; Zou et al. 2000). For at least some of these interactions, evidence indicates that the oversulfated disaccharide ΔDi4,6S [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose] is critical (Kawashima et al. 2002; Zou et al. 2003). Because virtually none of this disaccharide is detected in human skin versican, it is expected that human skin versican does not participate in interactions that require ΔDi4,6S. The different disaccharide compositions of fetal and adult versican may result in differences in the interactive properties of this proteoglycan, which may affect the biological properties of human skin.

Versican has also been demonstrated to influence cell migration (Evanko et al. 2001; Dutt et al. 2006). A chondroitin/dermatan sulfate proteoglycan, later determined to be the murine homolog of phosphacan, has been shown to promote neurite outgrowth due to its GAG structure (Faissner et al. 1994). The important component of the GAGs for this activity was determined to be ΔDi2,6S (Clement et al. 1998), which is present in human skin versican at all ages examined. Thus, versican in skin may be involved in promoting cell migration. In addition, elevated levels of versican have been found in highly proliferative tissues, such as tumors (Isogai et al. 1996; Paulus et al. 1996; Nara et al. 1997; Brown et al. 1999; Sakko et al. 2001; Ricciardelli et al. 2002; Touab et al. 2002). Versican from tumors has been shown to contain a higher percentage of ΔDi6S and ΔDi0S relative to versican from normal tissues (Tsara et al. 2002; Theocharis et al. 2003). These differences in the amount and disaccharide composition of versican from tumors and non-tumor tissue correlate with the differences between fetal skin versican and adult skin versican. Hence, the relative amount and the sulfation pattern of fetal skin versican may contribute to higher cell proliferation in fetal skin. Little information is available in relation to possible effects of versican on dermal repair. Excisional wound studies in mice have shown that ADAMTS5 knockouts do not generate a normal collagenous matrix in the subepithelial region during repair (Velasco et al. 2010). This abnormal repair is preceded by the accumulation and subsequent removal of versican V1. This might be explained if the versican accumulation represents a dysregulated response of reparative cells to proinflammatory factors.

With regard to the disaccharide composition of human skin versican, it is important to note that immunohistochemistry with antibodies that recognize discrete structural motifs in chondroitin sulfate indicate that there are spatially specific localizations of distinct chondroitin sulfate structures (Sorrell

et al. 1999). Restricted distributions of carbohydrate epitopes, which were attributed to versican, have also been reported for other tissues (Cattaruzza et al. 2002). Thus, although the biochemical analysis of the GAGs of extracted human skin versican measures only the disaccharide pattern of the total population of this tissue's versican molecules, within the tissue, there is a regionalization of versican molecules having specific GAG structural compositions. These spatial variations in the structure of the GAGs of versican may be involved in regional differences in the interactive or other functional properties of dermal versican, which are expected to be different for fetal and adult skin because of the age-related differences in versican's disaccharide composition. Although human skin versican shows clear age-related differences in its disaccharide composition, no major age-related differences are detected for human skin decorin. The decrease in decorin GAG length correlates with a previously observed age-related decrease in overall size of human skin decorin (Carrino et al. 2000). Dermatan sulfate from decorin and biglycan of mouse tendon displays an age-related decrease in size and an increase in the oversulfated disaccharide ΔDi2,4S (Derwin et al. 2001). The same may be true for human skin decorin if the small but consistent increase in ΔDi2,4S/ΔDi2,4,6S observed for adult skin decorin is due to ΔDi2,4S.

The results presented herein add further details to the analysis of the age-related changes in the proteoglycans of human skin. The age-related differences in proteoglycan structure and catabolism may play a role in age-related differences in the biological properties and the injury response of skin.

Materials and methods

Materials

The sources of reagents and other supplies have been reported previously (Carrino et al. 2000, 2003). Samples of human skin of various fetal and adult ages were obtained in accordance with the policies established by the Institutional Review Board of Case Western Reserve University (Carrino et al. 2000, 2003). Samples of fetal human skin were procured through the Central Laboratory for Human Embryology at the University of Washington, Seattle, WA, and samples of adult human skin were obtained through the Tissue Procurement Core Facility, Cancer Center, Case Western Reserve University, Cleveland, OH.

Proteoglycan extraction, isolation and fractionation

Proteoglycans were extracted from minced human skin and isolated by anion exchange chromatography on DEAE-Sephacel as described previously (Carrino et al. 2000). The anion exchange columns were eluted in a stepwise manner with 0.25 M and then 1.0 M sodium chloride (Carrino et al. 2000). The lower salt eluate, designated DEAE pool 1, contains hyaluronan and some loosely bound glycoproteins, whereas the higher salt eluate, designated DEAE pool 2, contains the sulfated proteoglycans (Carrino et al. 2000).

The various proteoglycans in DEAE pool 2 (versican, decorin, decorunt and biglycan) were separated by Sepharose CL-2B and octyl-Sepharose chromatography as reported previously (Carrino et al. 2003). All of the different lots of octyl-Sepharose were obtained from Sigma-Aldrich, catalog number O6001. Fractions for both columns were assayed by dot blots with appropriate antibodies. For the Sepharose CL-2B column, fractions that contained versican and fractions that contained the small proteoglycans (decorin, decorunt and biglycan) were pooled separately. The latter pool was further fractionated by octyl-Sepharose chromatography to generate separate pools that contain decorin, decorunt or biglycan. The amount of GAG in each of the column pools was determined by a Safranin O assay (Carrino et al. 1991). The fractionated samples of versican, decorin and decorunt were used for GAG analysis; GAG analysis was not done for biglycan due to insufficient material.

SDS–PAGE and immunoblotting

Electrophoresis on polyacrylamide gels, electrotransfer to Immobilon-P and immunoblotting were performed as described (Carrino et al. 2000). Samples were examined either as intact proteoglycans or as core proteins after treatment with chondroitinase ABC (Carrino et al. 2000). All the proteoglycan samples were subjected to core protein immunoblot analysis, but results are shown only for some of the samples. Those samples for which core protein results are not presented gave results like those for samples in the same age group (fetal or adult).

To analyze the catabolic fragments of decorin and their core proteins, the different pools from the octyl-Sepharose column were electrophoresed on tris-tricine polyacrylamide gels in order to increase the resolution of small proteins. For immunoblots of these gels, a procedure was used that enhances the binding of small proteins to the blotting membrane. This procedure was used because decorin catabolic fragments and their core proteins are not retained well on Immobilon-P immunoblotting membrane under the conditions routinely used for immunoblots (Carrino et al. 2000, 2003). The tris-tricine gels were electrotransferred onto Immobilon-P^{SQ} and the blots were air dried for 1 h at room temperature. Then the blots were moistened briefly in methanol, rinsed with water and blocked for 30 min in 3% enzyme-linked immunosorbent assay ELISA-grade bovine serum albumin (BSA) in TBS-Tween [tris-buffered saline containing 0.05% Tween-20 (polyoxyethylenesorbitan monolaurate)]. The blots were then incubated for 1 h in primary antibody diluted into 3% BSA in TBS-Tween, rinsed with TBS-Tween, incubated for 1 h in alkaline phosphatase–conjugated secondary antibody diluted into TBS-Tween, rinsed with TBS-Tween and finally incubated in alkaline phosphatase substrate.

GAG analysis

The disaccharide compositions of GAGs were determined as reported previously (Calabro et al. 2000a, 2000b). Samples of individual proteoglycans were treated with a combination of chondroitinase ABC and hyaluronidase SD (*Streptococcus dysgalactiae*). The digestion products were fluorescently labeled and then fractionated and quantified by a fluorophore-assisted carbohydrate electrophoresis procedure (Calabro, Benavides, et al. 2000; Calabro, Hascall, et al. 2000). Because this procedure does not resolve ΔDi2,4S and ΔDi2,4,6S, the band containing these disaccharides is designated ΔDi2,4S/ΔDi2,4,6S to indicate that this band may

contain either of these or a mixture of these. In addition to disaccharide compositions, this procedure also allows estimation of the number averaged size of the GAGs by quantification of the internal disaccharides and the non-reducing termini. The GAG analyses were performed for one aliquot of each sample that was analyzed. To assess reproducibility, some of the samples (a total of six) were analyzed in duplicate, and these duplicate analyses showed no significant differences (data not shown). GAG analysis was performed only for those samples for which results are presented.

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Conflict of interest

None declared.

Abbreviations

ΔDi6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi2,6S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi2,4S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-d-galactose; ΔDi4,6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose; ΔDi2,4,6S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BSA, bovine serum albumin; DEAE, diethylaminoethyl; EGA, estimated gestational age; GAG, glycosaminoglycan; TBS-Tween, tris-buffered saline containing 0.05% Tween-20 (polyoxyethylene-sorbitan monolaurate).

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