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CHARACTERIZATION OF OLIGOSACCHARIDE UNITS OF p-N-COLLAGEN TYPE III FROM DERMATOSPARACTIC BOVINE SKIN

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p-N-collagen type III was extracted from dermatosparactic and normal fetal bovine skin and purified by ion-exchange chromatography using DEAE- and CM-cellulose. Asparagine-linked sugar chains were fractionated by high voltage paper chromatography from the products obtained after hydrazinolysis and reduction with NaB^3H_4. These oligosaccharides composed of neutral and acidic components are mannose-containing oligosugars of the complex type. Their abundance is much higher in dermatosparactic p-N-collagen type III.

Introduction

Collagen is first produced as a biosynthetic precursor molecule, procollagen bearing at both NH_2- and COOH-terminal ends globular precursor sequences. Although there exists no *N*-glycosidically-linked sugar chains in interstitial collagen molecule, mannose and glucosamine containing sugar chains were detected in the COOH-terminal peptide of procollagen by metabolic labelling [1–5]. Mannose-containing sugar chains are linked to an asparaginyl residue included in the sequence -Asn-X-Thr (or Ser) [6]. This amino acid sequence was found in the COOH-terminal peptide of procollagen type I on the basis of DNA sequencing [7]. Neutral and amino sugars were also found in NH_2-terminal peptide of type II procollagen but these oligosugars were not characterized [8].

We have purified p-N-collagen type III from dermatosparactic bovine skin. By using exoglycosidases, we have determined the structural sequence of the asparagine-linked oligosaccharides released by hydrazinolysis and labelled by reduction with NaB^3H_4. A similar oligosaccharide moiety has been found in much lower concentration in p-N-collagen type III isolated from normal fetal calf skin.

Materials and Methods

Enzymes and chemicals. Sialidase from *Arthrobacter Ureafaciens* was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). α-Mannosidase and β-*N*-acetylhexosaminidase were purified from jackbean meal [9]. β-Galactosidase was purified from jackbean meal [10] and from culture media of *Diplococcus pneumoniae* [11]. α-L-fucosidase from *Charonia lampas* was purchased from Seikagaku Hogyo Co. (Tokyo, Japan).

1 unit of glycosidase was defined as the amount of activity which released 1 μmol of the saccharide from the substrate per min. NaB^3H_4 (100 mCi/mmol) was obtained from CEA (Gif-Sur-Yvette, France). Hydrazin anhydrate was purified from

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hydrazin hydrate by distillation in the presence of CaO and toluene at 95°C.

Preparation of p-N-collagen type III. p-N-collagen type III was extracted from fetal and dermatosparactic calf skin by 0.15 M NaCl, 0.05 M Tris-HCl buffer (pH 7.4) containing protease inhibitors (20 mM ethylendiamine tetraacetic acid disodium salt, 0.5 mM *N*-ethylmaleimide and 0.1 mM phenylmethanesulphonyl fluoride). The p-N-collagen type III and collagen type III were collected by differential salt fractionation as previously reported [12]. Collagen and p-N-collagen were separated by DEAE-cellulose (DE-52; Whatman Ltd., Kent, U.K.) chromatography as previously reported [13]. The p-N-collagen was further purified by using CM-cellulose chromatography according to the method of Piez et al. [14]. The purity of the preparation was monitored by SDS-polyacrylamide gel electrophoresis using the procedure of Laemmli [15], modified by the presence of 6 M deionized urea in the 6% separation gel.

Relase of asparagine-linked oligosaccharides by hydrazinolysis. The p-N-collagen type III was suspended in 0.4 ml of anhydrous hydrazine and heated in a sealed glass tube at 100°C for 12 h. The reaction mixture was evaporated to dryness under reduced pressure over concentrated H_2SO_4. After solubilizing the dry residue in saturated $NaHCO_3$ it was completely acetylated with acetic anhydride. The reaction mixture was passed through a Dowex 50 W (H^+) column and washed with 6 bed volumes of distilled water. The eluate and washing were combined and evaporated to dryness. The residue was dissolved in a small volume of water, spotted on a Whatman No. 3MM paper (5 × 60 cm) and subjected to paper chromatography for 16 h at room temperature using as solvent ethylacetate/pyridine/acetic acid/water (5 : 5 : 1 : 3). The oligosaccharides were recovered from the origin to 5 cm and eluted from the paper by water. This oligosaccharide fraction was reduced with 75 μmol of NaB^3H_4 in 200 μl of 0.05 M NaOH at 30°C for 4 h as reported by Mizuochi et al. [16]. The reaction was stopped by addition of acetic acid and the reaction products were passed through a small column of Dowex 50W (H^+). The column was washed with 5 bed volumes of distilled water. Eluate and washing were combined and evaporated to dryness. The residue was dissolved in water, spotted on Whatman No. 3MM paper and subjected to chromatography with *n*-butanol/ethanol/water (4 : 1 : 1) for 40 h. The radioactive area were monitored by a radiochromatoscanner (Packard Model 7220). The major radioactive peak which remained at the origin was eluted from the paper by distilled water.

Fractionation of oligosaccharides according to their anionic charges. The radioactive oligosaccharides were spotted on Whatman No. 3MM paper and subjected to paper electrophoresis using pyridine/acetic acid/water (3 : 1 : 387) at pH 5.4, 80 V/cm for 90 min. The radioactive peaks were eluted with distilled water and evaporated to dryness.

Determination of reducing terminal sugar. The radioactive oligosaccharides were hydrolyzed in 4 M HCl at 100°C for 2 h, and the hydrolysates were analyzed by paper electrophoresis using 0.06 M borate buffer, pH 9.5 for 2.5 h at 40 V/cm [17].

Molecular sieve chromatography. Bio-gel P-4 (200–400 mesh) column (2.5 × 90 cm) chromatography equilibrated in distilled water was performed at 50°C with a flow rate of 10 ml/h. Fractions of 3.5 ml were collected. A dextran hydrolysate was used as a standard mixture [18]. Differential refractometer type 98 (Knauer GmbH, Berlin, West Germany) was used for monitoring the glucose oligomers that eluted from the column. The elution of the radioactive oligosaccharides was monitored by liquid scintillation counting.

Results

Quantification and fractionation of asparagine-linked sugar chains in dermatosparactic p-N-collagen type III

After hydrazinolysis, *N*-acetylation and reduction with tritiated borohydride of p-N-collagen type III, three radioactive peaks were detected by high voltage paper electrophoresis (Fig. 1a), one neutral (N) and two acidic (A-1 and A-2). A similar pattern was observed for the fetal p-N-collagen type III (not illustrated). As shown in Table I the amount of these components estimated as a function of the specific activity of NaB^3H_4 was 67, 20 and 26 nmol for 96 nmol of dermatosparactic and 1.7, 1.1 and 1.8 nmol for 52 nmol of fetal calf skin p-N-collagen type III. Assuming a complete

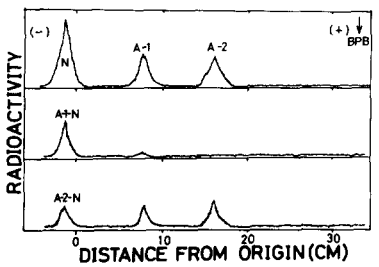


Fig. 1. Radioelectrophoretogram of the oligosaccharides derived from dermatosparactic p-N-collagen type III by hydrazinolysis. Tritium-labelled oligosugars were subjected to paper electrophoresis at pH 5.4, 80 V/cm for 90 min. The arrow indicates the position of Bromophenol blue (BPB). Upper panel (a): intact oligosaccharides. Middle panel (b): sialidase digestion of A-1. Lower panel (c): mild sialidase treatment of A-2.

reduction (or at least a reduction to a similar extent in the two types of p-N-collagen type III) and a complete recovery of the fractions the data would indicate that approximately one asparagine-linked oligosaccharide chains is present per p-N-collagen type III polypeptide in dermatosparaxis and less than 10% of that amount in fetal calf skin.Further analysis of the various oligosaccharides was only completed for the p-N-collagen type III from dermatosparactic skin. Digestion of the A-1 fraction with sialidase (20 mU/20 μl of 0.1 M acetate buffer, pH 5.0, at 37°C for 24 h) completely converted it to neutral fraction (Fig. 1b). By mild sialidase digestion of the A-2 component (1 mU/20 μl of 0.1 M acetate buffer, pH 5.0, for 30 min at 37°C) three components were detected by high voltage paper electrophoresis. One peak migrated at the A-2 position and two other peaks appeared corresponding to the N and A-1 fractions (Fig. 1c). These results suggest that A-1 contains one sialic acid and A-2 two sialic acid residues at the non-reducing extremity of the oligo sugars. The neutral radiolabelled oligosaccharides derived from A-1 and A-2 fractions after sialidase digestion (A-1-N and A-2-N) were recovered from paper electrophoresis by elution with water.

An aliquot of neutral components from N, A-1-N and A-2-N was hydrolyzed by HCl and analysed by paper electrophoresis in a borate buffer system for the detection of reducing termini of oligosaccharides. *N*-Acetylglucosaminitol was observed as the single radioactive component from each oligosugar (data not shown). These results indicated that the reducing terminal of these oligosaccharides was *N*-acetylglucosamine.

TABLE I

CONTENT OF ASPARAGINE-LINKED OLIGOSACCHARIDE CHAINS IN DERMATOSPARACTIC AND FETAL p-N-COLLAGEN TYPE III

The amount of oligosaccharide (in nmol) in each fraction has been obtained by idviding their radioactivity by the specific activity of the terminal sugar released by hydrazinolysis, acetylated and reduced ty tritiated sodium borohydride.

	Dermatosparactic	Fetal
Molecular weight (p-N-α_1 III)	$125 \cdot 10^3$	$125 \cdot 10^3$
Weight of sample	12 mg (96 nmol)	6.5 mg (52 nmol)
Oligosaccharide chains in	in nmol or (%)	in nmol or (%)
N fraction	66.8 (60%)	1.68 (36%)
A-1 fraction	19.6 (17%)	1.10 (24%)
A-2 fraction	26.0 (23%)	1.84 (40%)
Total	112.4 (100%)	4.62 (100%)
Oligosaccharide chain/p-N-α_1 III (mol/mol)	1.17	0.09

Analysis of the sequence of the oligosaccharides

Neutral oligosugars were analyzed by sequential exoglycosidase digestion and sieve chromatography.

The intact radioactive neutral oligosugar (N) was subjected to a Bio-Gel P-4 column chromatography. Three radioactive peaks were collected at an elution volume of 16, 14.5 and 13.5 glucose units oligomers (Fig. 2a). By incubation of the (N) fraction with jack-bean β-galactosidase, the radioactive components converted to 13, 12.2 and 11.2 glucose units (Fig. 2b). The size difference between degalactosyl-oligosugars and the intact oligosaccharides indicated that from maximum 5 (16–11) to 2 (14.5–11.2) galactosyl residues were liberated from the N component. One galactose residue almost behaves like 1 glucose unit on the Bio-Gel P-4 column.

When the peak corresponding to the 16-glucose units was digested with diplococcal pneumoniae β-galactosidase, the radioactivity was recovered at

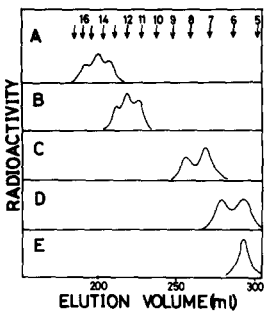


Fig. 2. Chromatographic patterns obtained by sequential exoglycosidase digestion of fraction N. The mixture of radioactive oligosugars and dextran hydrolysate was subjected to Bio-Gel P-4 column (2.5×95 cm) chromatography. The arrows indicate the eluting positions of glucose oligomers and the numbers indicate the glucose units. A: intact oligosaccharide from N. B: oligosaccharide N treated with β-galactosidase. C: radioactive peaks from B incubated with β-*N*-acetylhexosaminidase. D: radioactive peaks from C digested with α-mannosidase. E: radioactive peaks from D treated with α-L-fucosidase.

the position of 13 glucose units (data not shown). This result indicates that three galactosyl residues at the non reducing termini of the oligosugars eluting in the position of the 16 sugar units should be linked at the C-4 position of *N*-acetylglucosamine residues since *Diplococcus pneumoniae* β-galactosidase cleaves Gal-β1-4GlcNAc linkage [19].

When the radioactive peaks of Fig. 2b were incubated with β-*N*-acetylhexosaminidase, radioactivity was eluted at the position of 8.3 and 7.3 glucose units (Fig. 2c). By chromatography on Bio-Gel P4 *N*-acetylglucosamine elutes at the position of 1.8 to 2.0 glucose units. Two *N*-acetylglucosamine residues (= 4 glucose units) were therefore released from the 12.2 and 11.2 glucose units equivalent fractions. A 7.3 glucose units fraction also derived from the 13 glucose units component after digestion with β-*N*-acetylhexosaminidase (Fig. 2c). This result indicates that oligosaccharides in the 13 glucose units has 3 *N*-acetylglucosamine residues at the non-reducing termini and is therefore composed of three side-chains. When the radioactive oligosaccharides of Fig. 2c were incubated with α-mannosidase, radioactive peaks were detected at the mobility of 6.5 and 5.5 glucose units (Fig. 2d). After treatment of the radioactive peaks of Fig. 2d with α-L-fucosidase the double fraction was converted into a single peak eluting in the position of 5.5 glucose units. These results indicated that two mannose residues were liberated from 8.3 and 7.3 glucose units, respectively, and that the oligosugars in 5.5 glucose units position was Man-GlcNAc-GlcNActol while the 6.5 glucose units was Man-GlcNAc-(Fuc-)GlcNActol by analogy with the classical structure of Asparagine-linked oligosugars $^{\text{Man}\,\alpha1\,6}_{\text{Man}\,\alpha1\,3}\!>$Man-β1-4GlcNAc β1-4GLcNAc [20]. The proposed structure of the N component is illustrated in Fig. 5.

Localization of the sialyl residues in the acidic components

A-1-N and A-2-N displayed basically the same profile on Bio-Gel P-4 column chromatography as shown in Fig. 3a and Fig. 4a. They also ressembled the pattern of component N (Fig. 2a). These data suggested that the neutral oligosaccharides after sialidase treatment of A-1 and A-2 displayed similar structural sugar chains as the N components. Further analysis supported this proposal. The A-1 component made of three peaks was first digested with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase. After inactivation of

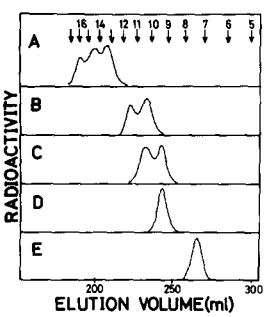


Fig. 3. Sequential exoglycosidase digestion of radioactive oligosaccharides A-1 and A-1-N. The labeled sugars and internal standards were applied to a Bio-Gel P-4 column (2.5×95 cm) and radioactivity in each tube was determined by liquid scintillation spectrometer (Beckmann LS-9000). The arrows indicate the elution positions of glucose oligomers of the internal standard and the numbers indicate the glucose units. A: intact labeled sugar of A-1-N. B: labeled oligosaccharide A-1 incubated first with a mixture of jackbean β-galactosidase and β-*N*-acetylhexosaminidase and then digested with sialidase. C: radioactive peaks of B treated with diplococcal β-galactosidase. D: radioactive peaks of C digested with α-L-fucosidase. E; peak of D incubated with β-*N*-acetylhexosaminidase.

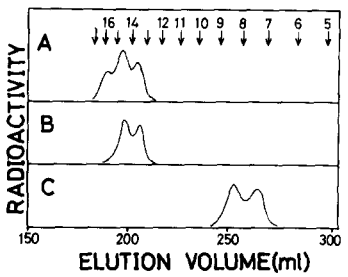
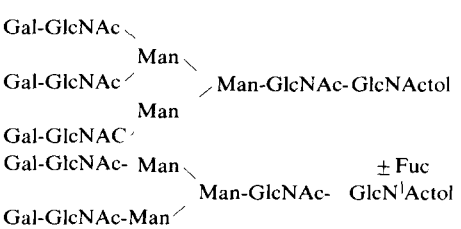
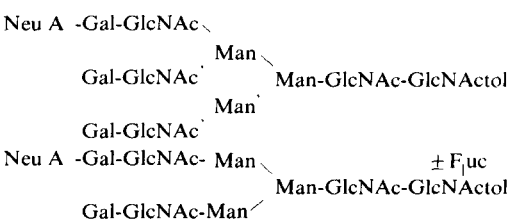


Fig. 4. Localization of the sialyl residues of A-2. Gel chromatography was performed on a Bio-Gel P-4 column (2.5×95 cm) at 50°C and effluent fractions of 3.5 ml were collected at the rate of 10 ml per hour. A: intact radioactive A-2-N. B: A-2 digested first with a mixture of β-galactosidase and β-N-acetylhexosaminidase and then treated with sialidase. C: peaks of B further incubated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase.

Components of N:



Components of A-1:



Components of A-2:

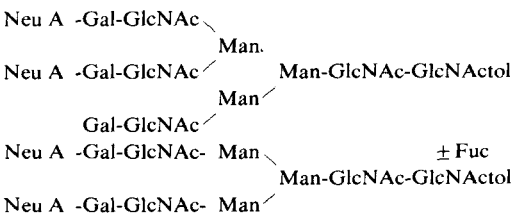
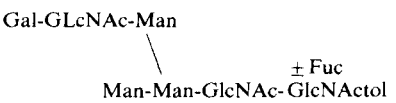
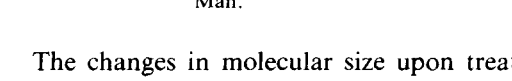


Fig. 5. Proposed saccharide sequence of N, A-1 and A2 of dermatosparactic p-N-collagen type III.

the enzymes by heating at 100°C for 10 min, the reaction mixture was incubated with sialidase. The peaks of oligosugars eluted from a Bio-Gel P-4 column as a double fraction at the position of 11.5 and 10.3 glucose units (Fig. 3b). These oligosugars were sensitive to β-galactosidase and 1 glucose unit was liberated from their molecule (Fig. 3c). This result suggested that one galactosyl residue was linked to *N*-acetylglucosamine per oligosaccharide chain in the 9.3 glucose units and in the 10.5 glucose units fractions. By incubating the oligosaccharides in position of 10.5 glucose units with α-L-fucosidase, the radioactivity was recovered at a mobility of 9.3 glucose units (Fig. 3d). When the radioactive oligosugar in position of 9.3 glucose units was treated with β-*N*-acetylhexosaminidase, it was noted to position of 7.3 glucose units (Fig. 3e). These results indicated that the oligosugar at 7.3 glucose units is Man_3-GlcNAc-GlcNAcol, that the oligosugar at 9.3 glucose units had one *N*-acetylglucosamine at the non-reducing terminal of the oligosugar and that the oligosugar at 10.5 glucose units had 1 fucose residue in supplement. Therefore, the oligosaccharide in the 10.3 glucose units fraction was strongly suggested as being



and the structure before sialidase digestion was inferred to be



The changes in molecular size upon treatment by the mixture of β-galactosidase and β-*N*-acetylhexosaminidase before sialidase provided evidence for the linkage of 2 and 1 disaccharide unit on the other mannose residue. These structures are illustrated in Fig. 5.

The A-2 component was first treated with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase and then digested with sialidase after heat inactivation of the enzymes. The radioactive oligosaccharides converted to components of 14.5 and 13.5 glucose units (Fig. 4b). These radioactive

oligosugars were sensitive to a mixture of β-galactosidase and β-*N*-acetylhexosaminidase and ere recovered in the position of 8.3 and 7.3 glucose units (Fig. 4c). The radioactive sugars in position of 16 glucose units were the only one sensitive to the first treatment by a mixture of β-galactosidase and β-N-acetylhexosaminidase as seen from Fig. 4a and b. These observations indicated that sialyl residues are linked to the non-reducing terminals of two mannose side chains in the 14.5 and 13.5 glucose units fractions. These oligosaccharides were sensitive to a second digestion with a mixture of β-galactosidase and β-*N*-acetylhexosaminidase which converted them in 8.3 and 7.3 glucose units components after sialidase treatment. The proposed structure of the A_2 component is illustrated in Fig. 5.

Discussion

Structurally, the asparagine-linked sugar chains of glycoproteins can be classified in at least three groups: 'high mannose', 'complex' and 'hybrid' types [20]. 'High mannose' type oligosaccharides were observed in the COOH-terminal precursor sequences of procollagen type I [1–5] and type II [8,21] and type III [5] by metabolic labelling in culture. From the sequence of the 3'-region of the pro-α_2-(I)-collagen gene it was possible to establish the amino acid sequence of the carbohydrate containing regions of its carboxyl propeptide [7,22]. It has been demonstrated that the N-propeptide of type I procollagen secreted by fibroblasts in culture does not contain oligosaccharide units [4]. In contrast, it has been shown that both the COOH- and NH_2-terminal propeptides of type II procollagen contain mannose [8]. Recently, we have detected asparagine-linked oligosugars in p-*N*-α_2-(I)-chain isolated from dermatosparactic calf skin (unpublished data). The present data demonstrate that 'complex' type oligosaccharides are bound to the NH_2-terminal precursor sequence of type III collagen isolated from dermatosparactic calf skin. One suitable sequence (-Asn-Tyr-Ser-) to support it is located very near (2 amino acids) the procollagen peptidase cleavage point in the N-propeptide sequence of the p-N-collagen type III [23].

Although the function of asparagine-linked oligosaccharide chains as those observed in the C-propeptide and in the amino-terminal precursor sequence of the dermatosparactic collagen type III are still speculative, it has been suggested that they are involved in the transport and adressing of the newly synthesized molecules from the endoplasmic reticulum to the Golgi apparatus. Inhibition of the glycosylation by tunicamycin [1] seems however to indicate that the carbohydrates in the propeptides are not essential for secretion of the procollagen to the extracellular medium. On the other hand, an excess of mannose in the C-propeptide of procollagen type I synthesized by fibroblasts from patients with osteogenesis imperfecta has been related to a slow rate of secretion of procollagen to the medium [5]. It is known that during the intracellular transport of the macromolecules, their oligosugars are modified by endoglycosidase processing before secretion [24].

The values presented in the results and given in Table I are mainly indicative. Radiolabelling by reduction by tritiated borohydride should indeed not be considered as a straightforward quantitative technique. However the large difference observed in the labelling of the p-N-collagen III isolated from normal fetal calves as compared to that collected from dermatosparactic animals is most probably related to variations in the amount of oligosugar bound to the N-propeptide. Although this excess of glycosylation in the defective calves might result of various causes, we favor the hypothesis that it depends on a defective endoglycosidase activity. In this hypothesis the oligosugars of the p-N-collagen type III in dermatosparaxis could be considered normal components of the intracellular collagen type III precursor.

In dermatosparaxis the p-N-collagen type I endopeptidase activity is missing mainly in skin [25]. It results in the accumulation of p-N-collagen type I in the extracellular space [26]. In the dermatosparactic skin, 90% of collagen type III also accumulates in the form of precursors bearing their amino-terminal propeptide as opposed to a significant processing of this procollagen (±50%) in the skin of age-matched normal calves [12]. In addition several properties of the dermatosparactic calf skin fibroblasts in culture differ from those of normal skin fibroblasts, increased cell density and altered morphology at confluence, defective capacity of contracting a collagen lattice supporting the

cells in culture [27], reduced collagen and protein biosynthesis in vitro (unpublished data), etc... The above mentioned hypothesis, i.e. a defective endoglycosidase activity could apply to various biosynthetic products (procollagen peptidase, membrane proteins, etc...) of the dermatosparactic cells to account for the pleiotypic expression of a single mutation transmitted as an autosomal recessive trait [28]. Further work is required to substantiate this proposal.

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