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Comparative Study of the *N*-Linked Oligosaccharides Released from Normal Human Esophageal Epithelium and Esophageal Squamous Carcinoma

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N-Linked sugar chains of normal human esophageal epithelium and esophageal squamous carcinoma were quantitatively released as oligosaccharides from their membrane preparations by hydrazinolysis. After being fractionated by serial lectin column chromatography using concanavalin A-Sepharose and *Datura stramonium* agglutinin-Sepharose, their structures were elucidated by exoglycosidase digestion in combination with methylation analysis. Both normal epithelium and esophageal carcinoma contained bi-, tri- and tetraantennary oligosaccharides as well as high mannose-type oligosaccharides. Interestingly, carcinoma had about 1.6 times larger amounts of tri- and tetraantennary oligosaccharides with the GlcNAcβ1→4Manα1→ and/or the GlcNAcβ1→6Manα1→ linkages than normal epithelium. Tri- and tetraantennary oligosaccharides with *N*-acetyllactosamine repeating units (the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc group) were also increased in carcinoma. These data indicated that the altered glycosylation of proteins previously found in transformed rodent cells also occurs widely in human esophageal carcinoma.

Key words: Sugar chain — Esophageal squamous carcinoma — Normal esophageal epithelium — *Datura stramonium* agglutinin — Membrane glycoprotein

Altered glycosylation of cell surface glycoproteins occurs in association with malignant transformation.[1] Evidence suggesting that the changes in sugar moieties are caused by the increase of the GlcNAcβ1→6 linkage attached to the Manα1→6 arm of the trimannosyl cores of complex-type sugar chains[2,3] has been accumulating during the past few years. It has also been reported recently that the changes in sugars may be associated with not only the tumorigenicity of rodent cells transformed by oncogenic virus[1] but also the metastatic potential of the cells.[4,5] Since the sugar moiety is known to affect the function of glycoproteins,[6] altered glycosylation has been suggested to modify several cellular properties such as cell adhesion[7–9] and acquisition of resistance to host immune surveillance.[10,11] Although the structures of sugar chains of purified glycoproteins produced by human carcinoma cells were studied,[12–15] information about the altered glycosylation of surface glycoproteins of human carcinoma is very limited. As the first step to apply the information obtained from the experimental model systems for clinical use, we have investigated the altered glycosylation of cell surface glycoproteins in human esophageal squamous carcinoma.

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[4] The abbreviations used are: Con A, concanavalin A; DSA, *Datura stramonium* agglutinin; Fuc, fucose; Sia, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose. All sugars mentioned in this paper were of D-configurations except for fucose, which was of L-configuration.

MATERIALS AND METHODS

Chemicals, enzymes and lectins NaB^3H_4 (360 mCi/mmol) and NaB^2H_4 were purchased from New England Nuclear, Boston, MA, and Merck Co., Darmstadt, respectively. β-Galactosidase and β-*N*-acetylhexosaminidase were prepared from jack bean meal[16] and the culture fluid of *Diplococcus pneumoniae*.[17] α-Mannosidases I and II from *Aspergillus saitoi*,[18] and endo-β-galactosidase from *Flavobacterium keratolyticus*[19] were purified according to the cited references. Sialidase from *Arthrobacter ureafaciens*[20] was purchased from Nacalai Tesque, Kyoto. Glycosidase digestion was carried out as reported previously.[2,21] Con A[4]-Sepharose[22] was purchased from Pharmacia, Ltd., Uppsala. *Datura stramonium* agglutinin (DSA)-Sepharose was prepared by the method of Yamashita *et al.*[24]

Analytical methods High-voltage paper electrophoresis was carried out at 80 V/cm for 2 h by using pyridine acetate buffer, pH 5.4. Descending paper chromatography was performed by using solvent I (1-butanol:ethanol:water=4:1:1), or solvent II (ethyl acetate:pyridine:acetic acid:water = 5:5:1:3). Con A-Sepharose column chromatography was performed by a modification of previous methods.[22,23] The sample dissolved in 100 μl of 10 m*M* Tris-HCl buffer (pH 7.4) containing 100 m*M* NaCl and 1 m*M* each of $MgCl_2$, $MnCl_2$ and $CaCl_2$ was applied to a Con A-Sepharose column (2 ml) and eluted

with 14 ml of the same buffer (Con A^- fraction). Radioactive oligosaccharides bound to the column were eluted with 14 ml of the buffer containing 5 m*M* methyl α-D-glucoside (Con A^+ fraction), and then with 14 ml of the buffer containing 100 m*M* methyl α-D-mannoside (Con A^{2+} fraction). DSA-Sepharose column chromatography was carried out as reported previously.[24] Bio-Gel P-4 column chromatography was carried out as described previously.[25] Methylation analysis was performed according to a published procedure,[26] using a DB5-30N column (0.25 mm×30 m) (J & W Scientific, Cordova, CA) and a JEOL DX-300 gas chromatograph-mass spectrometer (JEOL, Tokyo).

Oligosaccharides Mono- and disialyl derivatives of Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2-Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$[5] (Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$) and mono-, di-, and trisialyl derivatives of Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)-Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Gal_3·$GlcNAc_3$·Man_3·GlcNAc·$GlcNAc_{OT}$) were prepared from fetuin by hydrazinolysis.[27] Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]-Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal_4·$GlcNAc_4$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$), and Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα-1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$) were obtained from hamster melanoma tyrosinase.[28] Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ-1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (2,4-branched Gal_3·$GlcNAc_3$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$) and Galβ1→4GlcNAcβ-1→6(Galβ1→4GlcNAcβ1→2)Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (2,6-branched Gal_3·$GlcNAc_3$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$) were obtained from human urinary ribonuclease U_L.[29] Non-fucosylated forms of 2,6-branched triantennary and tetraantennary oligosaccharides were also obtained as described in the previous papers.[28, 29] Other standard oligosaccharides were prepared by exoglycosidase digestion of the oligosaccharides listed above.

Isolation of normal esophageal epithelium and esophageal squamous carcinoma cells Specimens of human esophageal epithelium and esophageal carcinoma were obtained by surgery. Three specimens of esophageal squamous carcinoma which were free from normal epithelium were obtained from three patients with esophageal squamous carcinoma (K.A., 58 years old, blood type B; Y.H., 56 years old, blood type A; K.H., 48 years old, blood type B) and designated as EC-1, EC-2 and EC-3. Two specimens of normal esophageal epithelium, which were free of tumor tissue, smooth muscle and connective tissue, were obtained as previously described[32] from two patients, K.A. and Y. H., and designated as NE-1 and NE-2.

Preparation of membrane glycoproteins Tissues were chopped finely with scissors and suspended in ice-cold 5 m*M* Tris-buffered saline, pH 9.0, for 30 min to be disrupted. The cell suspension was subjected to centrifugation as described[2] for the preparation of the membrane fraction. About 60 mg (dry weight) of the delipidated and dehydrated membrane preparation thus obtained from each sample was used for the present study.

Release of *N*-Linked Oligosaccharides from Membrane Glycoproteins The membrane preparations obtained from normal epithelium and esophageal carcinoma were subjected to hydrazinolysis as described previously[30] to release *N*-linked oligosaccharides. After *N*-acetylation, the oligosaccharide mixtures were purified by descending paper chromatography as described previously.[2] One-third of each purified oligosaccharide mixture was reduced with NaB^3H_4 and the remainder was reduced with NaB^2H_4 for methylation analysis. The yields of radioactive oligosaccharides were 1.3–1.5×10^6 cpm per 20 mg of each sample.

RESULTS

Fractionation of oligosaccharides by paper electrophoresis The redioactive oligosaccharide mixtures released from NE-1, NE-2, EC-1, EC-2 and EC-3 were subjected to paper electrophoresis at pH 5.4. As shown in Fig. 1, all radioactive samples were separated into neutral (N) and acidic peaks (A). Percent molar ratios of the fractions N and A from each sample calculated from their radioactivities were as follows: NE-1, 32:68; NE-2, 30:70; EC-1, 40:60; EC-2, 38:62; EC-3, 42:58. The acidic fractions obtained from five samples were mostly converted to neutral oligosaccharides by sialidase digestion (data not shown) indicating that the acidic nature of oligosaccharides in fractions A can be ascribed to sialic acid residues.

Fractionation of oligosaccharides by serial lectin column chromatography and Bio-Gel P-4 column chromatography Both fractions N and desialylated fractions A (AN) were further fractionated by serial lectin column chromatography. The samples were first subjected to chromatography on a Con A-Sepharose column, and separated into three fractions: Con A^-, Con A^+ and Con A^{2+}. As summarized in Table I, all esophageal carcinomas contained significantly smaller amounts of the Con A^+ fraction of AN than normal epithelia: the values of

[5] Subscripts OT and OD indicate NaB^3H_4-reduced and NaB^2H_4-reduced oligosaccharides, respectively.

this fraction in EC-1, EC-2 and EC-3 were approximately 60% of those of normal epithelia NE-1 and NE-2. On the contrary, the contents of the Con A^{2+} fraction of N and the Con A^- fraction of AN in esophageal carcinomas were significantly higher than those in normal epithelia. It is well known that biantennary oligosaccharides are recovered in the Con A^+ fraction, and high mannose-type and hybrid-type oligosaccharides in the Con A^{2+} fraction, while tri- and tetraantennary oligosaccharides pass through a Con A-Sepharose column.[23] Thus, the data suggested that the ratios of tri- and tetraantennary oligosaccharides as well as high mannose-type oligosaccharides are increased in carcinoma.

The Con A^- fractions of N and AN were then separated into passed-through (DSA^-), retarded (DSA^r) and bound (DSA^+) fractions by chromatography on a DSA-Sepharose column. As shown in Table I, carcinoma contained 1.6 times more DSA^+ fraction and 1.8 to 2.2 times more DSA^r fraction than normal epithelium. Since oligosaccharides with the Galβ1→4GlcNAcβ1→6(Galβ-1→4GlcNAcβ1→2)Man group bind strongly while those with the Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Man group are retarded in a DSA-Sepharose column,[24] the data indicated that tri- and tetraantennary oligosaccharides with these groups are highly expressed in esophageal carcinoma.

The samples fractionated by serial lectin column chromatography were then subjected to gel permeation

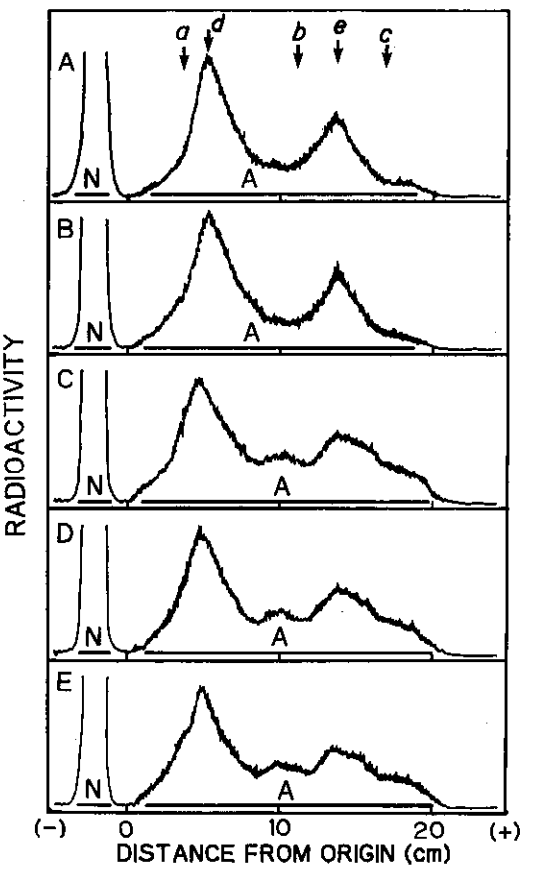

Fig. 1. Paper electrophoretograms of the radioactive oligosaccharides released from the membrane preparations of normal esophageal epithelium and esophageal carcinoma by hydrazinolysis followed by reduction with NaB^3H_4. A, B, C, D and E represent the data obtained from NE-1, NE-2, EC-1, EC-2 and EC-3. Arrows *a*, *b* and *c* indicate the migration positions of authentic mono-, di- and trisialylated $Gal_3{\cdot}GlcNAc_3{\cdot}Man_3{\cdot}GlcNAc{\cdot}GlcNAc_{OT}$, respectively, and arrows *d* and *e* indicate those of mono- and disialylated $Gal_2{\cdot}GlcNAc_2{\cdot}Man_3{\cdot}GlcNAc{\cdot}GlcNAc_{OT}$, respectively.

Table I. Fractionation of Oligosaccharides Liberated from Normal Esophageal Epithelium and Esophageal Squamous Carcinoma by Serial Lectin Column Chromatography

Fraction[a]	NE-1		NE-2		EC-1		EC-2		EC-3	
	N	AN	N	AN	N	AN	N	AN	N	AN
Con A^{2+}	9.4[b]	3.0	10.3	3.2	17.5	2.6	18.5	2.3	18.2	1.7
Con A^+	14.2	46.8	12.3	45.8	11.9	29.1	9.3	27.1	13.5	29.1
Con A^-	8.4	18.2	7.4	21.0	10.6	28.3	10.2	32.6	10.3	27.2
DSA^+	3.4	11.9	3.4	13.8	5.8	18.8	5.5	22.2	5.0	17.8
DSA^r	0.5	1.5	0.1	2.1	0.6	3.8	0.4	3.5	0.6	3.9
DSA^-	4.5	4.8	3.9	5.1	4.2	5.7	4.3	6.9	4.7	5.5

a) Symbols indicate fractions obtained by affinity chromatography on a Con A-Sepharose column (for naming, see the text). The Con A^- fractions were further separated by affinity chromatography on a DSA-Sepharose column into three fractions: passed-through (DSA^-), retarded (DSA^r) and that bound and eluted from the column with buffer containing 1% *N*-acetylglucosamine oligomers (DSA^+).
b) The number indicates the percent molar ratio of oligosaccharides included in each fraction calculated on the basis of their radioactivities.

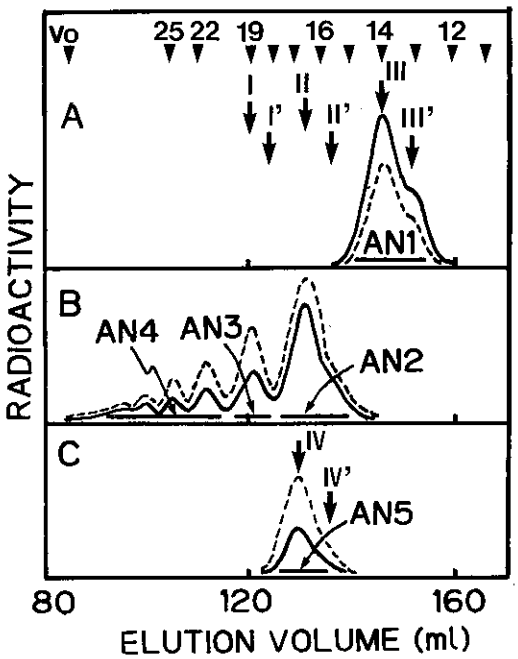

Fig. 2. Bio-Gel P-4 column chromatography of asialo-oligosaccharides. Asialo-oligosaccharides AN of NE-1 (solid line) and EC-1 (dotted line), fractionated with lectin columns as shown in Table I, were analyzed by Bio-Gel P-4 column chromatography. A, the Con A^+ fraction of AN; B, the Con A^- DSA^+ fraction of AN; C, the Con A^- DSA^r fraction of AN. Black arrowheads indicate the elution positions of glucose oligomers (the numbers indicate the glucose units), and the void volume (Vo). Black arrows I to IV are the elution positions of authentic oligosaccharides; I, $Gal_4 \cdot GlcNAc_4 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; II, 2,6-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; III, $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; IV, 2,4-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$, and black arrows I′ to IV′ are those of the non-fucosylated forms of I to IV, respectively.

on a Bio-Gel P-4 column. No qualitative difference between normal epithelium and carcinoma was detected in the elution patterns of the Con A^+ fraction, the Con A^-DSA^+ fraction and the Con A^-DSA^r fraction of AN. However, significant quantitative differences were detected between the two groups of samples. Since no significant difference was detected within the same sample group, only data for NE-1 (solid line) and EC-1 (dotted line) are shown in Fig. 2 as representatives. Fractions thus obtained and named AN1 to AN5 as indicated by bars in Figs. 2A to C were recovered and subjected to structural analysis in detail. There was only a slight difference in the contents of Con A^-DSA^- fractions of AN among the samples (Table I) and their elution patterns from a Bio-Gel P-4 column were quite similar to each other (data not shown). Therefore, no further analysis was done with these fractions.

In the neutral oligosaccharides, the amounts of Con A^{2+} fractions of EC-1, EC-2 and EC-3 were twice those of NE-1 and NE-2 (Table I). The oligosaccharides in this fraction were elucidated to be a high mannose-type series in the same manner as previously described[2] (data not shown). Carcinoma contained higher amounts of DSA^+ Con A^- fractions of N than normal epithelium as in the case of AN (Table I), but these fractions were not analyzed because of their low contents. In other fractions, no definite change was detected.

Structural analysis of oligosaccharides in fractions AN1 to AN5 The structures of the oligosaccharide fractions AN1 to AN5 were studied by exoglycosidase digestion. Since the analytical data obtained for the five samples were the same, only the results for NE-1 will be described below.

The Con A^+ fraction of AN (AN1) was eluted as a major component with a shoulder from a Bio-Gel P-4 column. The elution positions of the major and the minor peaks were the same as those of authentic $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$ and $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, respectively (Fig. 2A). AN1 was converted to the fucosylated and the non-fucosylated trimannosyl cores ($Man_3 \cdot GlcNAc \cdot \pm Fuc \cdot GlcNAc_{OT}$) with release of two residues each of galactose and *N*-acetylglucosamine by sequential digestion with diplococcal β-galactosidase (Fig. 3A, solid line) and β-*N*-acetylhexosaminidase (Fig. 3A, dotted line). It is known that diplococcal β-galactosidase cleaves only the Galβ1→4GlcNAc linkage, but not the Galβ1→3 or 6GlcNAc linkage[17] and that diplococcal β-*N*-acetylhexosaminidase cleaves the GlcNAcβ1→2Man linkage at non-branched points or in the GlcNAcβ1→4(GlcNAβ1→2)Man group, but not in the GlcNAcβ1→6(GlcNAcβ1→2)Man group.[31] Thus, these results indicated that AN1 consists of typical biantennary complex-type sugar chains as shown in Table II.

The Con A^-DSA^+ fraction of AN was separated into several components, which were pooled as AN2, AN3 and AN4 as shown in Fig. 2B. AN2 was composed of a major component and a minor shoulder with the same elution positions as authentic 2,6-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$ and $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, respectively (Fig. 2B). By incubation with diplococcal β-galactosidase, three galactose residues were removed from both components (Fig. 3B, solid line). The products were then converted to trimannosyl cores ($Man_3 \cdot GlcNAc \cdot \pm Fuc \cdot GlcNAc_{OT}$) by diplococcal (Fig. 3C, solid line) and jack bean β-*N*-acetylhexosaminidase (Fig. 3D, solid line) digestion, releasing one and two *N*-acetylglucosamine residues, respectively. The latter enzyme is known to have a broad substrate specificity cleaving all β-*N*-acetylglucosaminyl

Table II. Structures of Asialo-oligosaccharides Liberated from Normal Esophageal Epithelium and Esophageal Carcinoma

Fraction	Oligosaccharide
AN1	Galβ1→4GlcNAcβ1→2Manα1↘6 Manβ1→4R[a)] Galβ1→4GlcNAcβ1→2Manα1↗3
AN2	Galβ1→4GlcNAcβ1↘6 Manα1↘6 Galβ1→4GlcNAcβ1↗2 Manβ1→4R Galβ1→4GlcNAcβ1→2Manα1↗3
AN3 (Galβ1→4GlcNAcβ1→3)	Galβ1→4GlcNAcβ1↘6 Manα1↘6 Galβ1→4GlcNAcβ1↗2 Manβ1→4R Galβ1→4GlcNAcβ1→2Manα1↗3
	Galβ1→4GlcNAcβ1↘6 Manα1 Galβ1→4GlcNAcβ1↗2 ↘6 Galβ1→4GlcNAcβ1↘4 Manβ1→4R Manα1↗3 Galβ1→4GlcNAcβ1↗2
AN4 (Galβ1→4GlcNAcβ1→3)$_n$[b)]	Galβ1→4GlcNAcβ1↘6 Manα1 Galβ1→4GlcNAcβ1↗2 ↘6 Galβ1→4GlcNAcβ1↘4 Manβ1→4R Manα1↗3 Galβ1→4GlcNAcβ1↗2
AN5	Galβ1→4GlcNAcβ1→2Manα1↘6 Galβ1→4GlcNAcβ1↘4 Manβ1→4R Manα1↗3 Galβ1→4GlcNAcβ1↗2

a) R=GlcNAcβ1→4(±Fucα1→6) GlcNAc$_{OT}$.
b) Based on the differences of effective sizes of the peaks in fraction AN4 (Fig. 2B) from that of Gal$_4$·GlcNAc$_4$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, the n value was estimated to range from 1 to 4. Some of the outer chains occur as Galβ1→(4GlcNAcβ1→3Galβ1→)$_n$4GlcNAc sequences (see the text).

linkages. Based on the substrate specificity of diplococcal β-*N*-acetylhexosaminidase as described above and the binding specificity of a DSA-Sepharose column, AN2 is supposed to have 2,6-branched triantennary sugar chain structures.

AN3 was eluted at the same area as authentic Gal$_4$·GlcNAc$_4$·Man$_3$·GlcNAc·± Fuc·GlcNAc$_{OT}$ (Fig. 2B). When incubated with *Flavobacterium keratolyticus* endo-β-galactosidese which cleaves the internal β-galactosyl linkages of the *N*-acetyllactosamine repeating units such as Galβ1→(4GlcNAcβ1→3Galβ1→)$_n$4GlcNAc,[19] about one-third of AN3 was converted to a smaller component *a* releasing one Gal·GlcNAc·Gal group while the remainder was not degraded by the enzyme digestion (Fig. 3E). Peak *a* in Fig. 3E was converted to peak *a'*, which showed the same elution position as authentic GlcNAc$_3$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, by diplococcal β-galactosidase digestion with release of two galactose residues (Fig. 3F, dotted line). The product at this stage was identified as degalactosylated 2,6-branched triantennary sugar chains in the same way as described for AN2. Diplococcal β-galactosidase digestion removed four galactose residues from peak *b* in Fig. 3E, which was resistant to endo-β-galactosidase digestion (Fig. 3F, solid line). The resultant peak *b'* in Fig. 3F was then converted to trimannosyl cores by sequential digestion with diplo-

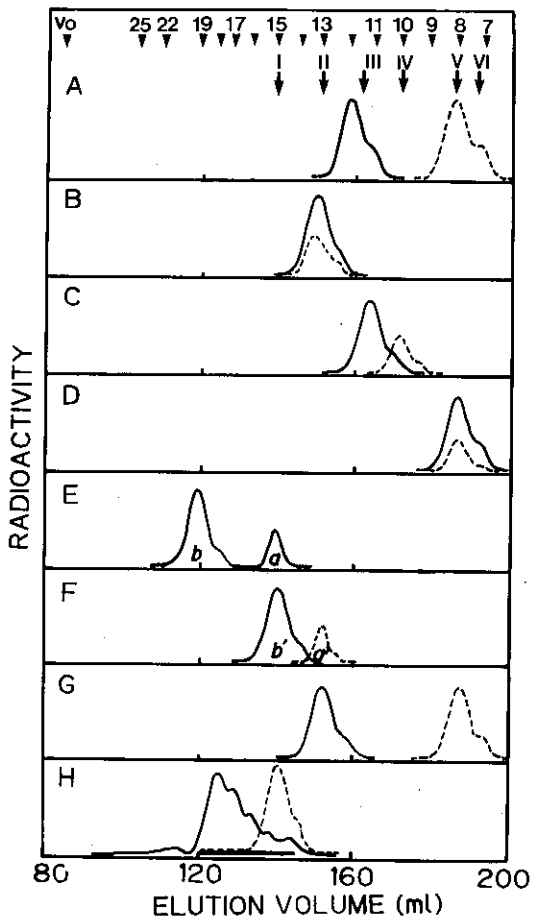

Fig. 3. Sequential glycosidase digestion of fractions AN1 to AN5. A, AN1 in Fig. 2A incubated with diplococcal β-galactosidase (solid line) and then with β-*N*-acetylhexosaminidase (dotted line); B, AN2 in Fig. 2B (solid line) and AN5 in Fig. 2C (dotted line) incubated with diplococcal β-galactosidase; C, the peaks from AN2 (solid line) and AN5 (dotted line) in (B) incubated with diplococcal β-*N*-acetylhexosaminidase; D, solid line and dotted line were obtained from the solid line and dotted line in (C) by digestion with jack bean β-*N*-acetylhexosaminidase, respectively; E, AN 3 in Fig. 2B incubated with *Flavobacterium keratolyticus* endo-β-galactosidase; F, dotted line and solid line represent, respectively, peaks *a* and *b* in (E) incubated with diplococcal β-galactosidase; G, peak *b'* in (F) incubated with diplococcal β-*N*-acetylhexosaminidase (solid line) and jack bean β-*N*-acetylhexosaminidase (dotted line); H, AN4 in Fig. 2B incubated with *Flavobacterium keratolyticus* endo-β-galactosidase (solid line) and then the resultant peaks indicated by a bar incubated with diplococcal β-galactosidase (dotted line). Black arrowheads are the same as in Fig. 2 and arrows I to VI are the elution positions of authentic oligosaccharides; I, $GlcNAc_4 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; II, $GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; III, $GlcNAc\beta1\rightarrow6(GlcNAc\beta1\rightarrow2)Man\alpha1\rightarrow6(Man\alpha1\rightarrow3)Man \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; IV, $Man\alpha1\rightarrow6(GlcNAc\beta1\rightarrow4Man\alpha1\rightarrow3)Man \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; V, $Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$; VI, $Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$.

coccal (Fig. 3G, solid line) and jack bean β-*N*-acetylhexosaminidase (Fig. 3G, dotted line) releasing one and three *N*-acetylglucosamine residues, respectively. These results indicated that AN3 consists of both tetraantennary sugar chains and 2,6-branched triantennary sugar chains with one *N*-acetyllactosamine repeating unit as shown in Table II.

AN4 contained a series of high-molecular-weight oligosaccharides with effective sizes larger than 22 glucose units (Fig. 2B). The size difference of each peak was approximately 3.0 glucose units suggesting the presence of different numbers of *N*-acetyllactosamine repeating units in the outer chain moieties. By incubation with endo-β-galactosidase from *Flavobacterium keratolyticus*, AN4 was converted to a mixture of smaller radioactive components as shown by the solid line in Fig. 3H. Diplococcal β-galactosidase digestion of these components gave a product which showed the same elution position as peak *b'* in Fig. 3F (Fig. 3H, dotted line). The product at this stage was identified as degalactosylated tetraantennary sugar chains by the same analytical procedures as described for the radioactive peak *b'* in Fig. 3F. The data indicated that AN4 consists of a mixture of tetraantennary sugar chains with *N*-acetyllactosamine repeating units.

In order to determine the structure of the *N*-acetyllactosamine repeating sequence, AN4 was digested with endo-β-galactosidase and the released oligosaccharides were labeled by reduction with NaB^3H_4. When the product was analyzed on a Bio-Gel P-4 column, two smaller radioactive peaks with effective sizes of 4.5 and 3.5 glucose units were obtained in addition to the peaks shown by the solid line in Fig. 3H (data not shown). The two additional peaks were proved to be $Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3Gal_{OT}$ (70%) and $GlcNAc\beta1\rightarrow3Gal_{OT}$ (30%) based on the results of sequential digestion with diplococcal β-galactosidase and jack bean β-*N*-acetylhexosaminidase (data not shown) and the finding that only C-3 substituted galactose residue (except for nonreducing terminal form) was detected in the asialooligosaccharide fraction by methylation analysis (Table III). Thus, AN4 is supposed to consist of tetraantennary complex-type sugar chains with linear *N*-acetyllactosamine repeating units. Detection of $GlcNAc\beta1\rightarrow3Gal_{OT}$ in the fragments released by endo-β-galactosidase digestion indicated that some of the *N*-acetyllactosamine repeating units are larger than $Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3Gal\beta1\rightarrow4GlcNAc$. Their proposed structures are shown in Table II.

The Con A^-DSA^r fraction of AN (AN5) was composed of a major component and a minor shoulder with the same elution positions as authentic 2,4-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$ and $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, respectively (Fig.

Table III. Methylation Analysis of Acidic (A) and Asialo-oligosaccharide (AN) Fractions Obtained from Normal Esophageal Epithelium (NE-1) and Esophageal Carcinoma (EC-1)

Methylated sugar	Molar ratio[a]			
	NE-1		EC-1	
	A	AN	A	AN
Fucitol				
2,3,4-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.7	0.8	0.7	0.7
Galactitol				
2,3,4,6-Tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.7	3.0	0.9	3.4
2,4,6-Tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	1.5	0.2	1.6	0.4
2,3,4-Tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	0.9	—[b]	1.2	—
Mannitol				
3,4,6-Tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.5	1.4	1.1	1.0
3,6-Di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.2	0.2	0.4	0.4
3,4-Di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0.3	0.3	0.6	0.6
2,4-Di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	*1.0*	*1.0*	*1.0*	*1.0*
2-Mono-*O*-methyl (1,3,4,5,6-penta-*O*-acetyl)	tr[c]	tr	tr	tr
2-N-Methylacetamido-2-deoxyglucitol				
1,3,5,6-Tetra-*O*-methyl (4-mono-*O*-acetyl)	0.2	0.2	0.2	0.2
1,3,5-Tri-*O*-methyl (4,6-di-*O*-acetyl)	0.7	0.7	0.7	0.7
3,4,6-Tri-*O*-methyl (1,5-di-*O*-acetyl)	0.1	0.1	0.1	0.1
3,6-Di-*O*-methyl (1,4,5-tri-*O*-acetyl)	3.8	3.8	4.5	4.6

a) Values were calculated by taking the value of 2,4-di-*O*-methylmannitol as *1.0*.
b) Not detected.
c) Less than 0.1.

2C). Diplococcal β-galactosidase digestion of AN5 released three galactose residues (Fig. 3B, dotted line). By sequential digestion with diplococcal (Fig. 3C, dotted line) and jack bean β-*N*-acetylhexosaminidase (Fig. 3D, dotted line), the resultant radioactive products were then converted to trimannosyl cores releasing two and one *N*-acetylglucosamine residues, respectively. According to the substrate specificity of diplococcal β-*N*-acetylhexosaminidase, which cleaves GlcNAcβ1→2 linkages in the GlcNAcβ1→2Man group and the GlcNAcβ1→4(GlcNAcβ1→2)Man group,[31] AN5 is supposed to have 2,4-branched triantennary sugar chain structures. That AN5 was recovered as the retarded fraction from a DSA-Sepharose column supported this structure, since 2,4-branched triantennary oligosaccharide is retarded in a DSA-Sepharose column while 2,6-branched triantennary oligosaccharide is strongly bound to the column and is recovered in the bound fraction.[24] Their proposed structures are given in Table II.

Methylation analysis To confirm the results of structural analysis by sequential glycosidase digestion, the acidic oligosaccharide fractions of NE-1 and EC-1 before (A) and after (AN) sialidase digestion were subjected to methylation analysis. As summarized in Table III, the results indicated that the sialic acid residues are linked to the C-3 and/or C-6 position of galactose, since the decrease of 2,4,6-tri-*O*-methylgalactitol and the disappearance of 2,3,4-tri-*O*-methylgalactitol were observed after desialylation. 2,4,6-Tri-*O*-methylgalactitol was detected even after desialylation, supporting the presence of *N*-acetyllactosamine repeating units as proposed for AN3 and AN4. Mannose residues occur in four forms, →2Man1→, $\overset{\rightarrow 6}{\rightarrow 2}$Man1→, $\overset{\rightarrow 4}{\rightarrow 3}$Man1→ and $\overset{\rightarrow 6}{\rightarrow 3}$Man1→, and *N*-acetylglucosamine residues occur in four forms, →4GlcNAc1→, $\overset{\rightarrow 6}{\rightarrow 4}$GlcNAc$_{OD}$, →4GlcNAc$_{OD}$ and GlcNAc1→. All fucose residues occur as non-reducing termini. The presence of a small amount of →4GlcNAc$_{OD}$ is consistent with the evidence that a non-fucosylated trimannosyl core is included in a small portion of oligosaccharides. In accordance with the increase of 2,4- and 2,6-branched triantennary and tetraantennary sugar chains in carcinoma tissues, values of 3,6-di- and 3,4-di-*O*-methylmannitols were higher in EC-1 than in NE-1. These data further confirmed the structural difference proposed for the *N*-linked sugar chains in the glycoproteins of normal and carcinoma tissues.

DISCUSSION

In this study, we comparatively analyzed the *N*-linked oligosaccharides of membrane glycoproteins obtained from normal esophageal epithelium and esophageal squamous carcinoma. It is important to obtain carcinoma tissues without any contaminating connective tissues and hematopoietic cells for biochemical analysis. Esophageal squamous carcinoma was virtually freed from contaminants and contained little mucinous substances. In addition, it is very easy to obtain normal esophageal epithelium by peeling the normal mucosal layer off the esophagus.[32] Thus, the samples obtained are suitable for the structural analysis of the sugar moieties of membrane glycoproteins.

Analysis by exoglycosidase digestion in combination with a methylation study revealed that both normal

esophageal epithelium and esophageal carcinoma contain bi-, tri- and tetraantennary complex-type oligosaccharides in common. Tri- and tetraantennary oligosaccharides with or without *N*-acetyllactosamine repeating units increase in all esophageal carcinoma, as compared to normal epithelium. This study indicated that the increment of these oligosaccharides is caused by the enhanced expression of both the GlcNAcβ1→6Manα1→ and the GlcNAcβ1→4Manα1→ linkages.

It has been reported that the enhanced expression of the GlcNAcβ1→6Manα1→ linkage occurs in association with oncoviral transformation of rodent cells[2, 3] and that the enzymatic basis of the change is an elevation of *N*-acetylglucosaminyltransferase V activity, which is responsible for the formation of this branching group.[33] In addition, our recent study on rat cells transformed by various gene fragments of adenovirus type 12 indicated that not only the GlcNAcβ1→6Manα1→ group but also the GlcNAcβ1→4Manα1→ group increases after malignant transformation of the cells and that this phenotypic change may be affected by the transactivation of some specific regions of the viral genome (unpublished results). The present study is unique in that normal and cancerous tissues of human origin have been used as materials, while the extensive studies so far reported were mainly carried out by using cultured rodent cells. The altered glycosylation observed in human esophageal carcinoma is consistent with that in the viral transformants of rodent cells. This observation suggests that the well-known glycosylation change observed in the *in vitro* rodent system is physiologically important in the establishment and the progress of human malignant cells.

Several lines of study have shown that the changes in cellular glycosylation induced by glycosidase inhibitors and cytotoxic lectins have a great influence on the susceptibility of tumor cells to cytolysis by macrophages and natural killer cells.[10, 11] It has also been suggested that altered glycosylation of tumor cells may take part in metastatic phenomena in the murine system.[4, 5] Humphries *et al.* reported that treatment of B16-F10 murine melanoma cells with swainsonine, an inhibitor of Golgi α-mannosidase II, results in inhibition of their lung colonization.[4] Furthermore, Dennis *et al.* showed that lectin-resistant mutants of a metastatic tumor cell line, which are deficient in *N*-acetylglucosaminyltransferase V activity, exhibit poor metastatic potential.[5] Accordingly, the change of sugar chain structures observed in esophageal carcinoma seems to be a phenotypic expression related to metastatic potential of carcinoma cells. Actually, regional lymph node metastasis has been detected in all of the patients from whom carcinoma tissues examined here were obtained. Whether the level of increased expression of the GlcNAcβ1→6Manα1→ group is parallel to metastatic ability in human malignancies or not will be clarified by the analysis of metastasized or far-advanced cancers.

In the present study, we have succeeded in detecting the difference in cellular glycosylation between normal esophageal epithelium and esophageal carcinoma by using a DSA-Sepharose column. As already described, DSA binds strongly to oligosaccharides with the Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man group and weakly to those with the Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Man group.[24] If altered glycosylation caused by an increase of the GlcNAcβ1→6-Manα1→ linkage or the GlcNAcβ1→4Manα1→ linkage is widely induced in several human carcinomas, DSA lectin may become a useful reagent for a histochemical approach to detect such a change in the sugar moieties of carcinoma.

It is important to accumulate information about the cellular glycosylation of human tumors by biochemical and histochemical studies in order to elucidate whether the altered glycosylation is associated with the malignant phenotypes of tumor cells or not.

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