Vol. 92, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 12, 1980 Pages 735-742

INCREASE OF ASPARAGINE-LINKED OLIGOSACCHARIDES WITH BRANCHED OUTER CHAINS CAUSED BY CELL TRANSFORMATION

Seiichi Takasaki, Hiroo Ikehira and Akira Kobata

From the Department of Biochemistry
Kobe University School of Medicine
Ikuta-ku, Kobe 650 Japan

Received November 13,1979

SUMMARY: By hydrazinolysis, oligosaccharides were released from fucose-labeled glycopeptides obtained from normal and polyoma-transformed baby hamster kidney cells, and their structures were comparatively analyzed. The oligosaccharides have the following structures, with different number of sialyl-galactosyl-N-acetylglucosaminyl outer chains: $(\pm Sia\alpha\rightarrow Gal\beta\rightarrow GlcNAc\beta\rightarrow)_n(Man\alpha\rightarrow)_2Man\beta\rightarrow GlcNAc\beta\rightarrow$ $(Fuc\alpha\rightarrow)GlcNAc$, (in normal cells, n=2,3 and 4, while in polyoma-transformed cells, n=2,3,4,5 and 6). Transformed cells are relatively rich in oligosaccharides with highly branched outer chains, as compared to normal cells.

One of the characteristic alterations associated with cell transformation is the appearance or increase of fucosyl glycopeptides of apparently large molecular weight. This phenomenon was found when the glycopeptide mixtures obtained from the surface of normal and transformed cells by exhaustive pronase digestion were analyzed by gel filtration (1-8). The molecular basis for the apparent size change is not clear. From the result of sialidase treatment, a higher content of sialic acid in the sugar chain moieties was said to be responsible for the increased molecular weight of the glycopeptides of transformed cells (3,5-6). However, Ogata et al. suggested that the key step leading to the large glycopeptides was not sialylation, but N-acetylglucosaminylation, which would lead to the formation of additional outer chain moieties in complex type asparagine-linked sugar chains (7). No exact structural analysis was performed to confirm this suggestion, because of the complex nature of the peptide moieties of the glycopeptides. We have recently developed a hydrazinolysis technique to release the carbohydrate moiety of a glycopeptide as an oligosaccharide (9). This technique was applied

0006-291X/80/030735-08$01.00/0

to investigate the nature of the structural change of the asparagine-linked fucosyl sugar chains of cells by transformation.

MATERIALS AND METHODS

Disialylated and monosialylated oligosaccharides were prepared from human transferrin by hydrazinolysis, and their structures were determined as follows; NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAc β1→4N-acetylglucosaminitol, and NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(3)[Galβ1→4GlcNAc β1→2Manα1→3(6)]Manβ1→4GlcNAcβ1→4N-acetylglucosaminitol, respectively (10).

β-Galactosidase, β-N-acetylhexosaminidase, and α-mannosidase from jack bean were purified according to the method of Li and Li (11). Sialidase from *Arthrobacter ureafaciens* (12) was purchased from Nakarai Chemicals, Ltd. Snail β-mannosidase (13) and *Charonia lampas* α-L-fucosidase (14) were kindly supplied by Seikagaku Kogyo Co.

Baby hamster kidney (BHK) cells and polyoma-transformed BHK cells were kindly donated by Dr. A. Hakura and Prof. K. Toyoshima, Institute of Infectious Diseases, Osaka University. The cells were plated at 1.0-2.0 X 10^4 cells/cm^2 in a stoppered TD-40 flask (surface area, 40 cm^2) and grown in 7 ml of Eagle's minimum essential medium (15) supplemented with 10% fetal calf serum, Streptomycin (100 μg/ml) and Penicillin (100 units/ml) at 37°. The medium was changed daily. After 4 days, 30 μCi of 6-[^{3}H]-fucose (17.1 mCi/mmol, The Radiochemical Centre) was added and the cells cultured one more day. At this stage the total number of BHK cells reached approximately 3 X 10^6 cells while that of polyoma-transformed BHK cells 5 X 10^6 cells. Then, the medium was removed and the culture was washed 3 times with Earle's solution (16) lacking $CaCl_2$, $MgSO_4$ and glucose (Solution I). The cells were detached from the flask by treatment with 1 mM EDTA in Solution I, and collected by centrifugation. The cells from 3 flasks were digested with 2 ml of 0.1% trypsin (DIFCO, 1:250) and 0.016% EDTA in Solution I at room temperature for 3 min, and centrifuged. The supernatant and the pellet suspended in 2 ml of Solution I were individually subjected to extensive digestion by daily adding 5 mg of pronase (Kaken Chemical Co., 70,000 p.u.k./g) at 37° for 3 days. After inactivating the pronase by heating at 100° for 2 min, the insoluble materials were removed by centrifugation. The resulting digests were designated as cell surface glycopeptides and cellular glycopeptides, respectively.

The cell surface and cellular glycopeptides were subjected to gel filtration on Sephadex G-50 (Fig. 1). Glycopeptide fractions (indicated by bars in Fig. 1) were collected and lyophilized.

Hydrazinolysis of the glycopeptide fractions was performed by the method previously reported (9) except that the reaction time was 12 hr and that the released sugar chains were reduced with $NaBH_4$ instead of NaB^3H_4.

Paper electrophoresis was carried out using pyridine-acetate buffer, pH 5.4 (pyridine:acetic acid:water=3:1:387). Descending paper chromatography was performed using the solvent; butanol/ethanol/water (4/1/1) for 20 hr.

RESULTS AND DISCUSSION

Fucose-labeled cell surface and cellular glycopeptides from normal and polyoma-transformed cells were subjected to gel filtration on Sephadex G-50. The glycopeptides with asparagine-linked sugar chains (fractions 45-85) were separated from those with mucin type sugar chains (fractions 35-45) by this fractionation. As shown in Fig. 1-A, cell surface glycopeptides with asparagine-linked sugar chains from transformed cells contained the fast-eluting components, in agreement

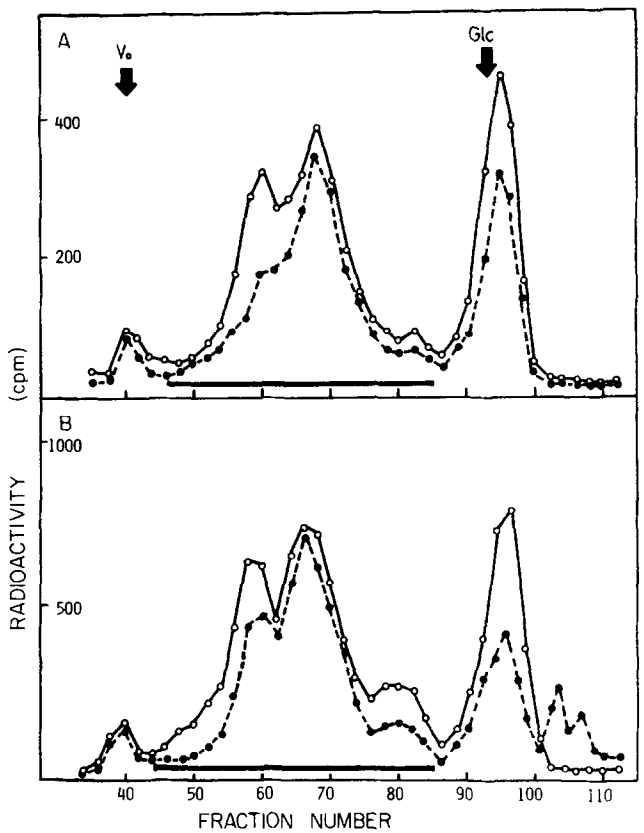

Fig. 1. Gel filtration of fucose-labeled glycopeptides from normal and polyoma-transformed BHK cells on Sephadex G-50.

The pronase digests were applied on a column (1 X 130 cm) of Sephadex G-50 equilibrated with 0.1 M puridine-acetate buffer, pH 5.5, and eluted with the same buffer. 1 ml-fractions were collected and aliquots were taken for counting the radioactivity. A; cell surface glycopeptides, B cellular glycopeptides, ●---● ; normal cells, o——o ; transformed cells.

with the results obtained before (1-8). Similar results were obtained in the case of cellular glycopeptides (Fig. 1-B). Fractions shown by bars were pooled, lyophilized and subjected to hydrazinolysis.

When the reaction products were first analyzed by paper chromatography, only one radioactive peak was detected at the origin of each sample indicating the complete absence of radioactive mono- and disaccharide fragments in the four preparations (data not shown). The radioactive components were extracted from paper by water, and subjected to paper electrophoresis at pH 5.4. The radioelectrophoreto grams obtained by radiochromatoscanning are shown in Fig. 2. The ratio of neutral and acidic oligosaccharides from cell surface glycopeptides was as follows N:A-1:

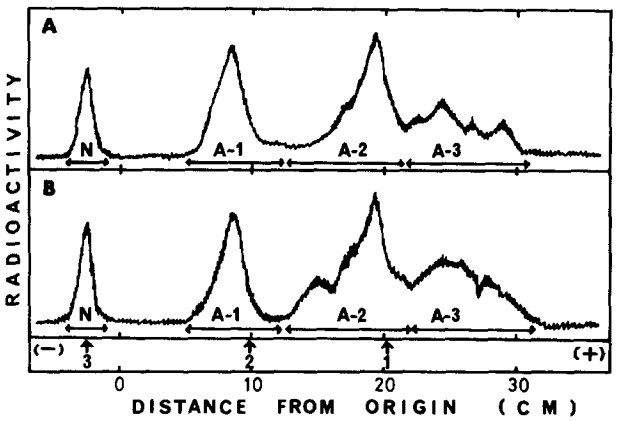

Fig. 2. Paper electrophoresis of the hydrazinolysis products.
Paper electrophoresis was performed at 73 V/cm for 70 min, and the radioactivity was detected by radiochromatoscanner. As the patterns were almost the same between cell surface and cellular origins, the former was shown in this figure. Arrows 1 and 2 indicate the positions of standard disialylated and monosialylated oligosaccharides from human transferrin, and arrow 3 is that of lactitol. A; normal cells, B; transformed cells.

A-2:A-3 equals to 18:37:35:10 for normal cells, and 17:22:35:26 for transformed cells, respectively. Thus, there is little difference of neutral oligosaccharide content between the two types of cells. However, an increase of fast-moving acidic oligosaccharides (A-3), and a decrease of slow-moving components (A-1) were observed. There was also a new component in A-2 region of transformed cells. The acidic nature of these oligosaccharides comes from sialic acid because all of them were completely converted to neutral oligosaccharides by sialidase treatment. These results indicated that sialylation of oligosaccharides from transformed cells was higher than that of normal cells. The increase of oligosaccharides with higher mobilities than an authentic disialylated oligosaccharide from human transferrin and the existence of shoulders located just after peak A-2 were supposed to reflect the structural change of the neutral portion as suggested by Ogata et al (7).

In order to clarify this point, the acidic oligosaccharides were desialized, and analyzed by gel filtration on Bio-Gel P-4. As shown in Fig. 3-A, different elution patterns were obtained from normal and transformed cells. The oligosaccharides from normal cells gave three radioactive peaks, and those from transformed cells gave five peaks. Peaks I, II and III from both cells were eluted at the same positions, but their ratio was different. When calibrated on Bio-Gel P-4 with standard glucose

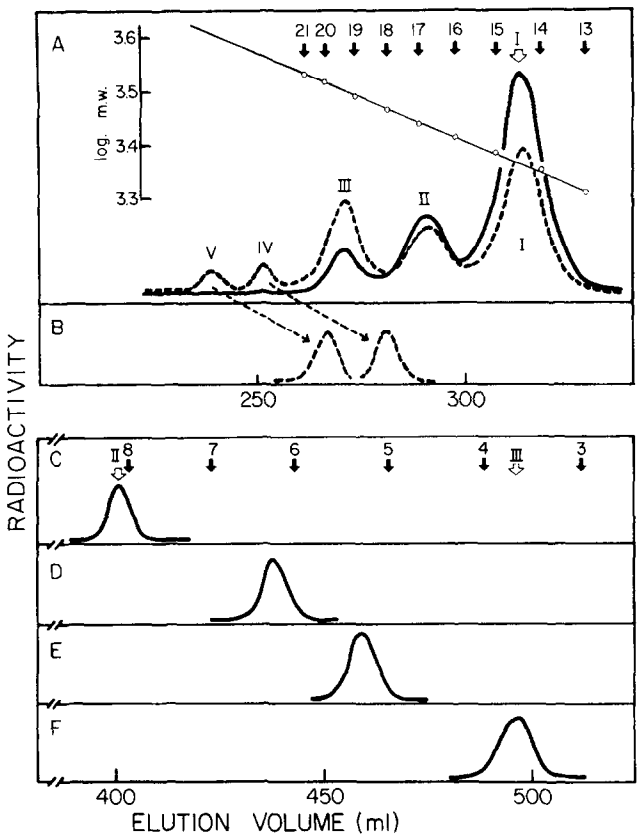

Fig. 3. Sizing of the neutral oligosaccharides by Bio-Gel P-4 and their sequential exoglycosidase digestion.

The samples were applied on a column (2 X 200 cm) of Bio-Gel P-4 (-400 mesh), eluted with distilled water at 55°, and fractionated by 3 ml per tube. As internal standards, partial acid hydrolysate of dextran composed of glucose oligomers (19) was used. The black arrows and numbers indicate the eluting posiitons and glucose units of the glucose oligomers, respectively. Logarithmic values of molecular weight of the standards were plotted against their elution volume. The white arrows indicate the positions of standard oligosaccharides (9, 15); I, $(Gal\text{-}GlcNAc)_2Man_3GlcNAc(Fuc)$N-acetylglucosaminitol II, $Man_3GlcNAc(Fuc)$N-acetylglucosaminitol III, Fuc-N-acetylglucosaminitol. A; The mixture of acidic oligosaccharides (A-1, A-2 and A-3) was digestes with 50 munits of sialidase in 100 µl of 0.1 M acetate buffer, pH 5.0 for 40 hr, followed by electrophoresis. The neutral products thus obtained were analyzed by Bio-Gel P-4. ——, normal cells, - - - -, transformed cells. B; Peaks IV and V in A were digested with 0.8 units of β-galactosidase in 50 µl of 0.05 M citrate-phosphate buffer, pH 3.5 for 45 hr, respectively. C; The mixture of peaks I to V in A from transformed cells was first digested with 0.8 units of β-galactosidase in 50 µl of 0.05 M citrate-phosphate buffer, pH 3.4 for 45 hr, and after desalting the product was further digested with 0.7 units of β-N-acetylhexosaminidase in 50 µl of 0.05 M citrate-phosphate buffer, pH 5.5 for 30 hr. D; The radioactive peak in C was digested with 0.6 units of α-mannosidase in 50 µl of 0.05 M acetate buffer, pH 4.5 containing 0.2 mM $ZnCl_2$ for 45 hr. E; The radioactive peak in D was digested with 10 munits of β-mannosidase in 50 µl of 0.05 M acetate buffer, pH 4.0 for 25 hr. F; The radioactive peak in E was digested with 0.35 units of β-N-acetylhexosaminidase in 50 µl of 0.05 M citrate-phosphate buffer, pH 5.5 for 40 hr. All enzymatic reaction was performed at 37° in the presence of 1 drop of toluene. The data for the oligosaccharides from the cellular glycopeptides of transofrmed cells and from the cell surface and the cellular glycopeptides from normal cells were not shown, but they gave the same results as in C, D, E, and F.

oligomers, I, II, III, IV and V were shown to have sizes of 14.4, 16.9, 19.5, 22.5 and 25.3 glucose units, respectively. Oligomers composed of over 22 glucose units were not available, and the sizes of IV and V were estimated by extrapolation.

After incubation with β-galactosidase and with β-N-acetylhexosaminidase, the five radioactive peaks from transformed cells and the three peaks from normal cells were all converted to a radioactive component with the mobility of 8.1 glucose units where the authentic $Man_3GlcNAc(Fuc)$N-acetylglucosaminitol eluted (Fig. 3-C). The structure of the radioactive oligosaccharide was confirmed as $(Man\alpha\rightarrow)_2Man\beta\rightarrow GlcNAc\ \beta\rightarrow(Fuc\alpha\rightarrow)$-acetylglucosaminitol by sequential exoglycosidase digestion. The products of sequented digestion with α-mannosidase, β-mannosidase and β-N-acetylhexosaminidase eluted at a position consistent with a loss of 2 moles of mannose, 1 mole of mannose, and 1 mole of N-acetylglucosamine, respectively (Fig. 3D. E and F), and the final radioactive product was eluted at the same position as authentic Fucα1→6N-acetylglucosaminitol. The radioactivity in this oligosaccharide was completely converted to fucose by incubation with *Charonia lampas* α-L-fucosidase (data not shown). Therefore, all the radioactive oligosaccharides shown in Fig. 3-A have the common core portion of the fucose containing complex-type asparagine-linked sugar chain Man_3·GlcNAc(Fuc)GlcNAc, and their size difference comes from the different content of galactose and N-acetylglucosamine residues in the outer chain moiety. As described already, the sizes from peak I to V are gradually enlarged by approximately three glucose units. Since one N-acetylglucosamine residue behaves as approximately two glucose units, the three glucose units may represents one Galβ→GlcNAc grouping. Because peak I showed the same mobility as authentic Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)N-acetylglucosaminitol obtained from human complement C1q (9) and from human chorionic gonadotropin (17), it should have two Galβ→GlcNAc groups in its outer chain moiety. Therefore, peaks II, III, IV and V are supposed to have one, two, three and four more Galβ→GlcNAc groups than peak I in their outer chain moieties. Since complex type sugar chains with five and six outer chains have never been reported, we have analyzed the structures of peaks IV and V in more detail.

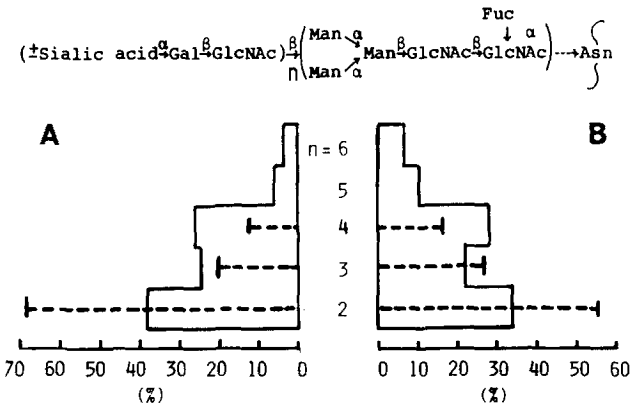

Fig. 4. The ratio of oligosaccharides with different numbers of outer chains.
A oligosaccharides from cell surface glycopeptides.
B oligosaccharides from cellular glycopeptides ⊢---⊣ ; normal cells,
▭ ; transformed cells.

β-Galactosidase digestion released approximately five galactose residues from peak IV and six galactose residues from peak V(Fig. 3-B). The resulting radioactive oligosaccharides from peaks IV and V were larger than the core portion (Fig. 3-C) by ten and twelve glucose units, respectively, which correspond to five and six N-acetylglucosamine residues. This result indicated that peaks IV and V actually have highly branched structures, and do not have any linear chain of Galβ→GlcNAc repeating unit in their outer chain moiety as recently found in human erythrocyte membrane glycoproteins (18). The ratios of the oligosaccharides which originate from the fucose containing asparagine-linked sugar chains of glycoproteins of normal and transformed cells are graphically presented in Fig. 4. It is obvious that the transformed cells are enriched in larger asparagine-linked fucosyl sugar chains in both cell surface and cellular glycoprotein. Since the larger sugar chains have a higher content of outer chain moieties, it can be finally concluded that the alteration is induced by a change in N-acetylglucosaminylation steps. The exact location of the extra outer chain groupings in peaks IV and V remains for future study.

ACKNOWLEDGEMENTS The authors wish to thank Dr. A. Hakura and Professor K. Toyoshima for providing us normal and transformed BHK cells. Thanks are also due to Miss J. Fujii for her expert secretarial assistance. This work has been supported in part by research grants from the Scientific Research Funds (1978-1979) of the Ministry of Education, Science and Culture of Japan and from the Mitsubishi Foundation.

REFERENCES

1. Buck, C. A., Glick, M. C. and Warren, L. (1971) Biochemistry, 9, 4567-4576
2. Buck, C. A., Glick, M. C. and Warren, L. (1971) Science, 172, 169-171
3. Warren, L., Fuhere, J. P. and Buck, C. A. (1972) Proc. Natl. Aca. Sci. U.S.A. 69, 1838-1842
4. Buck, C. A., Fuhere, J. P., Soslau, G. and Warrem, L. (1974) J. Biol. Chem., 249, 1541-1550
5. Van Beek, W. P., Smets, L. A. and Emmelot, P., (1973) Cancer Res., 33, 2913-2922
6. Van Beek, W. P., Smets, L. A. and Emmelot, P. (1975) Nature, 253, 457-460
7. Ogata, S., Muramatsu, T. and Kobata, A. (1976) Nature, 259, 580-582
8. Van Beek, W. P., Emmelot, P. and Homburg, C., (1977) Br. J. Cancer, 36, 157-165
9. Mizuochi, T., Yonemasu, K., Yamashita, K. and Kobata, A. (1978) J. Biol. Chem., 253, 7404-7409
10. Mizuochi, T., Yamashita, K. and Kobata, A. manuscript in preparation.
11. Li, Y. -T. and Li, S. -C. (1972) Methods Enzymol. 28, 702-713
12. Uchida, Y., Tsukada, Y. and Sugimori, T. (1974) Biochim. Biophys. Acta, 350, 425-431
13. Sugawara, K., Okumura, T. and Yamashina, I. (1972) Biochim. Biophys. Acta, 268, 488-496
14. Nishigaki, M., Muramatsu, T., Kobata, A. and Maeyama, K., (1974) J. Biochem. (Tokyo) 75, 509-517
15. Eagle, H., (1959) Science, 130, 432-437
16. Earle, W. R., (1943) J. Natl. Cancer Inst. 4, 165-212
17. Endo, Y., Yamashita, K., Tachinama, Y., Tojo, S. and Kobata, A. (1979) J. Biochem. (Tokyo) 85, 669-679
18. Jarnefelt, J., Ruth, J., Li, Y. -T. and Laine, R. A. (1978) J. Biol. Chem. 253, 8006-8009
19. Nishigaki, M., Yamashita, K., Matsuda, I., Arashima, S. and Kobata, A. (1978) J. Biochem. (Tokyo) 84, 823-834