The analysis of N-glycolylneuraminic acid(NeuGc) of hepatoma tissue and K562 cell ferritins using HPLC and mass spectrometry

By Hideo ASAKAWA,^{*1),†)} Masataka SASABE,^{*1)} Ryuunosuke MIYAZAKI,^{*1)} Haruo MATSUDA,^{*2)}
Fumio FUKAI,^{*3)} Kazuki HANADA,^{*4)} Hisashi HIRANO,^{*4)} and Seiichi TAKASAKI^{*5)}

(Communicated by Tamio YAMAKAWA, M. J. A.)

Abstract: Ferritin is an iron-storage protein and its serum level is known to increase in the patient of with inflammation and malignant tumor. To further elucidate the difference between ferritins from normal human liver tissue and that of cancer cells, their sialic acids were analyzed. The Western blot analysis and the cytochemical staining using anti-NeuGc antiserum indicated that ferritins from the human hepatocarcinoma tissue and malignant K562 cells contain NeuGc, but that from the normal liver does not. The result was also confirmed by HPLC analysis and MALDI-TOF/MS analysis of sialic acids which were derivatized by the DMB method. It was also shown that the sialic acid content in hepatocarcinoma ferritin was much higher than that in the normal liver ferritin. These results suggest that normal and cancerous liver ferritins are qualitatively and quantitatively different in sialylation. In addition, K562 cells were shown to express NeuGc even if the cells were cultured in serum-free media which lack NeuGc. This is of interest from the current concept that expression of NeuGc in human cells is due to uptake and utilization of exogenous NeuGc.

Key words: Hepatoma; K562 cell; ferritin; WB; HPLC; MS.

Introduction. Ferritin is an iron-storage protein which widely exists in animals and plants. In vertebrates, its cytosolic form consists of two different subunits, termed H (21 kDa) and L (19 kDa). Twenty-four ferritin subunits assemble to form the apoferritin (450 kDa). The ferritin content in human serum often increases in the patients with inflammation and malignant tumor. Therefore, its serum level has been noticed as the signal which shows the pathology of those diseases, and espe-

cially as a tumor marker.¹⁾ It is also known that ferritin in the malignant tumor tissue shows more acidic property.²⁾ Lee & Richter³⁾ supposed that the acidic isoform differs from the normal ferritin in amino acid composition. The isoform was also found to exist in the normal fetal tissue, and named carcino-fetal isoform by Alpert *et al.*⁴⁾ In addition, Makino & Konno,⁵⁾ and Marcus & Zinberg⁶⁾ reported that such isoform is also found in mammary cancer and pancreas cancer tissues. Urushizaki *et al.* have made the liver cancer in the rat, and pointed out the difference between normal and cancer liver ferritins.⁷⁾ Drysdale also detected the acidic isoform in cultured HeLa cells.⁸⁾ However, Arosio *et al.* showed that the isoform is detected in the heart, kidney, and pancreas of the normal adult.⁹⁾ The earlier proposal for the difference in amino acid composition between normal and neoplastic ferritins was denied by the molecular biological technique.^{10),11)} Our earlier observation suggested that the neoplastic isoform prepared from human hepatoma tissue shows more acidic property in electrophoresis and contains less amounts of iron. We suspected that there might be the

^{*1)} Kudanzaka Hospital, Department of Internal Medicine, Tokyo, Japan.

^{*2)} Department of Microbiology and Hygiene, Faculty of Applied Biological Science, Hiroshima University, Hiroshima, Japan.

^{*3)} Department of Patho-Physiology, Faculty of Pharmaceutical Science, Tokyo University of Science, Tokyo, Japan.

^{*4)} Yokohama City University, International Graduate School of Arts and Science, Kanagawa, Japan.

^{*5)} Division of Biochemistry, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

^{†)} Korokudai 143-4, Matsudo, Chiba 271-0075, Japan (cd93nd@bma.biglobe.ne.jp).

Abbreviations: NGNA, glycolyl neuraminic acid; NANA, acetyl neuraminic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; HpF, hepatoma ferritin; HLF, human liver ferritin.

difference in post-translational modification between the normal and the neoplastic ferritins. First, we examined the phosphate content, but could observe no decisive difference between them.¹²⁾ Then, we analyzed glycosylation, and found that there is a difference in the sialic acid content.^{13),14)} Considering the previous report that the Hanganutziu-Deicher antigen known as a heterophil antigen^{15),16)} are also found in tumor and the antibodies against the antigen recognize N-glycolylneuramic acid (NeuGc),¹⁷⁾ we focused on the qualitative difference in sialylation in this study. To begin with, we performed the Western blot analysis of ferritin purified from normal human liver (HLF), that from the hepatoma tissue (HpF), and that from K562 cells (K562F), using chicken monoclonal antibody (mAb) Hu/Ch2-7¹⁸⁾ recognizing NeuGc. Then, we histochemically analyzed the expression of NeuGc in the hepatoma tissue and K562 cells. In addition, biochemical approaches such as HPLC and mass spectrometry of sialic acids were introduced to detect NeuGc more clearly. Taking it in consideration that NeuGc might be expressed in human cells and tissues which lack CMP-NeuAc oxygenase by utilizing the exogenously incorporated NeuGc, we carefully examined if NeuGc is expressed by K562 cells cultured in the serum-free media.

Materials and Method. *Western blot analysis.*

Ferritin was purified from liver and K562 cells according to the procedures of Marcus & Zinberg⁶⁾ and Drysdale.⁸⁾ To check the purity, the ferritin preparations were subjected to polyacrylamide gel electrophoresis (PAGE) by the method of Cleveland & Laemmli¹⁹⁾ and were detected by silver staining. The protein content was measured by the Lowry's method. Each ferritin sample was resolved into the H and L subunits by SDS-PAGE using 15.4% gel, according to the method of Drysdale²⁰⁾ and transferred to PVDF membranes after SDS-PAGE using 15.4% gel. The membranes were stained with CBB or immunologically with the ECL plus Western blot detection system (Amersham Co.). The mAb HU/Ch2-7¹⁸⁾ was used as the first antibody to detect NeuGc. The peroxidase (POD)-labeled goat anti-chicken IgG antibody was used as the secondary antibody.

Cytochemical staining of tissues. The slide sample was made using cytospin with the K562 culture cells and the hepatoma tissue. The blocking of endogenous peroxidase was carried out as follows: After the 5 minutes steep in methanol containing 0.3% H₂O₂, the slides were washed three times with PBS containing Tween 20. And then the slides were blocked with 2% chicken albumin in PBS for 30 minute at room temperature, and

immunologically stained using the mAb HU/Ch2-7, the POD-labelled goat anti-chicken IgG antibody above mentioned, and 3,3'-diaminobenzidine (DAB) as a developing dye.

HPLC analysis of sialic acids. The ferritin samples were heated in 25 mM H₂SO₄ solution at 80 °C for 1 hour, and then stand on ice for 1 hour. The acid hydrolysates were labeled using a fluorescent labeling kit (DMB labeling kit, TAKARA) according to the manufacturer's direction. The labeled samples were centrifuged at 3,000 rpm for 10 minutes, and the aliquots were analyzed by HPLC. K562 cells (5 × 10⁶) cultured in RPMI 1640 containing 10% fetal calf serum (FCS) were washed with PBS, and then cultured in the serum-free media, ASF104 (Ajinomoto Co.) or ASF104N (Ajinomoto Co.). The cells were transferred to new culture flasks and cultured in the serum-free media. The culture in the new serum-free media was repeated three times. The washed cell pack was acid hydrolyzed and labeled as described above. HPLC of the labeled sialic acids was performed using a column (4.6 × 250 mm) of TSK-ODS80T (TOSOH). Elution was done with a mixture of methanol, acetonitrile, and water, 7/9/84(V/V/V), and at a flow rate of 0.8 ml/min. The fluorescence (excitation at 373 nm, emission at 448 nm) was monitored using a fluoromonitor (FS8000, TOSOH).

Mass spectrometry of sialic acid. 2, 5-Dihydroxybenzoic acid (DHB, Sigma) was dissolved at a concentration of 10 mg/ml in a mixture of acetonitrile and water (70:30 V/V) and used as a matrix solution for mass spectrometry. Two ml each of the sample solution and the matrix solution were mixed, spotted on the sample plate, and solidified in a desiccator. One µl of ethanol was added on the spot, and the sample/matrix was recrystallized. The ToF spec2E (Micro Mass), a matrix assisted laser ionization time flight (MALDI-TOF) type mass spectrometer, was used for the sample measurement. Ionization was done at 20 kV with 337 nm pulse laser, and positive and negative ions in the range of 0-600 m/z were measured in the reflectron mode.

Results. *Western blot analysis of purified ferritin and enzyme-immunostaining of tissues.* Ferritins were purified from normal liver and hepatocarcinoma liver, and were analyzed by the Western blot method. The result was shown in Fig. 1. The immunostaining image obtained using the mAb Hu/Ch2-7 recognizing NeuGc showed that the subunits of ferritin from K562 cells (lane 6) and HpF (lane 7) were clearly stained. On the other hand, the normal liver tissue ferritin was not stained (lane 8). Next, enzyme-immunos-

Fig. 1. The Western blot analysis of purified ferritin. Lanes 2 and 6 are ferritins from K562 cells, lanes 3 and 7 are those from hepatocarcinoma, and lanes 4 and 8 are those from normal liver, respectively. Lanes 1 and 5 are molecular weight marker proteins. Lanes 1 to 4 were stained using CB, and lanes 5 to 8 were immunostained with Hu/Ch2-7 anti GM3 chicken antibody recognizing NeuGc.

taining of hepatocarcinoma tissue (Fig. 2) and K562 cells (Fig. 3) was performed. The images showed positive staining of cytoplasm. On the other hand, the normal liver was not stained (data not shown). These results indicate that NeuGc exists in malignant tumor cells and tissue but not in the normal tissue.

Analysis of sialic acids of ferritin by HPLC and MALDI-TOF/MS. Sialic acids were released from ferritins, derivatized by the DMB labeling method, and analyzed by HPLC (data not shown). Two peaks corresponding to standard derivatives of NeuAc and NeuGc were detected from HpF, while only the peak corresponding to NeuAc was detected from HLF. Both peaks were also detected from ferritin (K562F) of K562 cells cultured in RPMI1640 containing 10% FCS. To confirm the results, MALDI-TOF/MS analysis was performed. As shown in Fig. 4A, the mass spectrum of the DMB derivatives of sialic acids from HpF indicated the presence of the ions at m/z of 425.05 and 441.05 corresponding to the $[M+H]^+$ values of the NeuAc and NeuGc derivatives. On the other hand, the ion at m/z of 425.72, the $[M+H]^+$ value of NeuAc derivative, was detected from HLF (Fig. 4B). Thus, the data indicate that HpF expresses both NeuAc and NeuGc, but HLF exclusively expresses NeuAc. The contents of NeuGc and NeuAc included in HLF, HpF and K562F were calculated on their peak areas in the HPLC

Fig. 2. Enzyme-immunostaining of hepatocellular carcinoma with Hu/Ch2-7 antiserum.

Fig. 3. Enzyme-immunostaining of K562 cells cultured in ASF104 (TAKARA Co. Not containing serum) with Hu/Ch2-7 antiserum.

analysis, and summarized in Table I. NeuGc accounts for approximately 13% of the total sialic acid in HpF, and 21% in K562F. It is of note that HpF contains very larger amounts of sialic acid than HLF.

Effect of culture media on the expression of NeuGc in K562 cells. As shown above, ferritin from K562 cultured in RPMI1640 containing 10% FCS was shown to express both NeuAc and NeuGc. Then, we examined if the expression of NeuGc by K562 cells is affected by the culture media. Two serum-free media, ASF104N and ASF104, were used for culture of the cells. First, we tested if the media contain sialic acid. The HPLC analysis of sialic acids derivatized by the DMB method showed no clear peak corresponding to NeuGc-DMB (data not shown). Furthermore, the spectra of MALDI-TOF/MS analysis did not show any ions corresponding to NeuGc-DMB and NeuAc-DMB (Fig. 5B and C). Then, K562 cells were grown in the serum-free media ASF104N and ASF104, and ferritins were purified

Fig. 4. MALDI-TOF/MS spectra of DMB derivatives of sialic acids released from hepatocarcinoma ferritin (A) and normal liver ferritin (B).

from the cells. Ferritin from the cells cultured in ASF104 was then subjected to the analysis by MALDI-TOF/MS. As shown in Fig. 5A, two ions at m/z 449.06 and m/z 465.06 corresponding to $[M+Na]^+$ of NeuAc-DMB and NeuGc-DMB were detected. More quantitative data were obtained by HPLC of DMB derivatives. As shown in Table II, the relative contents of NeuGc decreased by serum deprivation, but considerable amounts of NeuGc

were still expressed. Thus, the result shows that NeuGc is expressed by K562 cells grown in the media lacking NeuGc.

Discussion. It has been demonstrated that the total ferritin content increases in the serum of patients with various malignancies. However, it is known that inflammation also causes the increase of serum ferritin. Another cancer-associated change is a shift to acidic fer-

Table I. Sialic acid contents of ferritins from normal liver, hepatocarcinoma and K562 cells

	NeuAc	NeuGc
	nmole/mg protein	
normal liver ferritin	0.26	not detected
hepatocarcinoma ferritin	19.4	2.83
ferritin from K562 cells	4.66	1.22

Table II. Sialic acids in ferritins purified from erythroleukemic K562 cells cultured in serum-containing and serum-free media

	NeuAc	NeuGc
	Percent molar ratio ^{a)}	
RPMI 1640 plus 10% FCS ^{b)}	82	18
RPMI 1640 plus 10% FCS ^{b)}	79	21
ASF104 without FCS	87	13
ASF104N without FCS	86	14

^{a)}The relative amounts of NeuAc and NeuGc were presented by taking the total amounts of sialic acid as 100.

^{b)}The results obtained in different experiments were given.

ritins enriched with H subunits. The ratio of H to L subunits in ferritin varies depending on the tissue type and physiological status of the cells. For example, H-subunit is predominant in heart and kidney and L-subunit is in liver. Serum ferritins are composed of two forms; one actively secreted from cells like cancer cells and the other passively released from damaged cells in various tissues. It is difficult to discriminate ferritins produced and secreted by cancer cells from those passively released from damaged cells. It is necessary to approach the problem from a different aspect. In this study, we could show that hepatocarcinoma tissue ferritin contains larger amounts of sialic acids (22 nmole/mg of protein) as compared with normal liver tissue ferritin (0.26 nmole/mg protein). The sialic acid contents of carcinoma and normal ferritin are calculated to be 9.9 mole and 0.12 mole/mole of ferritin (450 kD) on an average, respectively. The present quantitative analysis supports the previous observation that incubation of human liver ferritin with neuraminidase did not change the isoelectric focusing patterns.²¹⁾ Interestingly, ferritins from spleen and heart have been shown to be also resistant to neuraminidase.²¹⁾ Thus, it is worthy of notice that the status of sialylation is clearly different between normal and cancer ferritins. In addition, we could show the clear difference in sialic acid species between normal and hepatoma ferritins. The Western blot analysis and the chemical analysis based on HPLC and mass spectrometry indicated that hepatocarcinoma tissue ferritin expresses considerable amounts of

NeuGc as well as NeuAc, while normal liver ferritin exclusively expresses NeuAc. This difference is of clinically great importance. It will be interesting to analyze sialylation of ferritins from various tissues in physiological and pathological conditions.

A gene encoding CMP-NeuAc hydroxylase that converts CMP-NeuAc to CMP-NeuGc resulting in incorporation of NeuGc to glycans has an exon deletion/frame shift mutation in humans.²²⁾ However, it has been shown that NeuGc is expressed in cancerous tissues of patients with breast cancer,²³⁾ melanoma,²⁴⁾ and large bowel cancer.²⁵⁾ In this study, we also presented the existence of NeuGc in human hepatocarcinoma tissue and an erythroleukemic cell line K562. The present consensus is that the expression of NeuGc in human cancer cells is due to uptake of non-human NeuGc from dietary sources and its incorporation into cellular glycoconjugates.²⁶⁾⁻²⁸⁾ Quite recently, it has been shown that hypoxic culture of human tumor cells induces expression of sialin, a sialic acid transporter which facilitate cellular incorporation of exogenous sialic acid of dietary origin, and results in increased expression of GM2 ganglioside containing NeuGc.²⁹⁾ It is known that fetal calf serum is a good exogenous source of NeuGc. Therefore, we examined how the expression of NeuGc by K562 cells changes by culturing in the absence of serum. Two serum-free media, ASF104 and ASF104N, were selected, and subjected to the HPLC analysis of sialic acids, resulting in the absence of no clear peaks corresponding to NeuGc-DMB and NeuAc-DMB. Furthermore, the

Fig. 5. MALDI-TOF/MS spectrum of DMB derivatives of sialic acids released from K562 cells cultured in serum-free medium ASF104 (A). The spectra of DMB-derivatives of ASF104 and ASF104N themselves were shown in (B) and (C), respectively.

spectra of MALDI-TOF/MS analysis did not show any ions corresponding to NeuGc-DMB and NeuAc-DMB. Furthermore, the spectra of MALDI-TOF/MS analysis did not show any ions corresponding to NeuGc-DMB and NeuAc-DMB (Fig. 5B and 5C). Then, K562 cells were grown in serum-free media, and cells were harvested after three passages. Both HPLC analysis and MALDI-TOF/MS analysis of sialic acids in ferritins indicated that NeuGc is still expressed in ferritin of K562 cells cultured in the serum-free media. The result is not explained by the uptake and utilization of exogenous NeuGc. However, we do not exclude the idea that expression of NeuGc in cancer tissue *in vivo* is at least partly due to the mechanism like the sialin-mediated incorporation from external milieu. Further study will solve the issue.

References

- 1) Mori, W., Asakawa, H., and Taguchi, T. (1975) J. Natl. Cancer Inst. **55**, 513-518.
- 2) Richter, G. W. (1965) Nature **207**, 616-618.
- 3) Lee, J. C., and Richter, G. W. (1971) Cancer Res. **31**, 566-572.
- 4) Alpert, E., Coston, R. L., and Drysdale, J. W. (1973) Nature **242**, 194-196.
- 5) Makino, Y., and Konno, K. (1969) J. Biochem. (Tokyo) **65**, 471-473.
- 6) Marcus, D. M., and Zinberg, N. (1975) J. Natl. Cancer Inst. **55**, 791-795.
- 7) Urushizaki, I., Ishitani, K., Natori, M., Yokota, M., Mitago, N., and Niitsu, Y. (1973) Gann **64**, 237-246.
- 8) Drysdale, J. W. (1974) Biochem. J. **141**, 627-632.
- 9) Arosio, P., Yokota, M., and Drysdale, J. W. (1976) Cancer Res. **36**, 1735-1739.
- 10) Costanzo, F., Colombo, M., Staempfli, S., Santoro, C., Marone, M., Frank, R., Delius, H., and Cortese, R. (1986) Nucl. Acids Res. **14**, 721-736.
- 11) Santoro, C., Marone, M., Ferrone, M., Costanzo, F., Colombo, M., Minganti, C., Cortese, R., and Silengo, L. (1986) Nucl. Acids Res. **14**, 2863-2876.
- 12) Yamaguchi, K., Asakawa, H., Teramura, Y., Nozawa, Y., Oka, T., Mori, W. (1997) Jpn. J. Electrophoresis **182**, 923-924 (in Japanese).
- 13) Asakawa, H., Yamaguchi, K., Miyauchi, S., and Mori, W. (1996) Igakunoayumi **176**, 521-522 (in Japanese).
- 14) Asakawa, H., Kubota, T., Kushi, Y., Teramura, Y., Hino, K., Usui, M., Kurimoto, M., and Mori, W. (1997) Igakunoayumi **182**, 923-924 (in Japanese).
- 15) Hanganutziu, M. (1924) Chaval. Cr. Seanac Soc. Biol. **91**, 1457-1459.
- 16) Deicher, H. (1925) Zhyg. Infection Krankh. **106**, 561-579.
- 17) Fujii, Y., Higashi, H., Ikuta, K., Kato, S., and Naiki, M. (1982) Mol. Immunol. **19**, 87-94.
- 18) Asaoka, H., and Matsuda, H. (1994) J. Vet. Med. Sci. **56**, 375-377.
- 19) Cleveland, D. W., Fischer, S. G., Kirshner, M. W., Laemmli, U. K. (1977) J. Biol. Chem. **255**, 1102-1106.
- 20) Drysdale, J. W., Adelman, T. G., Arosio, P., Casareale, D., Fitzpatrick, P., Hazard, J. T., and Yokota, M. (1977) Semin. Hematol. **14**, 71-88.
- 21) Cragg, S. J., Wagstaff, M., and Worwood, M. (1980) Clin. Sci. (Lond) **58**, 259-262.
- 22) Irie, A., Koyama, S., Kozutsumi, Y., Kawasaki, T., and Suzuki, A. (1998) J. Biol. Chem. **273**, 15866-15871.
- 23) Marquina, G., Waki, H., Fernandez, L. E., Kon, K., Carr, A., Valiente, O., Perez, R., and Ando, S. (1996) Cancer Res. **56**, 5165-5171.
- 24) Hirabayashi, Y., Hamaoka, A., Matsumoto, M., Matsubara, T., Tagawa, M., Wakabayashi, S., and Taniguchi, M. (1985) J. Biol. Chem. **266**, 13328-13333.
- 25) Higashi, H., Hirabayashi, Y., Fukui, Y., Naiki, M., Matsumoto, M., Ueda, S., and Kato, S. (1985) Cancer Res. **45**, 3796-3802.
- 26) Varki, A. (2000) Year book Phys. Anthropol. **44**, 54-69.
- 27) Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A., and Muchmore, E. (2003) Proc. Natl. Acad. Sci. USA **100**, 12045-12050.
- 28) Bardor, M., Nguyen, D. H., Diaz, S., and Varki, A. (2005) J. Biol. Chem. **280**, 4228-4237.
- 29) Yin, J., Hashimoto, A., Izawa, M., Miyazaki, K., Chen, G. Y., Takematsu, H., Kozutsumi, Y., Suzuki, A., Furuhashi, K., Cheng, F.-L. *et al.* (2006) Cancer Res. **66**, 2937-2945.

(Received April 21, 2006; accepted May 12, 2006)