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Screening of *N*-acylneuraminic acids in serum and tissue specimens of mouse C57BI with Lewis' lung cancer by high-performance liquid chromatography

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Abstract

Serum and tissue specimens from healthy C57BI mice and from mice with Lewis' lung cancer after metastasis were analyzed for *N*-acetyl- and *N*-glycolylneuraminic acid by high-performance liquid chromatography. Both neuraminic acids were present, while *N*-glycolylneuraminic acid was the predominant sialic acid in all tissues. Samples from mice with metastatic cancer showed a significant increase (67–200%) of total sialic acids mainly as a result of increased *N*-glycolylneuraminic acid synthesis. These results suggest that cancer metastasis in various tissues is closely associated with increased synthesis of the predominant neuraminic acid and may help to understand the underlying mechanisms of tumor development. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *N*-Acetylneuraminic acid; *N*-Glycolylneuraminic acid; Sialic acids

1. Introduction

Sialic acids (SAs) is the generic name given to a family of compounds derived from the 9-carbon sugar neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosic acid). They are present in high concentrations as components of glycoproteins, polysaccharides, glycolipids such as gangliosites, and are usually located on the outer cell membrane linked to other sugars by glycosidic bonds. The most commonly occurring sialic acids (Fig. 1) are the *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). They can also be *O*-substituted at the 4, 7, 8 and 9 positions, giving rise to a wide variety of compounds and isomers [1,2]. SAs act as biological masks by preventing ligands from recognizing receptors [3]. The high sensitivity of sialic acid as a tumor marker has been reported in a variety of cancerous conditions [4,5]. Increased serum levels of SAs have been observed in patients with cancer [6] and various other diseases [7–11] as well as in patients with congenital metabolic disorders [12,13] or after myocardial infarction [14,15]. However, the mechanism of SA elevation in serum is not clear. During neoplastic transformation, the carbohydrate chains in glycolipids and glycoproteins are frequently altered. A close relationship between the expression of certain carbohydrate antigens and tumor development also exists [16]. It has been demonstrated that

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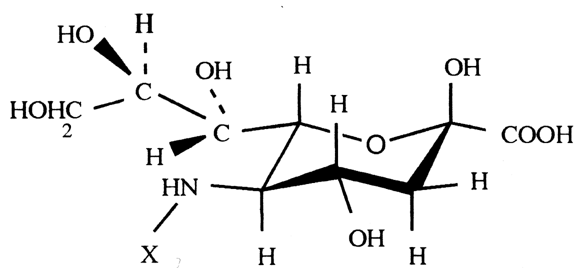

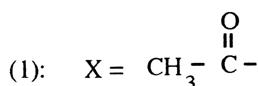

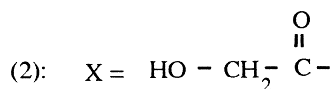

Fig. 1. Structure of *N*-acetylneuraminic acid (1) and *N*-glycolylneuraminic acid (2).

not all sialic acids are specific to cancer and significant variations exist in the cancer specificity depending on the difference in linkage in sialic acid residues [17].

Whereas serum SA is of limited use in the initial diagnosis of different types of cancer, serial measurements may be helpful in detecting the development of secondary metastases or to monitor the tumor bulk in response to treatment. A variety of methods are available for the measurement of SAs but very few have been reported for Neu5Ac and Neu5Gc [18–20]. High-performance liquid chromatography (HPLC) procedures can detect them at picogram levels and are relatively free of interference which is usually seen with classical methods.

The most common form of serum SA in humans and chicken is Neu5Ac synthesized from glucosamine by a series of chemical reactions involving phosphoenolpyruvate. Neu5Gc is synthesized oxidatively from Neu5Ac by Neu5Ac monooxygenase (EC 1.14.99.18) both before and after transfer to glycoconjugates [21,22]. Synthesis of Neu5Gc recognized in many animals, even in the primate monkey [23], was blocked during the evolution process to human beings. This block is broken, however, to a significant degree in various cancers. Thus, human and chicken cancerous tissues often synthesize this type of SA as a tumor-associated Hanganutziu–Deicher antigen [24–26].

There are two basic procedures to liberate SAs from glycosidic linkages; enzymatic and acid hydrolysis. In the former case, a variety of sialidases are used that differ in specificity for SA linkage or species [27,28]. In the latter case, several methods for acid hydrolysis are available [29–32]. With most acids the liberation is followed by simultaneous removal of *O*-acetyl groups, whereas the *N*-acyl linkages are more stable.

During the last decade, a number of molecules have been proposed as tumor markers [4,5,16,17]. SA levels in serum have been proposed as an evaluation marker of the cancer progress. Recently, we have reported on a simple procedure for the removal of interfering substances and a HPLC method for determining Neu5Ac and Neu5Gc in serum samples and/or tissue specimens [33]. In the present study, a comparative evaluation of Neu5Ac and Neu5Gc content in various tissues from C57BI mice versus tissues from mice with Lewis' lung cancer after metastasis was performed. Results obtained mainly from the lungs of mice which developed metastasis suggest that cancer metastasis is closely associated with increased synthesis of the predominant SA.

2. Experimental

2.1. Chemicals and biological material

Neu5Ac, Neu5Gc and *N*-acetylneuraminyl-α-(2,3)-lactose from human milk were purchased from Sigma (St. Louis, MO, USA). Sep-Pac C_{18} cartridges were obtained from Waters (Milford, MA, USA). All other chemicals used were of analytical grade.

A benzoylation mixture was prepared by dissolving 1 g of benzoic anhydride and 0.5 g of *p*-dimethylaminopyridine in 10 ml of pyridine. The solution was stored in dark at 4°C and was stable for more than one week.

2.2. Animal studies

Three male 6–8-week-old C57BI mice inoculated with Lewis' lung cancer were obtained from the Experimental Laboratory of Theagenian Anticancer Institute (Thessaloniki, Greece). All mice were fed

animal chow and had water ad libitum. Animals with tumors ranging between 0.8–1.5 cm^3 were sacrificed by cardiac exsanguination under ether anesthesia and tumors were excised under sterile conditions. A suspension in normal saline over ice was subsequently obtained by mincing the tumor and passing it through a series of sequentially smaller hypodermic needles (22–30 gauge). The final concentration was adjusted to $2 \cdot 10^7$ tumor cells/ml and this suspension was used for implantation of C57BI mice. The right hind leg of six C57BI mice was inoculated subcutaneously with 0.1 ml of cell suspension following local disinfection. The animals were similarly sacrificed 14 days later and the tumors and lungs were excised and weighed. Part of the lung was fixed in formalin for histological examination. Blood was also collected and serum was kept at −20°C until assayed. Similarly, serum and respective tissues of three normal C57BI mice were obtained serving as control.

2.3. Histological examination of lungs

Fixed lung specimens were embedded in paraffin according to standard histological procedures used in the clinical laboratory (Dept. of Pathology, University Hospital of Patras, Greece). Four sections were made through each sample and stained with haematoxylin-eosin. Evaluation of metastasis was performed by microscopic examination.

2.4. Sample treatment and preparation of standards

Tissue specimens from control and inoculated animals, i.e., muscle from the hind leg (480–560 mg), lungs (81–95 mg) and tumors (500–1500 mg) were collected and 20–100 mg of the respective tissue in 3 ml distilled water were homogenized at 0°C for 1 min (three 20 s pulses) by the use of a Polytron homogenizer (Kinematica, Switzerland). The homogenizing system was cleaned with an additional 1 ml of water and the total suspension was centrifuged at 11 000 *g* for 5 min. The supernatant was brought up to 5 ml with water in a graduated flask and kept at −20°C until SA analysis.

Two volumes of saturated ammonium sulfate, pH 10, were added to 100 μl of serum and the mixture was kept at 0°C for 20 min. Following centrifugation at 11 000 *g* for 5 min, the obtained precipitate was dissolved in water (200 μl) and the solution was chromatographed on a Sephadex G-25 (30 mm×13 mm I.D.) prepacked column (PD-10, Pharmacia, Sweden). The column was washed with 0.8 ml of water, and the SA-containing macromolecules were recovered by elution with 1.5 ml of water. The eluates were transferred to a graduated 2-ml flask, brought up to volume with water and kept at −20°C until SA analysis.

Glycoconjugates containing 5–50 μg of total SAs were hydrolyzed in 500 μl of 25 m*M* trifluoroacetic acid (TFA) at 80°C for 2 h in screw-capped polypropylene microtubes. Standards were prepared by treating known amounts of Neu5Ac and Neu5Gc under the same conditions. Hydrolyzates were lyophilized and the obtained residues were dissolved in 200 μl of 2× distilled water. The solutions were chromatographed on a Dowex 50X8 (200–400 mesh, H^+ form, 40 mm×3 mm I.D.) column. Cations were held back on the column, while the rest of the molecules, including the SAs, were eluted with 1 ml of water. The eluates were partly neutralized to a pH 3–5 with 0.5 *M* NH_4OH and then were rechromatographed on a Dowex 1X8 (200–400 mesh, $HCOO^-$ form, 40 mm×3 mm I.D.) column [34]. Neutral and positive charged molecules were removed by washing the column with 1 ml of water, while SAs were recovered by elution with 2 ml of 2 *M* HCOOH. The latter fraction was collected, lyophilized and taken for benzoylation.

2.5. Derivatization procedure

Per-*O*-benzoylated derivatives were prepared as previously described [19]. In particular, 100 μl of benzoylation mixture were added to the dry hydrolysates and the mixture was heated at 80°C for 20 min. The remaining amount of benzoic anhydride was destroyed by adding 0.9 ml of water and heating the mixture for a further 10 min at 80°C. Excess reagents and under-benzoylated derivatives were removed by passing the mixture through a Sep-Pak C_{18} cartridge which had been equilibrated with 5 ml of methanol and 10 ml of water. After sample addition, the cartridge was washed with 5 ml of water and the per-*O*-benzoylated derivatives of SAs

were eluted with 5 ml of acetonitrile. Following evaporation of the latter fraction, the residue was dissolved in 200 μl of acetonitrile and aliquots of 10–20 μl were taken for HPLC analysis.

2.6. Instrumentation, HPLC conditions and quantitation

An LDC constametric III pump equipped with a Reodyne Model 7125 injector unit with a 50-μl loop and an LDC spectromonitor 1204 A UV detector with a 8-μl flow cell was used. Separation was performed on a Supelcosil LC-18 column (250 mm× 4.6 mm I.D.), particle size 5 μm (Supelco, Bellfonte, PA, USA), equipped with a RP-18 pre-column (30 mm×4.6 mm I.D., Brownlee Labs., Santa Clara, CA, USA). Samples were chromatographed with 67% (v/v) aqueous acetonitrile at ambient temperature and at a flow-rate of 1.5 ml/min. Eluted peaks were recorded at 231 nm. Quantitation was performed by comparing the peak areas obtained from the samples with those of standards, treated under the same hydrolytic conditions. Homogenization of the tissue specimens was carried out in a Polytron homogenizer type PT 10/35 (Kinematica).

3. Results and discussion

The two major SAs, Neu5Ac and Neu5Gc, were analyzed as per-*O*-benzoylated derivatives [19]. Molecules in biological samples usually interfering with analysis were efficiently removed [33]. SA-containing macromolecules of serum were precipitated with saturated ammonium sulfate, pH 10, at 0°C. The precipitate was collected by centrifugation, dissolved in water, chromatographed on a Sephadex G-25 column (PD-10) and the void volume fractions were pooled. Analysis of these fractions showed that approximately 98.5% of total SA was recovered. Tissue specimens (20–100 mg of lung, muscle and/ or tumor) were homogenized in the presence of water at 0°C. Following centrifugation, the water-soluble SA-containing glycoconjugates were recovered in the supernatant and analyzed for Neu5Ac and Neu5Gc.

The release of SAs present in the non-reducing terminal of glycoconjugates was performed by mild acidic hydrolysis with 25 m*M* TFA [19]. In order to eliminate false negative results due to destruction of SA even under this mild treatment, standard solutions of Neu5Ac and Neu5Gc were subjected under the same hydrolytic conditions as samples [19,33]. Neutral monosaccharides and hexosamines present in hydrolysates were removed by ion-exchange chromatography on Dowex 50X8 (H^+ form) and Dowex 1X8 ($HCOO^-$ form) [34].

Benzoylation was performed according to the method of Karamanos et al. [19], with benzoic anhydride in pyridine in the presence of *p*-dimethylaminopyridine. Separation of per-*O*-benzoylated derivatives of Neu5Ac and Neu5Gc was successfully carried out on a reversed-phase Supelcosil LC-18 column eluted with 67% (v/v) aqueous acetonitrile and detection at 231 nm.

As shown in Fig. 2A, per-*O*-benzoylated derivatives of Neu5Ac and Neu5Gc with retention times 8.4±0.4 and 16.8±0.8 min, respectively, were completely separated. The sensitivity and linearity of the method were tested by using Neu5Ac and Neu5Gc, hydrolyzed with 25 m*M* TFA at 80°C for 2 h, as

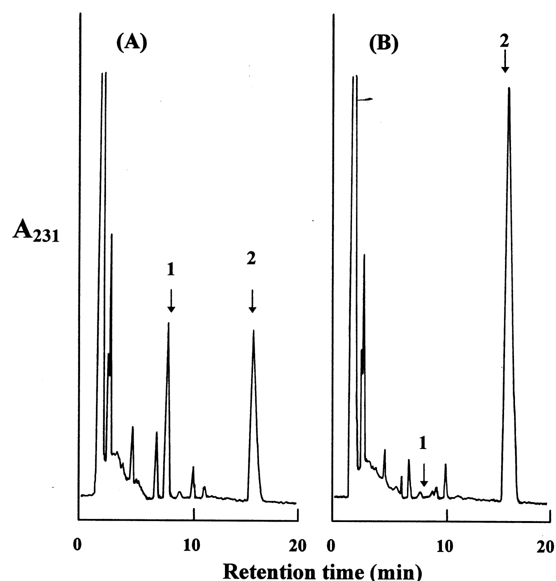

Fig. 2. Typical chromatogram of per-*O*-benzoylated derivatives of Neu5Ac (peak 1) and Neu5Gc (peak 2) (A) and chromatogram obtained by analysis of SAs in mouse serum with Lewis' lung cancer (B). Chromatographies were performed on a reversed-phase Supelcosil LC-18 column eluted with 67% (v/v) aqueous acetonitrile at 1.5 ml/min. Eluted peaks were recorded at 231 nm.

standards for SA analysis. The linearity of UV–Vis detector response was performed by injecting increasing amounts (0.05 to 50 μg) of Neu5Ac and Neu5Gc standards. Each level was analyzed in triplicate. The relationship between peak area and concentration of aqueous Neu5Ac or Neu5Gc standard solution was linear within this range with a correlation coefficient of 0.9995 (n=8) for the former and 0.9992 (n=8) for the latter SA, in excellent agreement with previously published values [19]. The detection limit expressed as twice the baseline noise corresponded to 10 ng for each SA type, when an injection volume of 10 μl was used. The precision of the method was determined by six repeated analyses of each neuraminic acid from healthy mouse serum. When 10 μl of the per-*O*-benzoylated derivatives of SA containing 0.9 μg of Neu5Ac or 7.7 μg of Neu5Gc were injected, the relative standard deviation was 2.4% for the former and 2.9% for the latter. The precision of the method was also determined by analyzing right hind leg muscle of the same healthy mouse. The relative standard deviation for Neu5Ac and Neu5Gc was 3.1% and 4.2%, respectively, when 10 μl of SA derivatives containing 1.2 or 8.3 μg of the respective neuraminic acid were injected.

All six inoculated C57BI mice developed regional tumors, while four of them developed metastatic focies in the lung upon histological examination. Serum and tissue specimens from healthy mice (n=3) and from mice which developed metastasis in the lung (n=4) were collected and analyzed for Neu5Ac and Neu5Gc. Representative chromatograms for Neu5Ac and Neu5Gc analyses of serum and tissues from healthy mice and animals which developed Lewis' lung cancer are shown in Fig. 2B and Figs. 3 and 4. Total SA, Neu5Ac and Neu5Gc values obtained from serum and tissues analyses in triplicate are summarized in Table 1. The determined standard deviation by analyzing each sample in triplicate was less than 5% of the mean value.

All tissues analyzed contained both types of SAs, while Neu5Gc was the major one. The obtained values showed an extensive variability, since a standard deviation of 16 to 26% of the target values was detected. Therefore, the variability recorded is due to biological rather than to analytical reasons. Total SA content in serum from mice with cancer was 850±220 μg/ml, whereas the respective value from healthy animals was 285±62 μg/ml. The increase of total SAs found in serum of animals with cancer may well be a result of enhanced sialylation due to elevated sialyltransferase activity, as it has been proposed for other types of cancer [3,35]. As shown in Table 1, no significant alteration in the

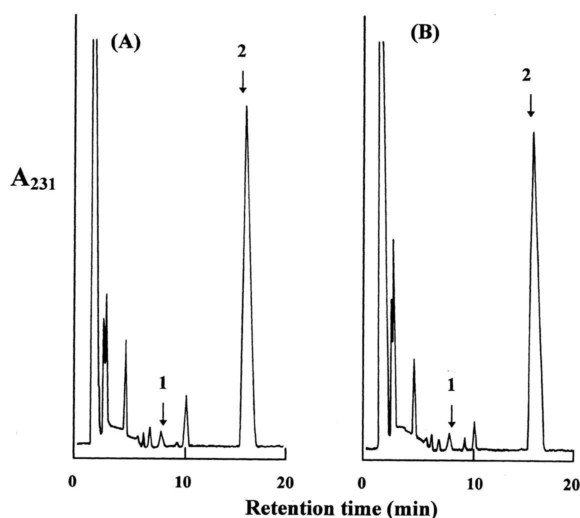

Fig. 3. Chromatographic analysis of SAs in mouse muscle (A) and in tumor developed in mouse with Lewis' lung cancer (B). Column, eluent and conditions as Fig. 2.

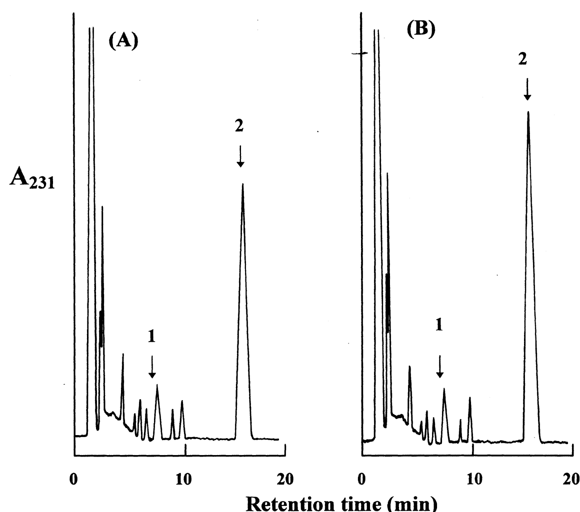

Fig. 4. HPLC profiles obtained by analyzing SAs in lung of control mouse (A) and in lung with Lewis' lung cancer (B). Column, eluent and conditions as in Fig. 2.

Table 1
Sialic acid content and Neu5Ac/Neu5Gc molar ratio in serum, tumor and lung from C57BI mice with Lewis' lung cancer

Subject group	n	Total SA	Neu5Ac (μg/ml of serum or μg/g of tissue)	Neu5Gc	Neu5Ac/Neu5Gc (mol/mol)
Serum	3	285±62[a]	1±0.1	298±65	0.4/99.6[b]
Ca Serum[c]	4	850±220	7±2	885±235	0.8/99.2
Lung	3	1412±234	282±52	1186±257	20/80
Ca Lung[c]	4	2487±518	350±78	2243±488	14.1/85.9
Muscle[d]	3	871±141	13±2	900±168	1.5/98.5
Tumor	4	1917±370	56±11	1954±399	2.9/97.1

[a] Average values (mean±S.D.).
[b] Average values ratio.
[c] Serum or tissue from mice which developed Lewis' lung metastatic cancer.
[d] Muscle from right hind leg from healthy mice.

Neu5Ac/Neu5Gc molar ratio in serum of control animals and mice with cancer was recorded, indicating a similar rate of Neu5Gc and Neu5Ac synthesis in both cases.

SA content in tumors was compared with those of muscle from hind leg of healthy mice. Total SAs in tumor were 1917±370 μg/g, showing a 2.2-fold increase as compared to muscle from control animals (871±141 μg/g). The Neu5Ac/Neu5Gc molar ratios were 2.9/97.1 and 1.5/98.5 for tumor and muscle, respectively, showing a similar content for both neuraminic acids.

Total SA content in lung from mice with cancer was higher than that of control mice (2487±518 μg/g vs. 1412±234 μg/g). In addition, the Neu5Ac/Neu5Gc molar ratio obtained for lungs of normal animals was 20/80 and for mice with Lewis' lung cancer 14.1/85.9, suggesting that the increase of total SAs in lungs corresponds, almost exclusively, to Neu5Gc synthesis. Taking into account that Neu5Gc is synthesized oxidatively from Neu5Ac by Neu5Ac monooxygenase either before or after transfer to glycoconjugates [21,22], it is plausible to suggest an extended Neu5Ac monooxygenase activity resulting in oxidation of the over produced Neu5Ac to Neu5Gc.

In conclusion, our results suggest that cancer metastasis in various tissues is closely associated with increased synthesis of the major SA. Furthermore, highly resolving power techniques may help in understanding the underlying mechanisms of cancer development.

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