Identification of 2-Keto-3-deoxy-D-glycero-D-galactonononic acid (KDN, Deaminoneuraminic Acid) Residues in Mammalian Tissues and Human Lung Carcinoma Cells

CHEMICAL EVIDENCE OF THE OCCURRENCE OF KDN GLYCOCONJUGATES IN MAMMALS

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Since the discovery of KDN glycoprotein in 1986, the occurrence of KDN (= 2-keto-3-deoxy-D-glycero-D-galactonononic acid) glycan chains has been reported for different organisms ranging from bacteria to lower vertebrates, including amphibians and fish. However, the presence of α2–8-linked oligo/polyKDN groups in mammalian tissues was shown by immunohistochemical and immunoblotting methods.

In this communication we report the detection and quantitation of the KDN residues in glycoprotein and glycolipid fractions of rat tissues and human lung cancer cell lines by a highly sensitive fluorometric high-performance liquid chromatography (HPLC) method. We now provide unequivocal chemical proof of the occurrence of KDN in mammals by isolation of KDN from pig submaxillary gland and by structural assignment using chemical methods including fast atom bombardment-mass spectrometry, fluorescence-assisted HPLC analysis, gas-liquid chromatography, and 1H NMR spectroscopy.

KDN was first discovered in rainbow trout egg polysialoglycosprotein as a capping residue of polysialyl chains (1). Subsequently, an increasing number of KDN glycoconjugates isolated from bacteria and lower vertebrates and the structures of their KDN glycan chains have been clarified (2–13). KDN represents a distinct type of sialic acid and not simply a derivative of N-acetylenuraminic acid by having a hydroxyl group instead of an aminoacyl group at position C-5.

We prepared a monoclonal antibody, mAb.kdn8kdn, which specifically recognizes the glycopeptide α2→8-linked oligo/polyKDN (14, 15). Recently, we also found and purified a novel type of sialidase, KDNase Sm, which specifically hydrolyzes KDN-ketosidic but not N-acetylenuraminyl linkages (16, 17). These two new reagents can be used as the important tools in studies aiming at identification, localization, and function of KDN glycoconjugates. By using mAb.kdn8kdn and KDNase Sm, we initiated a systematic search for the presence of α2→8-linked oligo/polyKDN in mammalian tissues (14, 18), and the following results have been obtained: (i) various organs of rat, golden hamster, pig, chicken, and human gave a positive reaction to mAb.kdn8kdn, which was abolished by pretreatment with KDNase Sm; (ii) interestingly, in some organs such as kidney, skeletal muscle, lung, and brain the expression of the α2→8-linked oligo/polyKDN glycoconjugate was developmentally regulated (18, 19); (iii) furthermore, in human lung oligo/polyKDN represented a new oncodevelopmental marker which was re-expressed in various histological types of lung carcinomas and cell lines derived therefrom (19). Despite these immunohistochemical results, the definite chemical proof of the presence of KDN residues is needed before drawing a final conclusion of their occurrence in mammalian cells and tissues.

We report here chemical evidence that KDN residues are indeed a constituent of glycoconjugates in mammalian cells and tissues by (i) application of a highly sensitive fluorometric HPLC method (20) and by (ii) isolation and unequivocal identification of KDN from the mild acid hydrolysates of pig submaxillary gland.

EXPERIMENTAL PROCEDURES

Materials—DMB was a product of Dojindo Laboratories (Kumamoto, Japan) and purchased from Wako (Osaka, Japan). Neu5Ac was purchased from Nakalai (Kyoto, Japan). KDN and Neu5Gc were isolated and purified from KDN glycoprotein (4) and polysialoglycoprotein (21), respectively. All columns for ion-exchange and gel chromatography were prepared from new batches of DEAE-Sephadex A-25 and Sephadex G-10 (Pharmacia Biotech Inc.), Bio-Gel P-2, Bio-Rad AG-1, and Dowex 1 (Dow Chemicals) in order to avoid possible contamination.

Preparation of Samples for Differential Sialic Acid Analysis by HPLC Method—Adult (200–300 g, body weight) and 7-day-old Wistar rats were anesthetized by an intraperitoneal injection of nembutal and the following organs dissected: forebrain, olfactory bulb, cerebellum, kidney, heart, lung, epididymal adipose tissue, thymus, spleen, submaxillary gland, and skeletal muscle. Lungs from adult and 7-day-old rats were also rinsed free of blood by perfusion with oxygenized Hanks balanced salt solution (pH 7.4, 37 °C) through the right heart ventricle. All tissues were immediately frozen with liquid nitrogen and kindly mailed frozen on dry ice from Prof. Jurgen Roth, Division of Cell and Molecular Pathology, Department of Pathology, University of Zurich, Switzerland to Tokyo where they were kept frozen until use. Human lung squamous cell carcinoma lines Calu and Hatz as well as lung adenocarcinoma cell lines A 125 and A 549 (kindly provided by Dr. R. zene; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; TBA, thio-
were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humid atmosphere containing 5% CO₂ and generously supplied to us by Prof. Roth. Pig submaxillary glands were purchased from a local slaughterhouse.

Tissues (0.5–3 g, wet weight) or pellets (about 0.2 ml) of cultured cells were homogenized in a Polytron homogenizer (Kinematica, Litau, Switzerland) with 3–4 volumes of 0.01 M Tris-HCl (pH 8.0). A portion of the homogenate corresponding to 0.15–0.3 g of wet cells or tissue was mixed with methanol and chloroform, keeping a chloroform/methanol/buffer solution ratio of 4:8:3 (v/v) (22), stirred for 30 min at room temperature, and centrifuged at 1,000 × g for 15 min. The precipitate was re-extracted with chloroform/methanol/0.01 M Tris-HCl (4:8:3) and centrifuged. The precipitate was re-extracted with chloroform/methanol/0.01 M Tris-HCl (4:8:3) and centrifuged. The precipitate (designated as glycoprotein fraction) was suspended in 0.5–1 ml of 0.1 M trifluoroacetic acid and heated at 80 °C for 1 h. The combined supernatants (designated as glycolipid fraction) were dried under reduced pressure and eluted successively with 3 volume each of 0.1 and 0.7 M formic acid. Eluates were dried under reduced pressure and derivatized for HPLC analysis. The majority of sialic acids was eluted with 0.7 M formic acid.

Preparation of Mild Acid Hydrolysate of Pig Submaxillary Glands—
Pig submaxillary gland (weighed 120 g, after removal of fat and connective tissue) was homogenized with 200 ml of 0.01 M Tris-HCl (pH 8.0) in a Waring blender. The homogenate was made acidic by the addition of 2 M HCl (final concentration, 0.1 M HCl) and heated at 80 °C for 90 min. The hydrolysate was cooled, neutralized with 2 M NaOH, diluted with 2 volumes of water, and clarified by centrifugation followed by filtration through glass wool. The filtrate were applied to a Dowex 1 × 8 column (4 × 30 cm, formate form). After washing the column with 2 volumes of water it was eluted successively with 0.1, 0.3, and 0.7 M formic acid (23). The eluate with 0.7 M formic acid was TBA-positive, and it was dried under reduced pressure below 37 °C. The pooled fractions from anion-exchange resins contained KDN, Neu5Ac, and Neu5Gc revealed by HPLC analysis. These sialic acids were partially separated from each other by gel chromatography on a Bio-Gel P-2 column (2.4 × 82 cm) equilibrated and eluted with 0.05 M pyridine-acetic acid (pH 5.0) (6): the order of elution was Neu5Ac, Neu5Gc, and KDN. The concentration of sialic acids in the eluate fractions was monitored by the TBA reaction and HPLC analysis. When material from Bio-Gel P-2 still contained Neu5Gc, the latter was removed after methanolysis as methyl ketoside of neuraminic acid by adsorption on Dowex 50 cation exchange resin (1).

Derivatization—DMB solution was prepared as described previously (20) except that we used 0.03–0.04 M trifluoroacetic acid as an acid. A 20–50-µl aliquot of the sample solution was mixed with an equal volume of DMB solution in a TedLok-lined screw-capped tapered glass tube and incubated for 2–2.5 h at 55 °C in the dark. The stoichiometric reaction of nonulosonates with DMB is shown in Reaction 1 (Neu5Ac, r = −NHCOCH₃; Neu5Gc, r = −NHCOCH₂OH; KDN, r = −OH).

The HPLC elution profiles of the DMB derivatives of sialic acids. a, authentic KDN, Neu5Ac, and Neu5Gc (5 ng each); b, the hydrolysate of rat adipose tissue glycoprotein fraction; c, the hydrolysate of human lung carcinoma cell (A549) glycoprotein fraction; d, KDN isolated from pig submaxillary gland. The ordinate expresses fluorescence detector response, and the abscissa represents retention time (minutes). Stoichiometric reaction of nonulosonates with DMB is shown in Reaction 1 (Neu5Ac, r = −NHCOCH₃; Neu5Gc, r = −NHCOCH₂OH; KDN, r = −OH).

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Gas-Liquid Chromatography (GLC)—Purified samples of KDN were subjected to methanolysis in 0.5 M methanolic HCl at 65 °C for 16 h and trimethylsilylation (24). A Shimadzu GC-17A gas chromatograph equipped with a capillary column CBP-5 (30 m × 0.32 mm) was operated

![Fig. 1. HPLC elution profiles of the DMB derivatives of sialic acids.](image)

![Fig. 2. Amounts of KDN, Neu5Gc, and Neu5Ac present in glycolipid fraction (a) and glycoprotein fraction of different tissues of rat (b).](image)
**RESULTS**

Detection of KDN and Determination of Three Different Sialic Acid Residues (KDN, Neu5Ac, and Neu5Gc) in Rat Tissues and Human Lung Carcinoma Cells by HPLC Analysis—We applied, with a minor modification, the fluorometric high-performance liquid chromatography originally reported by Hara et al. (20, 28) for detection of KDN, and quantitation of three different sialic acid residues in the samples originated from mammalian cells and tissues. Fig. 1a (see also Reaction 1) shows the elution profiles of the DMB derivatives of KDN, Neu5Ac, and Neu5Gc. One picomole of KDN in the hydrolysate of crude material was easily detectable, while the detection limit for authentic sialic acids was 100 fmol or less. The peak area was proportional to the amount of each sialic acid over wide range (practically up to 4 nmol), and the results were highly reproducible when the conditions of derivatization were kept constant. Precise identification of each peak was made by the retention time relative to that of Neu5Ac set equal to 1.00 (KDN, 0.74; Neu5Gc, 0.80). The retention time of 2-keto-3-deoxy-o-manno-octonic acid (KDO) was 0.79. Typical chromatograms for rat adipose tissue and human lung carcinoma cells are reproduced in Fig. 1, b and c, respectively.

Quantitative Determination of KDN, Neu5Ac, and Neu5Gc in Rat Tissues and Human Lung Carcinoma Cells—The amounts of KDN, Neu5Gc, and Neu5Ac in glycolipid (extractable with chloroform/methanol/aqueous) and glycoprotein (non-extractable with the above solvent) fractions of various adult and P7 (7 days postnatal) rat tissues determined by the present HPLC method are shown in Table I. Expression of KDN was found in all tissues examined.

Isolation of KDN from the Mild Acid Hydrolysate of Pig Submaxillary Gland—Submaxillary gland was one of the tissues that contained relatively high proportion of KDN as determined by the HPLC method. We isolated KDN from the mild acid hydrolysate of pig submaxillary gland. Three different kinds of sialic acid present in the pooled fraction obtained by anion-exchange chromatography were eluted separately on Bio-Gel P-2 column as shown in Fig. 3a. The elution profile was monitored by the HPLC analysis. Two KDN-containing peaks were detected and the second peak coincided with that of authentic KDN (200 ng) (b). Galactitol (50 ng) was an internal standard added to both samples. The abscissa and ordinate express retention time (minutes) and detector response, respectively.

KDN Residues in Rat, Pig, and Human

**TABLE I**

| Carcinoma cells | Glycolipid | Glycoprotein |
|-----------------|-----------|-------------|
|                 | KDN | Neu5Gc | Neu5Ac | KDN | Neu5Gc | Neu5Ac |
| A125            | 0.35 | 2.4    | (51.0) | 0.27 | 2.2    | (52.0) |
|                 | (0.65)| (4.5)  | (94.9) | (0.50)| (4.0)  | (95.5) |
| A549            | 0.08 | 4.0    | (24.0) | 0.25 | 1.8    | (51.0) |
|                 | (0.52)| (4.0)  | (95.7) | (0.47)| (3.4)  | (96.1) |
| Calu            | 0.10 | 1.1    | (29.0) | 0.34 | 4.3    | (77.0) |
|                 | (0.33)| (3.6)  | (96.0) | (0.42)| (5.3)  | (94.3) |
| Hotz            | 0.22 | 1.0    | (50.0) | 0.38 | 2.0    | (120.0) |
|                 | (0.43)| (2.0)  | (97.6) | (0.31)| (1.0)  | (98.0) |

**FIG. 3.** Column chromatographic separation of sialic acids on Bio-Gel P-2. The elution of each sialic acid was monitored by the HPLC analysis after derivatization with the DMB reagent. a, the sialic acid fraction obtained by anion-exchange chromatography of the mild acid hydrolysate of pig submaxillary gland; b, rechromatography of the KDN-rich fraction. Column sizes: 2.4 × 82 cm for a and 1.5 × 70 cm for b. Fractions of 2 ml were collected for a, whereas 1-ml fractions were collected for b.

with a temperature gradient at 6 °C/min from 180 to 280 °C. Galactitol (1.25 μg) was added to the sample solution as an internal standard.

**1H NMR Spectroscopy**—One-dimensional 500-MHz 1H NMR spectra were recorded with a JNM-LA500 spectrometer (Jeol).

**Fast Atom Bombardment Mass Spectrometry (FAB-MS)**—Fast atom bombardment mass spectra of a methyl glycoside of KDN were measured using a JMS-SX102A mass spectrometer (Jeol) equipped with an MS-IP 7000 data system operated at 10 kV in negative ion mode.

Other Analyses—The presence of sialic acids in the crude sample solutions or in the column eluates was monitored by the TBA method (25, 26).

![Image](image-url)
**KDN Residues in Rat, Pig, and Human**

**Table II**

| Chemical shift & Coupling constant**a** | KDN Residues in Rat, Pig, and Human |
|---------------------------------------|------------------------------------|
|                                       | Hlax, Hseq, H4, H5, H6, H7, H8, H9, H9'| |
| Authentic**b**                        | 1.82, 2.24, 4.00, 3.59, 3.97, 3.86, 3.76, 3.87, 3.67 |
| Isolated                              | 1.83, 2.22, ~4.0, 3.63, 3.97, 3.88, 3.81, 3.91, 3.70 |

**a** Chemical shifts are given in ppm downfield from the methyl proton signal of sodium 3-trimethylsilyl)propionate-2,2,3,3-d₄, for solutions in D₂O at 28°C, and at pH ~7. Coupling constants are given in Hz, and n.d. indicates value could not be determined.

**b** All measurements for authentic KDN were made on a Bruker AM-400 spectrometer at 400 MHz at 25°C. Assignments were based on two-dimensional DQF-COSY spectrum (27).

**DISCUSSION**

The question as to whether or not KDN is present in mammals remained unanswered until now, although the occurrence of α-2→8-linked oligo/poly KDN glycotopes in mammalian tissues was indicated by immunohistochemistry and immunoblotting method (14, 18). In the previous immunostaining experiments we used mAb, kdn 8kdn that recognizes α-2→8-linked (KDN)ₙ (n ≥ 2), but not α-2→8-linked (Neu5Ac)ₙ or α-2→6-linked (Neu5Gc) (14, 15) and a new bacterial sidialidase (KDNasesφ) that is highly specific to KDN ketosidic linkages (16, 17) to show that the immunostaining was abolished by pretreatment of the sample with this enzyme (18). Although our data based upon these specific reagents strongly suggested the occurrence of KDN residues in mammalian tissues, it was important to obtain the chemical evidence before drawing the final conclusion because KDN is a new building block of glycan chains. Demonstration of the occurrence in a variety of rat organs and some human lung cancer cells of very minute amounts of KDN other than Neu5Ac and Neu5Gc has been made possible by the introduction of the fluorescein-assisted HPLC method for identification and quantitation.

Because KDN is thus far an unusual component, not previously encountered as a component of any glycoconjugates from mammals and because, in the above HPLC method, the identification is merely based on the elution position, KDN was identified as a minor but definite component of mammalian glycoproteins and glycolipids. The present identification of KDN residues in mammalian systems raises several interesting future studies, including (a) structural elucidation of KDN-containing glycan chains, (b) their biochemistry, and (c) biological function. It may be noted that we were successful in identification of CMP-KDN synthetase and KDN transferases in rainbow trout (29, 30), suggesting that this sugar nucleotide and the KDN-specific transferase(s) are also present in mammals.

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