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Identification and partial characterization of soluble and membrane-bound KDN(deaminoneuraminic acid)-glycoproteins in human ovarian teratocarcinoma PA-1, and enhanced expression of free and bound KDN in cells cultured in mannose-rich media

Sadako Inoue · Geetha L. Poongodi · Nimmagadda Suresh · Tschining Chang · Yasuo Inoue

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Abstract KDN (Deaminoneuraminic acid, or deaminated neuraminic acid) is a minor but biosynthetically independent member of the sialic acid. Human occurrence of KDN has already been established, although its level is so little that it is often undetectable by conventional sialic acid analysis. Elevated expression of KDN in fetal cord blood cells and some malignant tumor cells have been reported. However, in mammalian cells and tissues KDN mostly occurs as the free sugar and little occurred conjugated to glycolipids and/or glycoproteins. A positive correlation between the ratio of free KDN/free Neu5Ac in ovarian adenocarcinomas and the stage of malignancy has been noted for diagnostic use. We hypothesized that elevated expression of KDN in mammalian systems may be closely related to elevated activities of enzymes involved in the formation of sialoglycoconjugates and/or aberrant supply of the precursor sugar, mannose, used in the biosynthesis of KDN. In this study we used human ovarian teratocarcinoma cells PA-1 to further analyze KDN expression in human cells. Major findings reported in this paper are, (i) a 30 kDa KDN-glycoprotein immunostainable with monoclonal antibody, mAb.kdn3G, (specific for the KDNα2 $\rightarrow$ 3Galβ1$\rightarrow$ epitope) and sensitive to KDNase was identified in the membrane fraction of the cell: (ii) a 49 kDa KDN-glycoprotein that is not reactive with mAb.kdn3G but is sensitive to KDNase was identified in the soluble fraction: and (iii) PA-1 cells showed unique response to mannose added to the growth medium in that the levels of both free and bound forms of KDN are elevated. This is the first report on the identification of mammalian KDN-glycoproteins by chemical and biochemical methods.

Keywords KDN · Human KDN-glycoprotein · Human ovarian teratocarcinoma PA-1 · Deaminoneuraminic acid · Mannose

Abbreviations

DMB	1,2-diamino-4,5-methylenedioxybenzene
HPLC	high performance liquid chromatography
CNBr	cyanogens bromide
KDN	2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid
PEP	phosphoenolpyruvate
PVDF	polyvinylidene difluoride

Introduction

Deaminoneuraminic acid (Deaminated neuraminic acid, KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid, or 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid) is a minor but biosynthetically independent member of the sialic acid that shares many features in common with *N*-acetylneuraminic acid (Neu5Ac). A distinct feature of KDN is in that while Neu5Ac-containing glycoconjugates are universally found in cells and tissues of mammals including human, KDN-containing glycoconjugates are only abundant in limited types of cells and mucus secretions of lower vertebrates [1,2].

Occurrence of KDN in mammals is so minute in amount that it is often undetectable by conventional sialic acid analysis. Immunohistochemical and immunoblotting studies showed the presence of multiple glycoproteins stainable by a monoclonal antibody, mAb.kdn8kdn that recognizes α2 $\rightarrow$8-linked oligoKDN structures in mammalian tissues

S. Inoue · G. L. Poongodi · N. Suresh · T. Chang · Y. Inoue
Institute of Biological Chemistry, Academia Sinica, Taipei 115-29, Taiwan

S. Inoue (✉)
Bioscience and Biotechnology Center, Nagoya University, Nagoya 464-8601, Japan
e-mail: sadako@agr.nagoya-u.ac.jp
Tel: 052-789-4297
Fax: 052-789-5228

[3–5]. Furthermore, some selective glycoproteins reactive with this antibody have been identified and their developmentally regulated expression has been shown [6–9]. Application of highly sensitive chemical methods gave firm evidence to the occurrence of KDN as a minor but ubiquitous sialic acid component in mammals including human [10]. Major important findings revealed by subsequent studies from our research group are (i) in mammalian cells and tissues KDN mostly occurs as the free sugar and little occurs conjugated or as cytidine 5′-KDN phosphate, (ii) elevated expression of KDN is evident in fetal cord red blood cells compared with adult red blood cells, and in ovarian tumor cells and tissues compared with the control normals, and (iii) a positive correlation was found between the ratio of free KDN/free Neu5Ac in ovarian adenocarcinomas and the stage of malignancy [2,11]. These studies suggest the presence of minute amount of KDN conjugated to glycan chains in mammalian cells during certain developmental stages. If KDN-containing glycoconjugates were identified in tumor cells they can be used as sensitive probes for diagnostic purpose, and they will also become key molecules in the basic studies of cancer biology. However, there has been no report on the identification and characterization of KDN-containing glycoconjugates based on chemical and biochemical techniques in mammalian systems.

Recent biochemical and molecular biological studies on biosynthesis of KDN and KDN-containing glycans established that KDN is synthesized *de novo* from mannose (Man) via the following three sequential reactions [12,13]:

$$\text{Man} + \text{ATP} \rightarrow \text{Man-6-P} + \text{ADP} \quad (1)$$

$$\text{Man-6-P} + \text{PEP} \rightarrow \text{KDN-9-P} + \text{Pi} \quad (2)$$

$$\text{KDN-9-P} \rightarrow \text{KDN} + \text{Pi} \quad (3)$$

Enzymes involved in the biosynthesis of KDN-containing glycans are CMP-KDN synthase and KDN-transferases. CMP-KDN synthase that effectively activates KDN has been identified in rainbow trout testis [14,15]. KDN-transferase that is involved in the formation of $\alpha 2 \rightarrow 8$ KDN-linkage was also suggested in the ovary of rainbow trout [16]. In mammals no enzyme that specifically recognizes KDN is known. However, it has been shown, both *in vivo* and *in vitro*, that KDN and KDN-containing glycans can be synthesized via reactions catalyzed by the enzymes involved in the biosynthesis of Neu5Ac and Neu5Ac-containing glycans in mammals, although the formation of KDN and CMP-KDN is far less efficient than that of Neu5Ac and CMP-Neu5Ac [13–15,17]. *In vitro* experiment using a commercial $\alpha 2$, 6-sialyltansferase showed transfer of KDN from CMP-KDN to other sugar residues appeared to proceed efficiently [18].

Now we hypothesized that elevated expression of KDN in mammalian systems may be closely related to elevated activities of enzymes involved in the formation of Neu5Ac-containing glycoconjugates and/or aberrant supply of the precursor sugar, mannose, used in the biosynthesis of KDN. In this study we used human ovarian teratocarcinoma cells, PA-1, which showed relatively high expression of KDN both in the free and bound forms to further analyze expression mode of KDN in human cells. Major findings reported in this paper are, (i) a 30 kDa KDN-glycoprotein immunostainable with monoclonal antibody, mAb.kdn3G, (specific for the KDN$\alpha 2 \rightarrow$3Gal$\beta 1 \rightarrow$ epitope) and sensitive to KDNase was identified in the membrane fraction of the cell: (ii) a 49 kDa KDN-glycoprotein that is not reactive with mAb.kdn3G but is sensitive to KDNase was identified in the soluble fraction: and (iii) PA-1 cells showed unique response to mannose added to the growth medium in that the levels of both free and bound forms of KDN are elevated. This is the first report on the identification of mammalian KDN-glycoproteins by chemical and biochemical methods.

Materials and methods

Cell culture

A cell line derived from a teratocarcinoma (PA-1) was obtained from American Type Culture Collection (ATCC) and was purchased from Food Industry Research and Development Institute, Hsinchu, Taiwan. PA-1 cells were cultured in minimal essential medium (MEM) with 0.1% non essential amino acids supplemented with 10% bovine fetal serum (heat inactivated). Cultures were maintained in a humidified CO_2-incubator (5% CO_2) at 37°C. Cells grew as monolayer and exhibited epithelial morphology. Cells were inoculated at the concentration of 5×10^4/ml and were collected at 5 stages (24, 36, 48, 60 and 72 h) after starting inoculation and incubation. The cells were photographed at each stage to examine changes in their morphological features.

Preparation of antibodies and immunoaffinity gel

The production and purification of monoclonal antibodies recognizing KDN$\alpha 2 \rightarrow$3Gal$\beta 1 \rightarrow$ epitope, mAb.kdn.3G, and ($\rightarrow$KDN$\alpha 2 \rightarrow$8)n$\rightarrow$ epitope, mAb.kdn8kdn were as reported [19,3]. To make immunoaffinity gel used for purification of KDN-containing glycoprotein, mAb.kdn3G was coupled to pre-swollen CNBr-activated Sepharose 4B gel (Pharmacia) at about 10 mg of antibody per ml of gel according to manufacturer's instructions.

Fractionation of cell extracts and sialic acid analysis in each fraction

All the procedures were carried out at 4°C unless otherwise stated. Cells were harvested by trypsinization after

washing with PBS (phosphate-buffered saline) and the wet weight of the cell pellets was determined after centrifugation. About a 50–100 mg portion of cell pellets was used for each analysis. Cells were homogenized with 0.5–1 ml of TBS (0.01 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl) using a polytron homogenizer (Kinematica, Littau, Switzerland). The homogenate was centrifuged at 60,000 rpm (100,000 × *g*) for 1 h. The pellet obtained was designated as the membrane fraction (analyzed for membrane-bound sialic acids after hydrolysis). The supernatant was mixed with one volume of ethanol, kept at −20°C, overnight and centrifuged at 3,000 rpm for 15 min. The ethanol soluble supernatant was evaporated and used for analysis of free KDN. The precipitate was designated as soluble protein fraction and used for analysis of soluble glycoprotein-bound sialic acids.

The amount of each sialic acid was quantified following derivatization with DMB (Dojimbo, Kumamoto, Japan) by HPLC with a fluorescence detector [10,11,20,21]. For the analysis of bound sialic acids, samples were hydrolyzed with 0.1 M TFA for 1 h at 80°C. In analyzing crude samples, the hydrolysates were applied to a Bio-Rad AG-1 column and sialic acids were eluted from the column with 0.7 M formic acid after washing the column with water. The acid was removed by evaporation and sialic acids in the residue were derivatized with DMB and resolved on a TSK-GEL ODS-120T (Tosoh, Tokyo, Japan) column.

The amount of KDNα2 →3Galβ1 → epitopes was estimated by ELISA. A 50 μl portion of samples from various stages of growth was separately added in a well of 96-well (maxisorp) plates and dried at 37°C overnight. Non-specific binding of proteins was blocked by incubating each well with 1% gelatin/PBS at 37°C overnight. A 50 μl portion of the mAb.kdn3G solution (2 μg/ml) were added to each well and incubated for 1 h at room temperature. After washing 3 times with 0.1% gelatin/PBS containing 0.05% Tween 20, 50 μl of peroxidase-conjugated affinity-purified goat anti-mouse (IgG) antibody dissolved in 1% bovine serum albumin /PBS (0.3 μg/ml) was added to each well and incubated at 37°C for 2 h. The enzyme activity remaining in each well, after washing the well 5 times with 0.1% gelatin/PBS containing 0.05% Tween 20, was visualized by incubation with *o*-phenylenediamine (0.5 mg/ml) for 30 min. Reaction was stopped by adding 100 μl of 2 N H_2SO_4 and absorbance at 492 nm was measured on a microplate reader.

Isolation and purification of KDN-containing glycoproteins

Membrane-bound KDN-glycoprotein—The membrane fraction obtained from 1 g of wet cells was extracted with 200 μl of PBS containing 1% Triton X-100 containing the following protease inhibitors; 0.3 U/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride and 40 mM leupeptin (all obtained from Sigma). After incubation for 10 min on ice the extract was centrifuged at 1000 × *g* at 4°C for 5 min. The pellet was re-extracted as above and centrifuged. The combined extracts were first applied to a Sephadex G-50 column and the void volume fraction containing KDN-glycoprotein was further purified by anion-exchange chromatography on a DEAE-Toyopearl 650 M (Tosoh, Tokyo, Japan) column. The fraction eluted with 0.1 M NaCl contained KDN-glycoprotein, which was then subjected to immunoaffinity column chromatography.

Protein sample was loaded on the immunoaffinity gel and mixed end to end for overnight at 4°C. The column was washed with 30 column volumes of PBS containing 1% Triton X100, 10 column volumes of PBS containing 300 mM NaCl, and finally with 10 column volumes of PBS. Bound fraction was eluted with buffer containing 100 mM diethylamine, pH 11.5, 150 mM NaCl, 2 mM EDTA, and 0.1% sodium deoxycholate [22]. The eluted solution was quickly neutralized by adding 500 mM Tris-HCl, pH 6.8, lyophilized, dialyzed, and used for further analysis.

Soluble KDN-glycoprotein—Material precipitated with ethanol from the soluble fraction of the cell extract was dissolved with 10 mM Tris buffer, pH 8 and subjected to anion–exchange chromatography on DEAE-Toyopearl 650 M. Sample eluted with 0.1 M NaCl contained KDN and this fraction was analyzed by SDS-PAGE after desalting.

SDS-PAGE and western blotting

The SDS-PAGE was performed routinely by using a NuPAGE electrophoresis system (Novex, San Diego, CA). All experimental procedures followed the protocol supplied by the manufacturer. In general, the gradient polyacrylamide gel (4–12%) was used, and MOPS SDS was used as the running buffer (final; 50 mM MOPS, 50 mM Tris-HCl, 3.5 mM SDS, and 1 mM EDTA, pH 7.7). Samples were mixed with an appropriate amount of the sample buffer (4X) to a final concentration of 0.29 M sucrose, 0.25 M Tris-HCl, 69 mM SDS, 0.5 mM EDTA, 0.22 mM Serva Blue G250, and 0.17 mM phenol red, and then heated at 70°C for 10 min. Electrophoresis was performed under a constant voltage of 200 V for 50 min. The protein samples resolved by polyacrylamide gels were transferred to PVDF (polyvinylidene difluoride) membrane using a semidry blotting apparatus. For immunoblotting, the membrane was blocked with TBS containing 1% skim milk and 0.1% Tween-20 for 1 h at room temperature and incubated with mAb.kdn3G (1.5 μg/ml) for 18 h at 4°C followed by alkaline phosphatase-conjugated, affinity-purified goat anti-mouse IgG + IgM antibodies (0.12 μg/ml). Color was developed using nitroblue tetrazolium chrolide/5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (Invitrogen).

Sialic acid analysis in samples on the PVDF membrane, and in KDNase- or exosialidase-treated samples

Samples were subjected to SDS-PAGE, transferred to the PVDF membrane and stained with Coomasie brilliant blue. The membrane pieces corresponding to the stained bands were excised and subjected to DMB/HPLC analysis after hydrolysis in 0.1 M TFA for 1 h at 80°C. The sample treated with KDNase or exosialidase was applied to a small column (1 × 7 cm) of Sephadex G10 to remove liberated sialic acid, and the void fraction was subjected to analysis after concentration.

Enzymatic treatments

A sample containing about 25 μg of protein was digested at 25°C for 18–24 h with 9 U of KDNase Sm generously provided by Seikagaku Kokyo, Co., in 20 μl of 100 mM Tris-acetate buffer (pH 6.0). The same amount of the sample was denatured for digestion with recombinant peptide *N*-glycanase (PNGase F, Takara) by heating at 100°C for 2 min in phosphate buffer (pH 7.5–8.0), containing 1 mM EDTA, 0.5% SDS, 5% β-mercaptoethanol). After cooling, 2 mU of the enzyme were added and the mixture was incubated for 18 h at 37°C. For exosialidase treatment, sample containing about 10 μg of membrane-bound glycoprotein was digested for 1 h at 37°C with 20 mU of *A .ureafaciens* exosialidase in 20 μl of 10 mM sodium acetate buffer (pH 5.5). As the control experiment, samples were treated under identical conditions without the enzyme.

Results

Increase of free and bound forms of KDN and Neu5Ac in PA-1 cells during cell culture

The intracellular levels of KDN and Neu5Ac both in the free and bound forms increased during cell culture until 72 h after inoculation when the cells reached full confluence, although the levels of KDN were 2–3 orders of magnitude lower than those of Neu5Ac. Marked increase with time was noted in the levels of free KDN and KDN conjugated to soluble glycoprotein, whereas the level of KDN conjugated to membrane-bound glycoprotein was low and showed only slow increment with culture time (Fig. 1A). This is in sharp contrast to the level of Neu5Ac that increased most prominently in the membrane-bound fraction (Fig. 1B). In this study we harvested cells by trypsinization, a procedure argued by some researchers as damaging to the cell surface proteins. Although, no significant problem was noted by the procedure so far as the distribution of KDN and Neu5Ac was concerned, precise localization of "soluble KDN-containing glycoprotein" described in this paper has not been defined. The presence

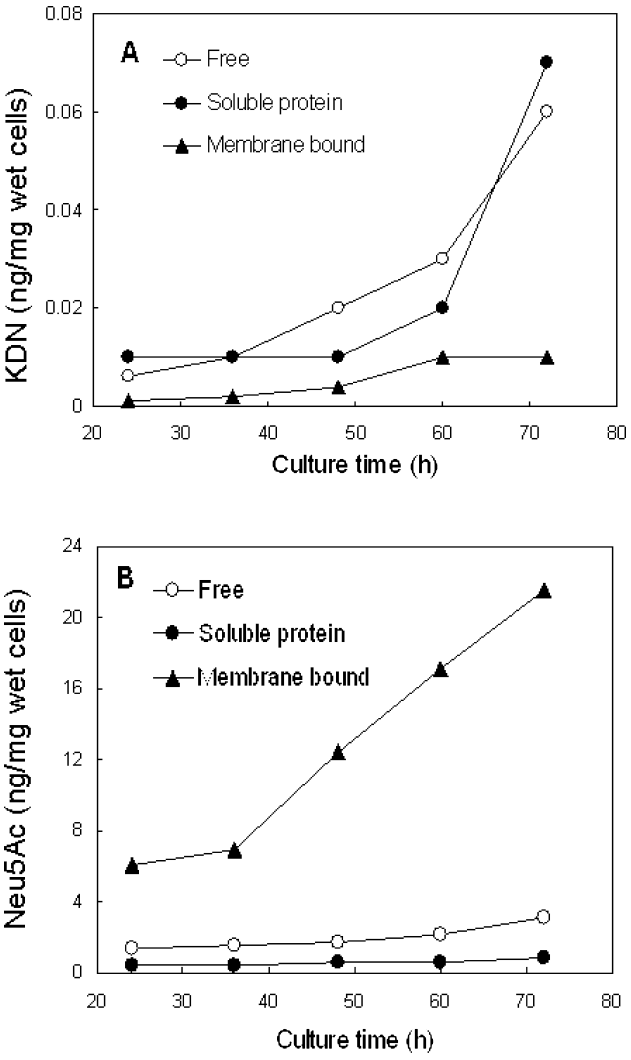

Fig. 1 Growth dependent changes in the expression levels of (A), KDN and (B), Neu5Ac in PA-1 cells. At each stage, 100 mg of wet cells were fractionated and used for sialic acid analysis as described in materials and methods. Expression levels are compared by ng of sialic acid per mg wet cells. (○) Free form; (●) soluble protein-bound; (▲) membrane-bound

of KDN in the membrane-bound fraction and the increment of its level with culture time was also shown by the results of ELISA, and specific immunofluorescence staining of the fixed cells grown at confluence using monoclonal antibody mAb.kdn3G (specific for the KDN α2 →3Galβ1 → epitope) (Data not shown).

Evidence for the presence and cell growth-dependent increase of membrane-bound KDN-glycoprotein by immunoblotting analysis

Membrane fractions (10 μg protein) extracted at various incubation times were subjected to Western-blot analysis using mAb.kdn3G. A single band with a size about 30 kDa appeared and the intensity increased with culture time (Fig. 2).

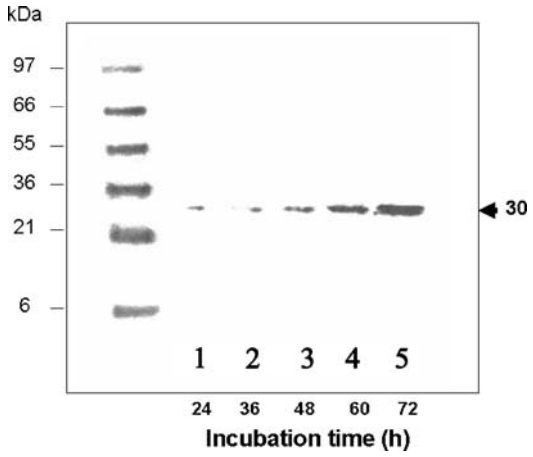

Fig. 2 Western blot analysis of the expression of KDN-glycoprotein in PA-1 cells during cell growth. At each stage (lane 1–5), the membrane fraction of the cell (10 μg of protein) was loaded on a lane and subjected to SDS-PAGE, blotted on PVDF membrane, and immunostained with mAb.kdn3G that recognizes KDNα2→3 Galβ1→ epitope

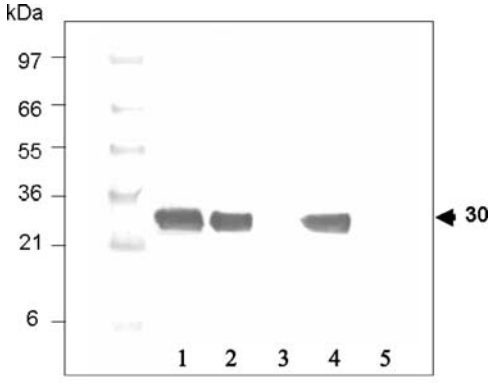

Fig. 3 Western blot analysis of the KDN-glycoprotein present in the membrane fraction of PA-1 cells after treatment with KDNase and PNGase F. (Lane 1) original sample; (lane 3) KDNase-treated sample; (lane 5) PNGase F-treated sample; (lane 2 and 4) control samples for KDNase and PNGase F treatment, respectively

Evidence showing the presence of KDN residues on the 30 kDa glycoprotein and that they are linked to *N*-linked glycan chain(s)

The membrane extract was subjected to digestion with either KDNase, *N*-glycanase, or exosialidase before Western-blot analysis. As clearly shown in (Fig. 3), the 30 kDa band was abolished by KDNase treatment (lane-3), giving evidence for the presence of KDN residues linked to the glycoprotein. The band also became undetectable by PNGase F treatment (lane 5), suggesting the KDNα2→3-epitope was on *N*-glycan(s). In control samples similarly treated without the enzymes, KDNase (lane 2) and PNGase F (lane 4), the 30 kDa bands appeared. Figure 4 shows HPLC profile for the samples before enzyme digestion (Fig. 4B) and after digestion with KDNase (Fig. 4C), and exosialidase (Fig. 4D).

Isolation and purification of the KDN-glycoprotein

When the membrane extract was subjected to gel filtration chromatography on Sephadex G50, the 30 kDa KDN-glycoprotein was eluted in the void volume fraction. This fraction was applied to a column of DEAE-Toyopearl 650 M. The sample eluted with 0.1 M NaCl was found to contain the KDN-glycoprotein by Western-blot analysis. The sample was then purified by immunoaffinity chromatography. The sample eluted from the immunoaffinity column was subjected to SDS-PAGE (Fig. 5, lane 1 and 2). Silver staining of the gel (Fig. 5, lane 2) revealed the presence of at least 3 protein bands in addition to the most intensely stained 30 kDa band.

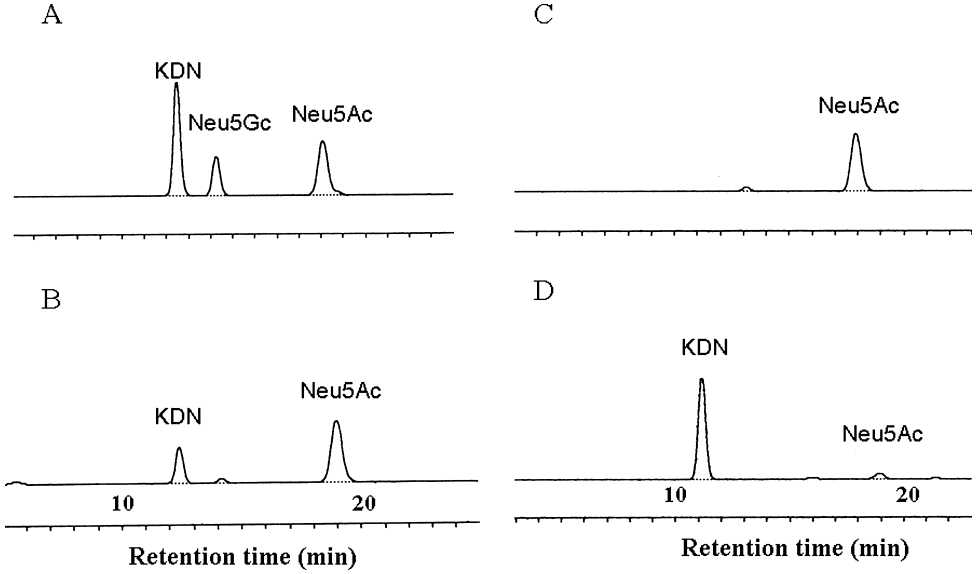

Fig. 4 Fluorometric HPLC sialic acid analysis of a partially purified sample derived from the membrane fraction of PA-1 cells. The membrane fraction was subjected to Sephadex G-50 and DEAE-Toyopearl 650 M chromatography, and the fraction containing KDN-glycoprotein was subjected to fluorometric HPLC analysis for sialic acid before and after treatment of enzymes. (A) standard sialic acids; (B) the sample before enzyme treatment; (C) the sample after KDNase treatment; (D) the sample after exosialidase treatment

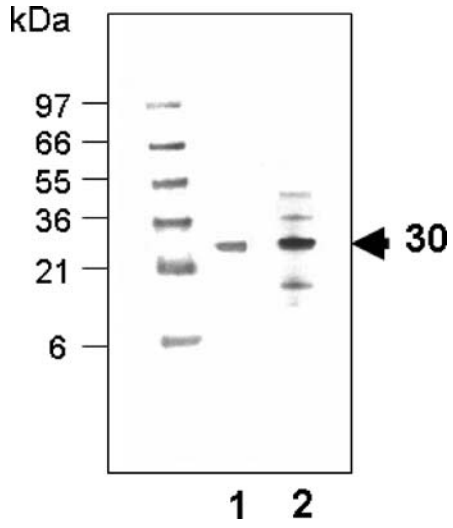

Fig. 5 SDS-PAGE analysis of the sample after immunoaffinity chromatography. (Lane 1) immunostaining with mAb.kdn3G; (lane 2) silver staining

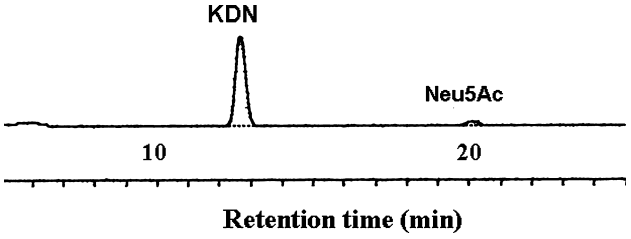

Fig. 6 Fluorometric HPLC sialic acid analysis of the sample dissected from the PVDF membrane. Immunopurified sample was subjected to SDS-PAGE and the Coommasie-stained band at 30 kDa was excised after transferred on to a PVDF membrane sheet, hydrolyzed and analyzed for sialic acids by fluorometric HPLC

Flurometric HPLC analysis of KDN in the 30 kDa band after transferred to the PVDF membrane

The immunoaffinity purified sample was subjected to SDS-PAGE and then transferred on to the PVDF membrane. The major band visualized by staining with Coomassie blue was excised, hydrolyzed and then derivatized with DMB for fluorometric HPLC analysis of sialic acids. A prominent peak showing the presence of KDN was seen together with a small peak of Neu5Ac (Fig. 6). The result showed that the purified material contained a KDN-glycoprotein having KDN as the major sialic acid component.

Identification of KDN in the soluble glycoprotein fraction of PA-1 cells

As shown in (Fig. 1) the soluble fraction of PA-1 contained larger amount of protein-bound KDN than the membrane-bound fraction. The sample (prepared by dissolving the ethanol precipitate of the soluble fraction) containing 25 μg of protein was subjected to fluorometric HPLC analysis for sialic acids after mild acid hydrolysis. The result showed the presence of KDN in amount about 20% of Neu5Ac (Data not shown).

Partial purification of KDN-containing glycoprotein in the soluble fraction and its characterization

Soluble protein fraction derived from PA-1 cells was applied to a DEAE-Toyopearl 650 M column and the column was eluted successively with 0.01 M Tris-HCl (pH 8), and 0.01 M Tris-HCl (pH 8) containing increasing concentrations of NaCl. When analyzed by fluorometric HPLC, KDN was found only in the fraction eluted with the buffer containing 0.1 M NaCl. This fraction also contained Neu5Ac and the ratio of KDN to Neu5Ac was about 1 : 2 (Fig. 7B). To obtain evidence showing that KDN was linked to glycan chain(s), the sample was treated with either KDNase or exosialidase before HPLC analysis of sialic acids. The peak of KDN disappeared and Neu5Ac was greatly reduced after KDNase- and exosialidase treatment, respectively (Figs. 7C and D). Western-blot analysis showed the absence of band stainable with mAb.kdn.3G or mAb.kdn8kdn, indicating the absence of KDNα2$\rightarrow$3Galβ1$\rightarrow$ and ($\rightarrow$8KDNα2$\rightarrow$)n$\rightarrow$ epitopes in the soluble fraction. SDS-PAGE revealed the presence of at least 7 bands stainable with Coomassie brilliant blue (Fig. 8, lane 2). Only the major band with molecular mass about 49 kDa contained KDN together with smaller amount of Neu5Ac (20 moler% of KDN) as revealed by HPLC analysis (Fig. 9B). When the same sample was treated with KDNase the peak of KDN was diminished (Fig. 9C), and when it was treated with exosialidase relative proportion of Neu5Ac decreased (Fig. 9D).

To see the effect of PNGase F on soluble KDN-rich glycoprotein, partially purified sample was digested with PNGase F and then analyzed by SDS-PAGE. The major band with molecular mass of about 38 kDa appeared at the expense of the 49 kDa band, suggesting the presence of multiple numbers of *N*-glycans in the 49 kDa glycoprotein (Fig. 8, lane 3). KDN residues are most likely bound to most of the *N*-glycan chains by α2 $\rightarrow$6-linkages but the definite conclusion was yet to be obtained.

Elevated expression of free and bound sialic acids in PA-1 cells cultured with mannose supplement

Elevated expression of free KDN in B16 mouse melanoma and COS-7 African green monkey kidney cells cultured in mannose-rich media was reported [23]. We examined if similar effects of mannose were observed on the levels of KDN in human ovarian teratocarcinoma cell PA-1 (Fig. 10A–C) and compared them with the effects in human melanoma cell A375 (Fig. 10D–F). Sharp increment was observed for PA-1 cells in the intracellular levels of both free

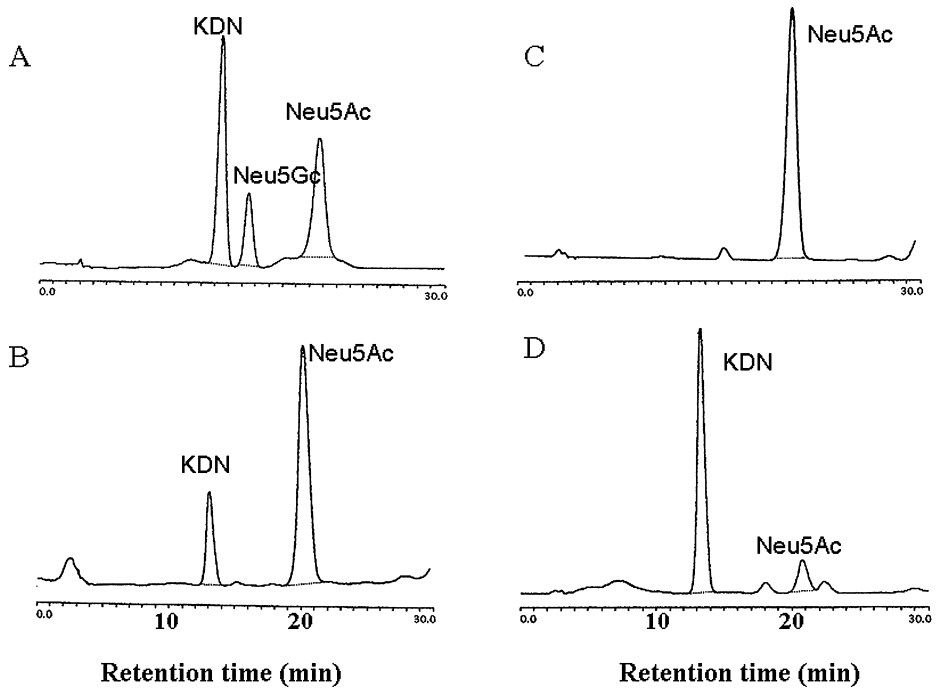

Fig. 7 Fluorometric HPLC sialic acid analysis of the partially purified sample derived from the soluble fraction of PA-1 cells before and after enzyme treatment. The fraction eluted with the buffer containing 0.1 M NaCl from a DEAE-Toyopearl 650 M column was analyzed before and after enzyme treatment. (A) standard sialic acids; (B) the sample before enzyme treatment; (C) the sample after KDNase treatment; (D) the sample after exosialidase treatment

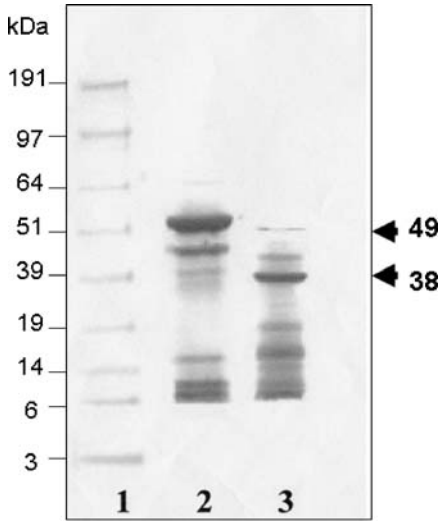

Fig. 8 SDS-PAGE analysis of partially purified KDN-containing soluble protein fraction derived from PA-1 cells. Soluble protein fraction was subjected to DEAE-Toyopearl 650 M chromatography and the fraction eluted with the buffer containing 0.1 M NaCl was analyzed before and after PNGaseF treatment. (Lane 1) molecular marker; (lane 2) the sample before PNGase treatment; (lane 3) the sample after PNGase treatment

(Fig. 10A) and bound forms of KDN and Neu5Ac (Fig. 10B), for soluble glycoprotein-bound forms, and (Fig. 10C) for membrane-bound forms) with increasing concentrations of external mannose. Similar effects were also observed for the melanoma cells but the increment of the KDN levels was smaller than that of PA-1 cells (Fig. 10D–F). Sharp increase of intracellular free KDN by increasing concentration of mannose added to the medium during cell culture was reported for B16 and COS-7 cells [23]. However, increase of the bound KDN by external mannose in these cells was much smaller than that observed for PA-1. The results indicate that biosynthesis of KDN and KDN-containing glycoconjugates occur more efficiently in PA-1 cells than in other cells examined. The results also show that KDN-containing soluble glycoprotein is synthesized in PA-1 more efficiently than KDN-containing membrane-bound glycoprotein in response to mannose supplement. That mannose added to the medium was used through the reactions for biosynthesis of KDN described in introduction was supported by the effect of mannose-6-phosphate added to the culture medium. Addition of 2 mM mannose-6-phosphate increased the levels of both free and bound forms of KDN and Neu5Ac in PA-1 cells to the levels 2-fold as high as the levels when 20 mM mannose was added to the medium.

Discussion

The report on the natural occurrence of KDN delayed 30 years from that of Neu5Ac [24]. This is because abundant occurrence of KDN in animal is limited to lower vertebrates [1,2]. It was after the development of monoclonal antibodies specific for KDN-linkages that human occurrence of KDN was suggested [3–9], and it was due to recent progress in chemical and instrumental analyses that human occurrence of KDN was substantiated [10,11]. Thus, it has already been established that KDN is ubiquitously found in almost all cells and tissues of mammals including human. Studies showed, however, >90% of KDN in mammalian cells and tissues

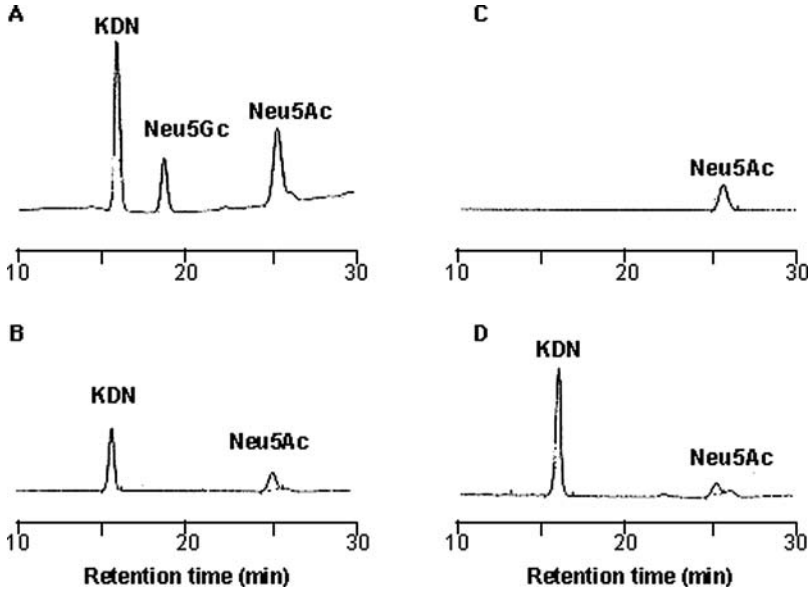

Fig. 9 Fluorometric HPLC sialic acid analysis of the 49 kDa band dissected from the PVDF membrane before and after enzyme treatment. The 49 kDa band separated by SDS-PAGE (Fig. 8, lane 2) was excised after transferred on a PVDF membrane sheet, and analyzed for sialic acids before and after enzyme treatment. (A) standard sialic acids; (B) the sample before enzyme treatment; (C) the sample after KDNase treatment; (D) the sample after exosialidase treatment

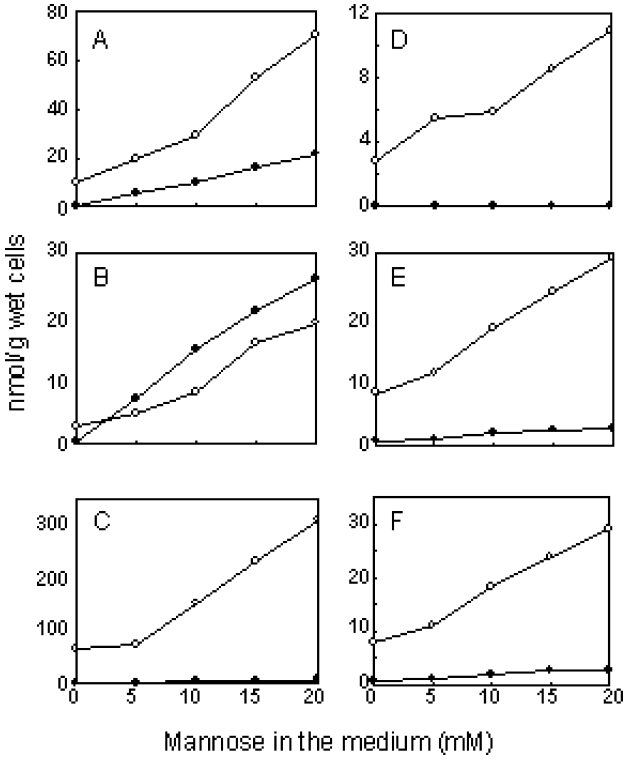

Fig. 10 Effects of mannose added to the culture medium on the intracellular levels of free and bound KDN (●) and Neu5Ac (○) in PA-1 and A375 cells. (A, B, C) PA-1 cells; (D, E, F) A375 cells. Panels (A) and (D), free forms; (B) and (E), soluble protein-bound forms; (C) and (F), membrane-bound forms

occurred in the form of free sugar and KDN conjugated to glycolipids and glycoproteins was almost negligible [11]. Nevertheless, our results of chemical analysis showing that the expression of free KDN in human cells and tissues is developmentally regulated led us to further study on the biological significance of human occurrence of KDN as a minor sialic acid component. Furthermore, the finding of enhanced expression of KDN in some tumor tissues such asovarian and breast cancers, and the malignancy-dependent increase in the ratio of free KDN *vs* free Neu5Ac in ovarian adenocarcinoma suggested the possibility that these elevated expressions of KDN can be used for diagnosis of cancer [2,11].

In this study, we could identify KDN-containing glycoproteins both in the soluble and membrane fractions of PA-1 cells. This is the first identification of KDN-containing glycoproteins in human cells by biochemical and chemical methods. However, we could not identify the proteins bearing KDN-containing glycan chains: it was difficult to purify KDN-containing glycoproteins to the state required for protein sequencing because repeated purification steps resulted in the cleavage of acid-labile KDN-linkages. Nevertheless, we have to overcome the difficulty to develop this study in the area of cancer biology. It is important that expression of these KDN-containing glycoproteins has so far been noted only in ovarian teratocarcinoma PA-1 cells. We have already shown that expression of KDN is high in many types of ovarian cancer tissues, and the ratio of free KDN *vs* free Neu5Ac could be used for diagnostic purpose. Furthermore, we have shown that this ratio is malignancy dependent in ovarian adenocarcinomas [11]. We examined if similar phenomena were observed in tumors of other tissues such as breast, colon, and liver. The results showed that elevated expression of KDN in tumor tissues was observed in breast cancer (S. Inoue, unpublished). However, in colorectal tissues and hepatic tissues the levels of free KDN were significantly lower in tumor compared with normal when matched pairs of normal and tumor tissues obtained from each individual patient were analyzed [2]. In these past experiments we quantified only free KDN because the levels of bound KDN were almost negligible.

As mentioned in the introduction, no mammalian enzyme that specifically recognizes KDN is known. However, human CMP-sialic acid synthase expressed in insect cells has broad substrate specificity, and CMP-KDN was shown to be formed both *in vivo* and *in vitro* in the presence of this enzyme [17]. In addition, at least one mammalian sialyltransferase was reported to recognize CMP-KDN and CMP-Neu5Ac almost equally [18]. Thus, incorporation of KDN in glycoproteins in human cells may occur when the precursor sugar, mannose, is abundantly supplied and/or the enzyme activities involved in sialylation of glycoproteins, *i.e.* sialic acid phosphate synthase, CMP-sialic acid synthase, and sialyltransferases, are elevated. We have shown, in the present study, that increase in the extra-cellular mannose levels during cell culture resulted in large increase of free and bound forms of both KDN and Neu5Ac in PA-1 cells. The affinity purified 30 kDa KDN-glycoprotein isolated from the membrane fraction of PA-1 cells contained mainly KDN (>80 molar%) as a sialic acid component. In this study we did not aim to identify a Neu5Ac-rich counterpart of this glycoprotein. But judging from the abundance of Neu5Ac over KDN in the membrane fraction (compare Fig. 1A with B), at least we can say this KDN-glycoprotein represents only a tiny portion of total sialoglycoproteins found in the membrane fraction of the cell. However, in the 49 kDa KDN-glycoprotein derived from the soluble fraction without using immunoaffinity column, KDN was the major sialic acid component (82% of total sialic acid). This band was the major protein band present in a fraction partially purified by anion-exchange chromatography, and KDN was found only in this band (Fig. 8, lane 2). Thus, it is conceivable that KDN was preferentially incorporated in the 49 kDa KDN-glycoprotein. Judging from the sialic acid composition (KDN; 36% of total sialic acid) of the crude extract of the soluble protein fraction (Fig. 7B), the 49 kDa KDN-glycoprotein represented relatively large portion of total sialoglycoproteins present in the soluble fraction. The mechanism and biological significance of the biosynthesis of these KDN-glycoproteins are important problems to be clarified in the future work.

As a mechanism of elevated expression of free KDN, Angata showed that high extra-cellular levels of mannose increased intracellular levels of mannose, and intracellular levels of free KDN increased proportionally to intracellular levels of mannose in mouse melanoma B16 and African green monkey kidney COS-7 cells [23]. In this paper we showed the effects of mannose on intracellular levels of sialic acids in human teratocarcinoma PA-1 and human melanoma A375 cells. High degree of enhancement of expression levels of free and soluble protein-bound KDN in response to mannose was marked in PA-1 cells. Interestingly, addition of mannose in culture media also increased intracellular levels of free and bound Neu5Ac in both PA-1 and A375 cells. These results suggest that local increase of intracellular mannose concentration changes the metabolism of sialic acids, although the elucidation of its mechanism is out of scope of this work. Over-expression of sialic acids (mainly Neu5Ac) on cell surface proteins may possibly change the fate of the cell from normal to tumor. Thus, the elevated cellular levels of KDN and Neu5Ac might be reflecting the same metabolic consequence. Nevertheless, if we use sensitive analytical methods such as fluorometric HPLC used in the present study, or more conveniently if specific antibodies against tumor-specific KDN-glycoproteins are established, detection of the minor component KDN becomes easier and more reliable than analyzing the levels of Neu5Ac quantitatively. The question remains to be answered if KDN-glycoproteins other than those identified in this study become detectable in the PA-1 cells, and if KDN-glycoproteins are found in other cell lines when the intracellular KDN levels are raised by culturing the cells with mannose supplement.

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