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REVIEW

KDN (Deaminated neuraminic acid): Dreamful past and exciting future of the newest member of the sialic acid family

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Abstract KDN is an abbreviation for 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid, and its natural occurrence was revealed in 1986 by a research group including the present authors. Since sialic acid was used as a synonym for *N*-acylneuraminic acid at that time, there was an argument if this deaminated neuraminic acid belongs to the family of sialic acids. In this review, we describe the 20 years history of studies on KDN (KDNology), through which KDN has established its position as a distinct member of the sialic acid family. These studies have clarified that: (1) KDN occurs widely among vertebrates and bacteria similar to the occurrence of the more common sialic acid, *N*-acetylneuraminic acid (Neu5Ac), but its abundant occurrence in animals is limited to lower vertebrates. (2) KDN is found in almost all types of glycoconjugates, including glycolipids, glycoproteins and capsular polysaccharides. (3) KDN residues are linked to almost all glycan structures in place of Neu5Ac. All linkage types known for Neu5Ac; α2,3-, α2,4-, α2,6-, and α2,8- are also found for KDN. (4) KDN is biosynthesized *de novo* using mannose as a precursor sugar, which is activated to CMP-KDN and transferred to acceptor sugar residues. These reactions are catalyzed by enzymes, some of which preferably recognize KDN, but many others prefer Neu5Ac to KDN. In addition to these basic findings, elevated expression of KDN was found in fetal human red blood cells compared with adult red blood cells, and ovarian tumor tissues compared with normal controls. KDNase, an enzyme which specifically cleaves KDN-linkages, was discovered in a bacterium and monoclonal antibodies that specifically recognize KDN residues in KDNα2,3-Gal- and KDNα2,8-KDN-linkages have been developed. These have been used for identification of KDN-containing molecules. Based on past basic studies and variety of findings, future perspective of KDNology is presented.

Keywords KDN · Deaminated neuraminic acid · Deaminoneuraminic acid · Sialic acid · Mannose · Ovarian tumor

Abbreviations

KDN	2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid
KDO	2-keto-3-deoxy-D-*manno*-octonic acid
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
PSGP	polysialoglycoprotein
Fuc	fucose
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GalNAcol	*N*-acetylgalactosaminitol
Glc	glucose
GlcUA	glucuronic acid
GlcNAc	*N*-acetylglucosamine
Cer	ceramide
Man	mannose
Man-6-P	mannose 6-phosphate
ManNAc	*N*-acetylmannosamine
ManNAc-6-P	*N*-acetylmannosamine 6-phosphate
CMP-KDN	cytidine monophospho-KDN
CMP-Neu5Ac	cytidine monophospho-Neu5Ac
Rib	ribose

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PEP	phosphoenolpyruvate
UDP-Glc	uridine diphospho-Glc
UDP-GlcNAc	uridine diphospho-GlcNAc
GC-MS	Gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
NMR	nuclear magnetic resonance
PNGase	peptide *N*-glycanase
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Two decades have passed since our discovery in rainbow trout eggs of deaminated neuraminic acid (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid, or 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid), now familiar to glycobiologists by the name of KDN. During these years we have continuously been involved with KDNology, *i.e.* studies on KDN from various aspects using various biological materials and using contemporary biological, chemical, and molecular biological techniques. Our studies as well as contributions from many other research groups have established that KDN is a new type of sialic acid, and that KDN and the other more common sialic acid, *N*-acetylneuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nononic acid, or 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid, Neu5Ac) are two biosynthetically independent precursors of a divergent number of modified sialic acids. In this review, we will summarize our past studies and speculate on the potential impact that KDNology may have on the future direction of KDN research. While a number of important contributions to the chemical synthesis and analytical methods for detecting KDN and KDN-containing glycans have also been reported, they are not extensively treated in this review.

Historically, the abundant occurrence of KDN was first shown in limited types of cells and mucus secretions of lower vertebrates, and several bacterial species. Studies of KDN-containing glycans from KDN-rich sources and determination of their precise chemical structures showed that KDN occurred in all types of sialo-glycoconjugates including glycolipids, glycoproteins, and bacterial polysaccharides. In almost all of these KDN-linked structures, one can find counterparts where KDN replaces Neu5Acyl residues, i.e. Neu5Ac and Neu5Gc (*N*-glycolylneuraminic acid). In addition to the development of biological probes, including monoclonal antibodies that specifically recognize KDN-structures, and KDNase that specifically cleaves KDN-linkages, application of highly sensitive chemical methods have provided firm evidence for the occurrence of KDN as a minor sialic acid component in mammals including human. Furthermore, recent progress in biochemical and molecular biological studies on the biosynthesis and metabolism of KDN and KDN-containing glycans established that KDN is synthesized *de novo* from more simple precursors. These studies have also revealed that some of the enzymes involved in the biosynthesis of KDN and KDN-glycans are specific to KDN, while others are common to Neu5Ac synthesis. These metabolic studies may account in part for the widespread but biased mode of distribution of KDN observed in living organisms.

The species-specific expression of KDN in glycoconjugates present in some lower vertebrate cells and excretions, and in the cell wall polysaccharides of pathogenic bacteria suggests that this selected expression may have biological and evolutionary significance that remains to be clarified. Elevated expression levels of free KDN in human red blood cells from umbilical cord, and some malignant tumors including ovarian and breast have also been described. Such aberrant expression may be a reflection of a defect or disorder in the metabolic pathway leading to the formation of KDN, but the molecular mechanism(s) responsible for the elevated expression, and the relationship of this elevation to the malignancy of tumor cells remains to be determined in future studies. Furthermore, recent studies show that in the human teratocarcinoma cell, PA-1, KDN occurs not only free but also in two different KDN-containing glycoproteins, one soluble and the other membrane-associated. These findings thus provide the basis for a promising and fruitful future for KDN research that should be supported by basic studies aimed at elucidating the chemical structures of KDN-containing glycoconjugates and the molecular mechanism regulating KDN expression at both the gene and enzyme levels.

Occurrence of KDN and its distribution in nature

Abundant KDN-containing glycoconjugates occur in lower vertebrates and bacteria

1. Gamete cells of Salmonidae fish

KDN was first discovered in cortical alveolar polysialoglycoprotein (PSGP) of rainbow trout eggs in 1986 [1]. In PSGP, occurrence of KDN as a capping residue attached $\alpha 2 \rightarrow 8$ to the nonreducing terminal Neu5Gc of oligo/polysialic acid chains made the chains resistant to bacterial sialidases. This capping reaction has been suggested to be important in protecting oligo/polysialic acid chains from bacterial sialidases during early embryonic development of salmonid fish in the natural environment. An oligosacharide alditol in which a single residue of KDN was attached ($\alpha 2 \rightarrow 3$ to an internal GalNAc and $\alpha 2 \rightarrow 6$ to proximal GalNAcol was also isolated after alkaline borohydride treatment of PSGP [2]. Thus, the KDN residues were shown to occur as the nonreducing terminal residues involved in $\alpha 2 \rightarrow 3, \alpha 2 \rightarrow 6, \alpha 2 \rightarrow 8$ linkages

in *O*-glycan chains of PSGP. It should be noted that the KDN residue incorporated in glycan chains cannot be an acceptor for Neu5Acyl and/or KDN in the biosynthesis of PSGP [65].

In subsequent studies, mucin-type acidic glycoproteins that contained only KDN as a sialic acid component were isolated from the egg vitelline envelope and ovarian fluid of rainbow trout [3,4]. These glycoproteins, designated as KDN-glycoprotein (KDN-gp), contained >50% by weight of KDN that was present in *O*-linked glycan chains. Structural analysis of the KDN-glycoproteins isolated from the vitelline envelope showed that the *O*-linked oligosaccharides were comprised of a common core trisaccharide Galβ1 → 3GalNAcα1 → 3GalNAc in which the terminal Gal residue was blocked α2 → 3 by a single residue of KDN, and the proximal GalNAc residue was substituted α2 → 6 by α2 → 8-linked oligoKDN chains (chain length, 2–5) [5]. The chemical structure of KDN-gp isolated from the ovarian fluid of rainbow trout appeared to be similar to that of KDN-gp isolated from the vitelline envelope, and KDN-gps were postulated to be synthesized by the same tissue during oogenesis, some of which were incorporated into the outermost layer of the vitelline envelope, while the rest were excreted into the ovarian fluid at ovulation. KDN-gp isolated from vitelline envelopes contained a small number of *N*-linked oligosaccharides in addition to a large number of *O*-linked glycan units. Structural analysis revealed the presence of fully KDNosylated (single KDN was linked α2 → 3 to non-reducing terminal Gal of each antennae) bi- and tri-antennary complex-type oligosaccharide chains, most of which were fucosylated on the innermost GlcNAc residue and bisected by the GlcNAc residue linked α1 → 4 to the β-Man residue [6]. While KDN-gp is a major glycoprotein component of the vitelline envelope and ovarian fluid of rainbow trout, chum salmon, and kokanee salmon, the vitelline envelope and ovarian fluid of cherry salmon contains little KDN-gp. An analogous family of mucin-type acidic glycoproteins that contain Neu5Gc instead of KDN was the major glycoprotein component found in cherry salmon [7].

KDN was also found as a sialic acid component of gangliosides isolated from rainbow trout sperm, testis, and ovarian fluid. First, KDNGM3, KDNα2 → 3Galβ1 → 4Glcβ1 → Cer, was isolated as a major ganglioside component of mature rainbow trout sperm [8]. Using monoclonal antibody, mAb.kdn3G (recognizes KDNα2 → 3Galβ1 →, see below) for detection, four KDN-gangliosides were found in immature testis. These gangliosides were not detected in mature sperm, whereas KDNGM3, a major ganglioside in mature sperm, was expressed in testis throughout all stages of maturation [9]. The structures of these gangliosides were established as (i) KDNGD1a, KDNα2 → 3Galβ1→3GalNAcβ1 →4(KDNα2 →3)Galβ1 → 4Glcβ1 → Cer; (ii) (KDN, Neu5Ac)GD1a, KDNα2 → 3Galβ1 → 3GalNAcβ1 → 4(Neu5Acα2 → 3)Galβ1 → 4Glcβ1 → Cer, and Neu5Acα2 → 3Galβ1 → 3GalNAcβ1 → 4(KDNα2 → 3)Galβ1 → 4Glcβ1 → Cer; (iii) KDN-GD1α, KDNα2 → 3Galβ1 → 3(KDNα2 → 6)GalNAcβ1 → 4Galβ1 → 4Glcβ1 → Cer; and (iv) (KDN, Neu5Ac)-GD1α, KDNα2 → 3Galβ1 → 3(Neu5Acα2 → 6)GalNAc β1 → 4Galβ1 → 4Glcβ1 → Cer. Of these gangliosides KDNGM3, KDNGD1a, and (KDN, Neu5Ac)GD1a were found in testis throughout all stages of spermatogenesis, whereas the α series of KDN-gangliosides, i.e. KDNGD1α, and (KDN, Neu5Ac)GD1α, emerged at a later stage of development, 2–3 months prior to spermiation. Furthermore, the difference in the sphingoid base suggested that, unlike the case with KDNGM3 and KDN-containing GD1a, GD1α gangliosides were not expressed in precursor cells of sperm, but most likely in Sertoli cells. The stage-specific and site-specific expression of different KDN-gangliosides suggests that these gangliosides may play an important, yet unknown role in spermatogenesis.

Two KDN-containing glycosphingolipids were also isolated from rainbow trout ovarian fluid and their structures were determined as KDNα2 → 3Galβ1 → 3GalNAcβ1 → 4(KDNα2 → 3)Galβ1 → 4Glcβ1 → Cer [KDNGD1a], and 9-O-AcKDNα2→ 3Galβ1→3GalNAcβ1→ 4(KDNα2 → 3)Galβ1 → 4Glcβ1 → Cer [KDNGD1a-(OAc+)] [10]. The 9-*O*-acetyl substitution of KDN had been identified on KDN capping oligo/polyNeu5Gc chains of PSGP from kokanee salmon (*Oncorhynchus nerka adonis*) [11]. Oligo/polyNeu5Gc chains of PSGP from this fish species were extensively *O*-acetylated at C4, C7, and C9, suggesting that *O*-acetylation of KDN was catalyzed by the same enzyme.

2. *Amphibian egg jelly coats*

KDN-containing *O*-linked oligosaccharides were isolated from amphibian egg jelly coats and their structures were determined by a French research group [12–15]. In these oligosaccharide chains KDN was linked α2 → 6 to the proximal GalNAc residues. In oligosaccharide chains isolated from Mexican axolotl, and *Xenopus laevis*, KDN occurred as an internal residue to which a single Fuc or Fucα1 → 3Fuc unit was linked α1 → 4. In the egg jelly coat of other axolotl species (*Ambystoma tigrinum, Ambystoma maculatum*) KDN also occurred as internal residues monofucosylated at C4 or difucosylated at C4, and C5 [16–18]. Structures containing internal sialic acid substituted by sugar other than sialic acid are unusual for animal oligosaccharide units. In egg jelly mucin of frog, *Bambina bambina,* KDN occurs as a nonreducing terminal residue, and in *Bambina variegate* mucin Neu5Gc (major) or Neu5Ac (minor) replaced KDN in the oligosaccharide units of similar structures, although these two species are the most closely related within the genus [19]. Thus, observed species specific diversity of glycan structures

of amphibian oviductal mucin reflects evolutional change in enzymes involved in the biosynthetic pathway. In amphibia as well as fish, embryonic development takes place outside of female body and in most cases in water.

3. *Skin mucus and other organs of fish*

The skin mucus of the loach (*Misgurnus anguillicaudatus*) contained ~40% by weight of sialic acid, >95% of which was found to be KDN [20]. This report first showed occurrence of KDN in a glycoconjugate produced by non-gamete cells. Two different structures of *O*-linked units, KDNα2 → 6GalNAc, and KDNα2 → 6(KDNα2 → 3)GalNAc, were presented. These authors also found that carp (*Cyprinus carpio*) and wrasse (*Halichoeres tenuispinis*) showed high KDN contents in the skin mucus (85 and 25% of total sialic acid, respectively). Minute amounts if any, of KDN were found in the skin mucus of eel (*Auguilla japonica*), rainbow trout (*Onchorinchus mykiss*), crucian carp (*Carassius auratus*), and stingray (*Dasyatis akajei*). The sialic acid components of the mucus of these fish were Neu5Ac and Neu5Gc [21].

KDN was found to occur in various organs of the loach (*Misgurnus anguillicaudatus*) as a sialic acid component of glycolipid and glycoprotein fractions in all organs examined, of which intestine, muscle, and ovary showed the highest proportion of KDN (20–30 mol% of total sialic acid). The proportion of KDN was low in brain, heart, liver, and kidney (3–7 mol% of total sialic acid) [22]. Thus, loach is unique in that it shows high expression levels of KDN in many organs as well as in gamete cells.

4. *Capsules and cell envelopes of pathogenic bacteria*

The first report of the occurrence of KDN in bacteria was made in 1989 in the acidic capsular polysaccharide from pathogenic bacterium, *Klebsiella ozaenae* serotype K4, that causes respiratory diseases in human [23]. A pentasacharide repeating unit, → 3Glcβ1 → 2(KDNα2 → 4)GlcUA(3-OAc)α1 → 3Manα1 → 3Glcα1 →, was characterized as the structure of this polysaccharide.

Bacterial occurrence of KDN was later reported in the capsular polysaccharide of a Gram-negative bacterium *Sinorhizobium fredii* strain SVQ293, a thiamine autotrophic mutant of *S. fredii* HH103 [24]. Rhizobium species are able to induce the formation of a nitrogen-fixing organ (nodule) on the root of leguminous plants. The polysaccharide (fatty acids were absent) fraction separated from the hot phenol/water extract consisted of the trisaccharide repeating unit → 2Galα1 → 2Ribα1 → 9(5-O-Me)KDNα2 →, where 25% of the KDN residues were not methylated. This KDN-containing polysaccharide was not present in the wild-type strain HH103, which produced a 3-deoxy-D-*manno*-2-octulosonic acid (KDO)-rich polysaccharide. These polysaccharides are believed to play an important role in the symbiotic bacterium-plant interaction, and the change of typical KDO-rich polysaccharide to KDN-rich polysaccharide as the result of mutation is proposed to be related to alterations in the nodulation host range observed in the mutant.

More recently KDN was reported to occur in the anionic cell wall polymers of *Streptomyces sp*. MB-8, the causative agent of potato scab [25]. The basic structure of the oligosaccharide was determined to be Gal(±3OMe)β1 → 9KDNα2 → 4[Gal(±3OMe)β1 → 9] KDN. It was not shown how this small oligosaccharide was associated with other cell wall polysaccharides. A polymer with a backbone of polyKDN (~20 repeating units) in the cell wall of a plant pathogen, *Streptomyces sp*. VKM Ac-2124 [26], has also been reported. The polyKDN structure was unusual in that KDN residues were linked β2 → 4 to each other. Glucose was linked β1 → 8 to each KDN residue. The amount of the KDN-containing polymer was 20% of total cell wall polysaccharide. The KDN-containing polymer, like the polymer containing KDO was suggested to be involved in an early step of the attachment of pathogenic bacteria to host plant cells. In this connection a new type of sialic acid derivative, 5,7-diamino-5,7,9-trideoxy-nonulosonic acid was isolated from a lipopolysaccharide fraction from *Pseudomonas corrugate*, the causal agent of tomato pith necrosis [27]. The presence of KDN appeared to be characteristic to plant pathogenic streptomycete strains causing scab disease of potatoes and root crops. In bacterial plant pathogens, KDN appears to be functionally and structurally related to the octulosonic acid, KDO.

Occurrence of KDN in mammals

1. *Confirmation that KDN occurs in mammalian tissue*

The occurrence of KDN in mammals was first suggested based on immunohistochemistry and immunoblot analysis of tissue extracts using mAb.kdn8kdn (see below). Chemical analysis using a highly sensitive fluorometric HPLC method also supported the presence of KDN in mammalian tissues. However, it has been difficult to isolate KDN-containing glycoconjugates in amounts sufficient for chemical analysis, as was the case for lower vertebrates. As a first step to obtain definitive chemical proof, we analyzed the free sialic acid fraction obtained by mild acid hydrolysis of pig submaxillary glands, a tissue rich in sialomucin, and found substantial amounts of KDN in the hydrolysate. Starting with 120 g of pig submaxillary gland, 300 μg of free KDN was isolated after mild acid hydrolysis, anion-exchange chromatography, and gel chromatography. The presence of KDN was confirmed by fluorometric HPLC, gas chromatography, mass spectrometry and ^{1}H-NMR spectroscopy [28]. The importance of this work

is that it was the first unequivocal evidence for the occurrence of KDN in mammalian tissue. In subsequent studies, various rat tissues were separated into the chloroform-methanol extractable and non-extractable fractions, and the sialic acid composition of each fraction was analyzed by fluorometric HPLC after mild acid hydrolysis. KDN was present in all tissues examined in both chloroform-methanol extractable (containing free and glycolipid-linked KDN), and non-extractable (containing glycoprotein-linked KDN) fractions. The relative amount of KDN was in the order of 1:100 and 1:1000 of the total sialic acid in the chloroform-methanol extractable and non-extractable fraction, respectively. KDN was also found in mild acid hydrolysates of chloroform-methanol extractable and non-extractable fractions obtained from human lung carcinoma cells. In this case, the proportion of KDN relative to total sialic acid was 0.3–0.5 mol%.

In a subsequent study we showed that KDN was present in human red blood cells and ovarian cancer cells [29]. Furthermore, we showed that in these cells most KDN occurred as the free sugar in a fraction soluble in 80% ethanol and little occurred conjugated or as CMP-KDN. The amount of free KDN in fetal red blood cells was 2.4-fold higher than in red blood cells from the mothers or from healthy non-pregnant women. Free KDN was also identified in normal human ovaries, in human tumor ovaries, and in ascites cells obtained from ovarian cancer patients. Importantly, as in fetal cord red blood cells, a distinguishing feature of KDN expression in ovarian tumor cells was an elevated level of free KDN compared with normal controls. To explain the phenomena observed in this study, several questions remained to be answered. First, where does free KDN come from, i.e. how is it synthesized? Second, what regulates intracellular level of KDN? Third, how are increased intracellular levels of free KDN related to the metabolism of Neu5Ac? Some of these questions have already been solved as described below.

The occurrence of KDN in mammalian tissues was reported in rat liver where the age-dependent change in the expression level was shown. In this case, the KDN content decreased from newborn to only trace amounts in adult rats, but increased again with aging [30]. The presence of KDN in human erythrocyte membranes was also shown using a new GC-MS based method [31].

2. *Development of monoclonal antibodies that recognize KDN-linkages in glycan chains and their application to detect KDN-glycoconjugates in mammalian cells and tissues*

Two different kinds of monoclonal antibodies that recognize KDN-sequences, mAb.kdn3G [32], and mAb.kdn8kdn [33] have been developed. The antigenic determinants for mAb.kdn3G and mAb.kdn8kdn were shown to be $\mathrm{KDN}\alpha 2 \rightarrow 3\mathrm{Gal}\beta 1 \rightarrow$ and $(\rightarrow 8\mathrm{KDN}\alpha 2 \rightarrow)_n$, $n \geq 2$, respectively. The minimum chain length for immunoreactivity of the anti-oligoKDN antibody, mAb.kdn8kdn, was determined using a series of lipid-conjugated oligoKDN with defined degree of polymerization [34].

(A) Widespread and developmentally regulated expression of glycoproteins reactive with mAb.kdn8kdn

mAb.kdn8kdn was used to search for the occurrence of oligo/polyKDN-containing glycoproteins in mammalian tissues. It was rather surprising that immunohistochemical staining was observed in many cells and tissues of rat, including ovary, testis, the endothelial cells of blood vessels, the smooth muscle fibers of skeletal muscles and the digestive, respiratory, and urogenital tracts; the thymus, spleen, and lymph nodes, the adipocytes of the white and brown adipose tissues; and the liver [35]. In sections of thymus, for example, the positive immunostaining was shown to be abolished by pre-treatment with KDNase (see below). Immunoblotting showed a single predominant band with an apparent molecular mass of 150 kDa in muscle, kidney, lung, and brain. Furthermore, expression of the epitope was developmentally regulated. In lung the mAb.kdn8kdn-positive 150-kDa band was postulated to represent a new oncodevelopmental antigen [36].

More recently, the >350-kDa immunoreactive protein in rat kidney was identified as megalin, a member of the low density lipoprotein receptor gene family [37–39]. These authors also reported that ceruloplasmin isolated from rat testis, thymus, ovary, blood serum, and postnatal day-2 lung and brain by immunoprecipitation with anti-ceruloplasmin was immunoreactive with mAb.kdn8kdn. Immunoreactivity was abolished by PNGase F treatment, leading to the suggestion that ceruloplasmin carries oligo/polyKDN on its *N*-glycan chains [40]. It is important to note, however, that in none of the reports was the presence of KDN confirmed by chemical analysis. These results should thus be considered preliminary until confirmed by chemical analysis.

(B) Occurrence of glycoproteins that are reactive with mAb.kdn3G in mammalian cultured cells

Some but not all cultured cell lines express chemically detectable levels of KDN. Human melanoma (A375 and B16-F1), and human ovarian teratocarcinoma (PA-1) showed relatively high levels of KDN expression. In these cells, the majority of KDN occurred as the free sugar and little, but still chemically detectable KDN was conjugated to glycoproteins found in the cytosolic and membrane fractions. The amount of KDN (both in the free and the conjugated forms) present in the same weight cells increased with time up to 72 h when the cells reached full confluency. At confluency, binding of the mAb.kdn3G to the cell surface was observed by immunofluorescence staining of fixed cells. Interestingly, the level of KDN in both the free and the conjugated forms increased when these cells were grown in the medium supplemented with 5–20 mM mannose [96].

KDN-rich glycoproteins were isolated from PA-1 cells, partially purified and characterized [96]. A 30-kDa KDN-glycoprotein reactive with mAb.kdn3G was isolated from the membrane fraction of PA-1 cells. The immunopurified protein still showed multiple bands on SDS-PAGE when tested by silver staining and needed further purification before protein sequencing. However, evidence showing that the 30-kDa band stainable with mAb.kdn3G contained a KDN-rich glycoprotein was as follows. First, when the band was excised from a PVDF membrane and analyzed by fluorometric HPLC analysis after mild acid hydrolysis, a peak identified as KDN appeared along with a much smaller peak for Neu5Ac. Furthermore, when the PVDF membrane was similarly analyzed after KDNase treatment, KDN disappeared from the HPLC profile. Second, KDN was most likely attached to *N*-glycans since the immunoreactive 30-kDa band disappeared after the sample was treated with PNGase F before Western-blot analysis. The soluble protein fraction from PA-1 cells contained a larger amount of KDNase-sensitive KDN than the membrane fraction, yet the fraction was negative to both mAb.kdn3G and mAb.kdn8kdn antibody staining, indicating absence of $\alpha 2 \rightarrow 3$- and $\alpha 2 \rightarrow 8$-linkages. A KDN-containing fraction obtained by anion-exchange chromatography of the soluble fraction was subjected to SDS-PAGE, and a 49-kDa band containing KDN and Neu5Ac in a ratio of $\sim 6:1$ was found. The peak of KDN disappeared when the 49-kDa band was treated with KDNase before mild acid hydrolysis and then subjected to fluorometric HPLC analysis. Similarly, when the same sample was treated with *Arthrobacter ureafaciens* sialidase and then subjected to sialic acid analysis, the peak of Neu5Ac relative to that of KDN was greatly reduced. When the KDN-containing fraction was digested with PNGase F, the 49-kDa band diminished and a new band appeared at $\sim$38 kDa, suggesting that the 49-kDa glycoprotein contained *N*-linked glycan chains. [41,42,96]. KDN-containing glycoproteins (38 kDa and 50 kDa) reactive with mAb.kdn3G were also isolated from the human melanoma cell line A375. Both KDN-containing glycoproteins were found in the soluble fraction. Evidence for the presence of KDN in the fractions partially purified by combination of gel-permeation and hydrophobic chromatography was obtained by the fluorometric HPLC method. However, the purified glycoprotein samples contained larger amounts of Neu5Ac than KDN, suggesting that KDN residues were minor sialic acid components replacing a part of Neu5Ac residues in the isolated glycoproteins (Lin YH *et al*. to be published).

Sialidases that cleave KDN-linkages

1. KDNase

A sialidase that specifically hydrolyzes KDN ketosidic but not *N*-acylneuraminyl linkages was discovered and purified from the bacterium (*Shingobacterium multivorum*) isolated from the sludge in a sewage pond at a local trout hatchery [43]. Activity of the affinity purified enzyme, designated KDNase SM, toward 4-methylumbelliferyl KDN (4-MU-KDN) was not inhibited by the *N*-acylneuraminidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acylneuraminic acid. KDNase SM released free KDN from the naturally occurring substrates, KDNGM3 and KDN-gp, which include KDN$\alpha 2 \rightarrow$ 3Gal, KDN$\alpha 2 \rightarrow$ 6GalNAc, and KDN$\alpha 2 \rightarrow$ 8KDN linkages. KDNase SM was found to be an inducible enzyme that was localized in the periplasm of *S. multivorum* [44]. Growth of the bacterium in the presence of KDN-containing oligosaccharides as a sole carbon source induced the enzyme activity 15–40-fold, compared with growth in the absence of inducer. KDNase SM activity resided in a single polypeptide chain with an estimated molecular weight of approx. 47,500. Enzyme activity was maximal at near neutral pH. KDNase SM was also induced when cells were grown in the presence of synthetic α-ketosides of KDN, all of which were good substrates for the enzyme [45].

The transition state analogue inhibitor, 2,3-didehydro-2,3-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid was a strong competitive inhibitor of KDNase SM ($Ki = 7.7\ \mu M$ versus $Km = 42\ \mu M$ for KDN-MU) [46]. KDNase SM activity was not inhibited by 2-deoxy-2,3-didehydro-*N*-acylneuraminic acids, strong competitive inhibitors of bacterial sialidases. Substrate specificity of KDNase SM was also studied using chemically synthesized KDN analogs [47]. These results suggested that the hydroxyl group at C-5 is important for recognition of the inhibitor by the enzyme. ^{1}H NMR spectroscopic studies clearly demonstrated that the thermodynamically less stable α-form is preferentially formed as the first product of the cleavage reaction and that isomerization rapidly follows, leading to an equilibrium mixture where the β-isomer is the major species. These findings led us to the proposal that KDNase SM catalysis proceeds via a mechanism common to the known exosialidases.

2. KDN-Sialidases

KDN-linkages are resistant to the action of bacterial and viral sialidases. Sialidases that cleave both KDN- and *N*-acylneuraminyl linkages (KDN-sialidases) have been reported in animals that contain KDN-glycoconjugates. In rainbow trout KDNase activity was found in kidney, spleen and ovary [48]. The highest activity was found in ovarian post-ovulatory follicles obtained from female fish at the time when the reproductive organ was undergoing natural effacement. Based on a kinetic study using mixed substrates, it was concluded that the cleavage of both KDN- and *N*-acylneuraminyl linkages was catalyzed by a single enzyme.

A sialidase capable of hydrolyzing α-ketosides of KDN, KDN$\alpha 2 \rightarrow$ 3Gal$\beta 1 \rightarrow$ 4GlcCer and KDN$\alpha 2 \rightarrow$ 6*N*-acetylgalactosaminitol as well as 4-methylumbelliferyl

α-ketoside of KDN was found in the liver of the loach, *Misgurnus fossilis*, a fish species in which KDN-containing glycoproteins were also found [49]. This enzyme also cleaves α-ketosidic linkages of Neu5Ac. Both KDN- and Neu5Ac-ketoside cleaving activities were co-eluted from a column and the pH optimum for both activities were 4.6, suggesting the same protein was responsible for both activities. The Km values using the substrates 4-methylumbelliferyl α-ketoside of KDN and 4-methylumbelliferyl α-ketoside of Neu5Ac were 0.07 and 0.12 mM, respectively.

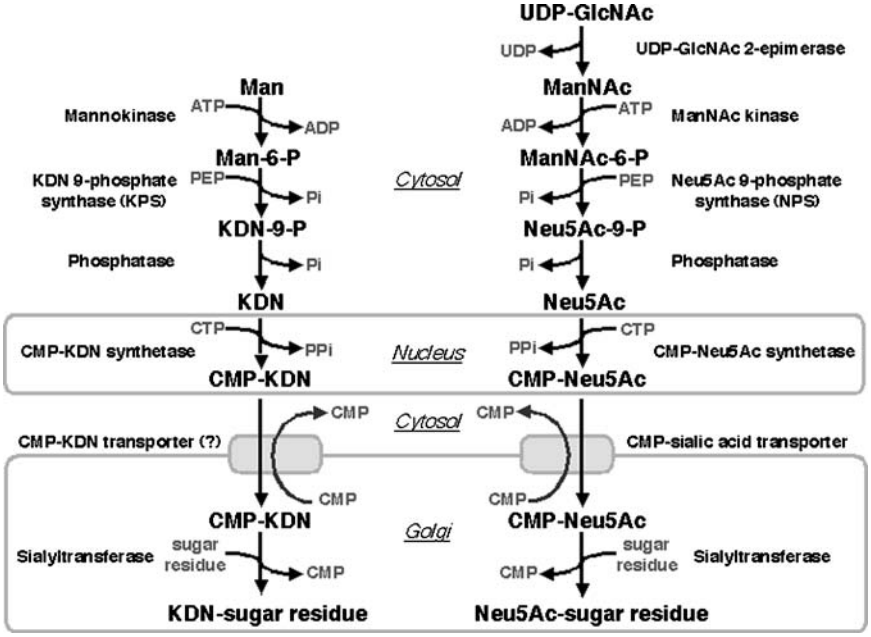
Two different sialidases, KDN-sialidase and conventional sialidase (sialidase that does not cleave KDN-ketoside), were partially purified from the entrails of *Asterina pectinifera,* a starfish species abundant in southern Japan [50]. Both enzymes differ in several properties other than glycon specificity, including molecular mass, isoelectric point and susceptibility to competitive and non-competitive inhibitors.

The hepatopancreas of the oyster, *Crassostrea virginica*, was found to contain a major sialidase, KDN-sialidase, and a minor sialidase, KDNase, but was devoid of conventional sialidase activity [51]. Oyster KDN-sialidase cleaves α-ketosidic linkages of KDN more efficiently than those of Neu5Ac. Kinetic analysis suggested that the KDN-sialidase contains two separate active sites for hydrolysis of KDN and Neu5Ac.

Metabolism of KDN and KDN-glycans

1. Identification and characterization of enzymes involved in biosynthesis of KDN

Two pathways are possible for KDN synthesis. First, it could be formed directly from Neu5Acyl by deacylation and deamination. However, no evidence for the direct conversion of Neu5Ac to KDN has been obtained in any tissue homogenates examined to date. An alternative pathway, *de novo* synthesis via enzymes different from those required for Neu5Ac synthesis, appeared to be plausible by analogy with synthesis of other ulosonic acids. For example, 3-deoxy-D-*manno*-octulosonic acid (KDO) and Neu5Ac are synthesized through a series of similar reactions involving a condensation of phosphoenolpyruvate (PEP) and the relevant sugar phosphate, either arabinose 5-phosphate for 3-deoxy-D-*manno*-octulosonic acid 8-phosphate, or *N*-acetylmannosamine 6-phosphate (ManNAc-6-P) for Neu5Ac 9-phosphate. Accordingly, the *de novo* biosynthesis of KDN is hypothesized to involve the condensation of PEP and mannose 6-phosphate (Man-6-P), giving rise to KDN 9-phosphate (KDN-9-P) (Scheme 1). Thus, a KDN-9-phosphate synthase, an activity that condenses Man-6-P and PEP was identified in rainbow trout ovaries and testis, tissues rich in expressing KDN-containing glycoconjugates [52]. The KDN-9-P synthase in the testis was co-purified with Neu5Ac-9-P synthase. However, the ratio of both activities changed significantly during purification, suggesting that syntheses of KDN-9-P and Neu5Ac-9-P were catalyzed by two separate synthase activities. The KDN-9-P synthase activity was also found in trout liver, but the ratio of specific activities of KDN-9-P to Neu5Ac-9-P synthase in the liver was markedly lower than in testis. This difference in the expression level of the two synthase activities in testis and liver suggested the presence of multiple enzyme proteins with different properties. However, it is still not clear if KDN-9-P synthase and Neu5Ac-9-P synthase activities are on separate enzyme proteins. Mannose could not replace Man-6-P, nor could pyruvate replace PEP



Sch. 1 *De novo* pathways for the synthesis of KDN and Neu5Ac in animal cells. Man-6-P is a key precursor monosaccharide for KDN, while ManNAc-6-P is that for Neu5Ac. The biosynthesis of KDN from Man-6-P follows the similar enzyme reactions to that of Neu5Ac from ManNAc-6-P. UDP-GlcNAc 2-epimerase and ManNAc kinase activities are on the same enzyme, and regulate the supply of Neu5Ac by a feedback mechanism. An analogous pathway for the synthesis of Man-6-P from UDP-Glc is not known

in the condensation reaction. No inhibition of the KDN-9-P synthase activity was observed by KDN (up to 1 mM), Neu5Ac (up to 1 mM), CMP-KDN (up to 0.5 mM), or CMP-Neu5Ac (up to 0.5 mM).

Man-6-P can be synthesized by at least two possible biosynthetic pathways in eukaryotic cells. First, Man-6-P is formed in the cytosol from fructose 6-phosphate by phosphomannoisomerase. Alternatively, mannose can be phosphorylated by a 6-phosphokinase after transport into the cell. Mannose is a normal component of the blood of higher animals. [53,54]. In contrast, ManNAc-6-P is synthesized from UDP-GlcNAc and ATP by a bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase [55–57]. An analogous pathway from UDP-Glc to Man-6-P has not been reported.

Cellular levels of free and bound forms of KDN were found to increase when mouse melanoma B16 and African green monkey kidney COS-7 cells were cultured in mannose-rich media [58]. The elevated expression of free KDN was proportional to the intracellular concentration of free Man. KDN-9-P synthase activity was detected in both cell lines. Interestingly, each cell line showed a different response to external mannose. Human teratocarcinoma PA-1 cells were particularly responsive to externally added mannose in the synthesis of both free and bound KDN [96]. Most recently, it has been demonstrated that the elevation of free KDN expression in response to high-dose administration of Man occurs ubiquitously in various mammalian cultured cells and mouse organs, although a full understanding of the mechanism for this phenomenon requires further study [97]. In this connection, the stage-specific expression of phosphomannose isomerase, an enzyme that interconverts Fru-6-P and Man-6-P in mouse testis may also raise the KDN level [59]. It was reported that both expression of this enzyme and that of protein-bound KDN in testis were higher than in liver [59].

Recently a cDNA of the mouse homologue of the *Escherichia coli* Neu5Ac synthase (neuB) was cloned [60]. The recombinant mouse homologue, which was expressed in HeLa cells as a 50-kDa protein, possessed Neu5Ac-9-P synthase activity but lacked Neu5Ac synthase activity. This enzyme was localized in the cytosol and was ubiquitously distributed in various mouse tissues. Notably, the Neu5Ac-9-P synthase could not catalyze the synthesis of KDN or KDN-9-P *in vitro*, suggesting that the enzyme may not be involved in the synthesis of KDN-9-P synthesis. However, the involvement of the Neu5Ac-9-P synthase in the synthesis of KDN *in vivo* has most recently been demonstrated based on the results of RNA interference experiments (Go *et al.*, unpublished results).

In 2000, a human sialic acid phosphate synthase gene, *SAS*, was cloned based on homology to the *E. coli* sialic acid synthase gene (*neuB*) [61]. The human gene was ubiquitously expressed in human tissues and encoded a 40-kDa protein. The Neu5Ac levels of Sf-9 insect cells grown in serum-free medium were extremely low. Transfection of Sf-9 cells with recombinant baculovirus (AcSAS) resulted in the appearance of KDN in the cell lysate but no increase in the low level of Neu5Ac. A 900-fold increase in the level of Neu5Ac was obtained, however, when the growth medium was supplemented with 10 mM ManNAc compared to the non-transfected cells grown in the absence of ManNAc. AcSAS transfection of Sf-9 cells also led to large increases in KDN levels, which increased slightly more upon the addition of Man or ManNAc, whereas both AcSAS transfection and ManNAc feeding were required to obtain substantial Neu5Ac levels. *In vitro*, the human enzyme used ManNAc-6-P and Man-6-P as substrates to generate Neu5Ac-9-P and KDN-9-P, respectively, but exhibited much higher activity toward the Neu5Ac-9-P product.

2. CMP-KDN synthetase

CMP-nonulosonate synthetase was partially purified from the homogenate of trout testis where various KDN-gangliosides were expressed in a developmentally regulated fashion [62]. Vmax/Km studies showed that KDN was the preferred nonulosonic acid substrate compared to Neu5Ac or Neu5Gc (4.4×10^{-3} min^{-1} for KDN *versus* 2.3 and 1.8×10^{-3} min^{-1} for Neu5Ac and Neu5Gc, respectively). A kinetic study using mixed substrates showed that both CMP-KDN and CMP-Neu5Ac synthetase activities in the partially purified enzyme were due to the same active site on a single polypeptide. In contrast, KDN was a poor substrate for the calf brain CMP-sialic acid synthetase. The expression of CMP-KDN synthetase was temporally correlated with development during spermatogenesis and with the developmental expression of KDNGM3. No CMP-KDN synthetase activity was found in spermiated mature sperm.

Recently, a cDNA encoding the rainbow trout testis CMP-KDN synthetase was isolated by PCR using degenerate oligonucleotide primers corresponding to the conserved motifs in the bacterial and murine enzymes [63]. The trout enzyme was shown to consist of 432 amino acids with two potential nuclear localization signals. The cDNA sequence showed 53.8% identity with the murine enzyme. Kinetic analyses showed that the recombinant trout enzyme had high activity towards synthesis of both CMP-KDN and CMP-Neu5Ac (Vmax/Km values, 1.1 versus 0.68 min^{-1}), whereas the recombinant murine enzyme had little activity towards synthesis of CMP-KDN (Vmax/Km values for CMP-KDN and CMP-Neu5Ac were 0.23 and 3.5 min^{-1}, respectively). Thus, the results obtained using the recombinant trout CMP-KDN synthetase support the previous conclusion obtained from the substrate competition experiment using the partially purified enzyme from rainbow trout testis. Northern blot analysis showed that several mRNA sizes were expressed in trout liver, testis, and ovary in a tissue-specific manner.

The occurrence of several transcripts for the trout CMP-sialic acid synthetase in immature and mature trout testis may be related to the developmental changes in expression of KDN-gangliosides in trout testis during spermatogenesis.

Occurrence of KDN in almost all mammalian cells and tissues has been shown, although the level is usually 2-3 orders of magnitude lower than that of Neu5Ac [28]. However, most of the KDN occurs as the free monosaccharide and little, if any, occurs conjugated to glycolipids or glycoproteins [29]. It has been found that in some mammalian cells the expression level of free KDN is elevated compared to that of free Neu5Ac when the cells are grown in media supplemented with mannose. Nevertheless, the expression level of bound KDN in cells grown in mannose-rich media was still 1–2 orders of magnitude lower than bound Neu5Ac [58]. Activity of the recombinant murine CMP-sialic acid synthetase was 15 times lower with KDN compared with Neu5Ac [63]. Although there are more reaction steps after the formation of CMP-KDN, including translocation of the sugar nucleotide into lumen of the Golgi apparatus by a CMP-KDN transporter and transfer of KDN to acceptor glycans by KDN-transferases, the formation of CMP-KDN appears to be a key factor limiting the biosynthesis of KDN-containing glycoconjugates in mammalian systems. Many of the steps in the pathway, however, remain to be determined.

cDNA for a human CMP-sialic acid synthetase was also identified and found to encode a protein with 94% identity to the murine enzyme [64]. When expressed in Sf9 insect cells, the encoded protein was functional and localized to the nucleus as in mammalian cells. When *CMP-SAS* was co-expressed with human sialic acid phosphate synthetase gene (*SAS*) in insect cells, the major nucleotide sialic acid product was CMP-KDN. CMP-Neu5Ac production was achieved only upon growth in the presence of ManNAc. The human enzyme appeared to have broad sialic acid substrate specificity. *In vitro* enzyme assays using a nuclear fraction from transfected Sf9 cells, showed that KDN, Neu5Ac, and Neu5Gc were all activated to their respective sugar nucleotide forms with comparative efficiency.

3. Sialyltransferases that catalyze transfer of KDN from CMP-KDN to the non-reducing termini of glycan chains

A sialyltransferase activity that catalyzes transfer of KDN from CMP-KDN to the non-reducing termini of oligo/polysialyl chains of polysialoglycoprotein (PSGP) was identified in the cortical vesicle fractions of the rainbow trout ovary [65]. Incorporation of the KDN residues into the oligo/polysialyl chains prevented their further elongation, resulting in capping of the oligo/polysialyl chains. Expression of KDN-transferase activity was developmentally regulated during oogenesis in parallel with expression of the $\alpha 2 \rightarrow 8$-polysialyltransferase, which catalyzes synthesis of the oligo/polysialyl chains in PSGP. Since expression was also temporally correlated with expression of KDN as indicated by KDN/Neu5Ac ratio in the ovary, it is clear that the observed KDN-transferase activity participates in incorporation of KDN into the oligo/polysialyl chains of PSGP as the capping residue during the maturation stage of oogenesis.

In accord with the broad specificity reported for the sialyltransferases toward donor substrate, a rat liver $\alpha 2$,6-sialyltransferase (Gal$\beta 1 \rightarrow$ 4GlcNAc $\alpha 2$,6-sialyltransferase, Boeringer Mannheim, Germany) was shown to transfer KDN from CMP-KDN to asialotransferrin and *N*-acetyllactosamine [66]. The incorporation efficiency of KDN from CMP-KDN into asialotransferrin was about half that of Neu5Ac from CMP-Neu5Ac, based on the Vmax/Km values for these substrates, 0.0527 min^{-1} and 0.119 min^{-1}, respectively.

Properties of KDN-containing glycans

To understand the potential biological significance of preferential expression of KDN in glycoconjugates of living organisms, studies on the interaction of KDN-containing glycoconjugates with other molecules will be of critical importance. To begin to address this problem, we determined calcium ion binding properties of oligo/polyKDN by equilibrium dialysis and circular dichroism [67]. KDN-gp (a glycoprotein containing $\alpha 2 \rightarrow$ 8-linked oligo/polyKDN on *O*-linked glycans) was shown to bind calcium ions with a Ka of 2.9×10^3 M^{-1}, which was essentially identical to that of PSGP (a glycoprotein containing $\alpha 2 \rightarrow$ 8-linked oligo/polyNeu5Gc on *O*-linked glycans). This Ka value was about 20-fold higher than the binding constants reported for the formation of the 1 :1 Ca^{2+}-sialic acid monomer complex. From these studies, the number of sialic acid residues required to bind 1 calcium ion was estimated to be 2 for both KDN-gp and PSGP.

Binding of rainbow trout sperm to egg was shown to be mediated by strong carbohydrate-to-carbohydrate interaction between KDNGM3 and Gg3-like epitope in model experiments using KDNGM3-containing liposomes binding to Gg3Cer-coated plastic plate [68]. The binding between KDNGM3 and Gg3Cer was much stronger than the binding between Neu5AcGM3 and Gg3Cer. Importantly, fresh trout sperm were also shown to adhere to Gg3Cer-coated plate under physiological conditions.

Carbohydrate-carbohydrate interaction between various gangliosides and artificial glycoconjugate polymers was also studied by surface plasmon resonance [69]. Strong interactions were observed between a Neu5AcGM3 monolayer and a Gg3 (GalNAc$\beta 1 \rightarrow$ 4Gal$\beta 1 \rightarrow$ 4Glc$\beta 1 \rightarrow$)-carrying polystyrene polymer. KDNGM3 also showed strong binding

with this polymer, but the affinity constant for KDNGM3 was one order of magnitude lower than that for Neu5AcGM3.

Development of analytical and synthetic methods for the study of KDN and KDN-glycoconjugates

The continued development of analytical and synthetic methods for the study of KDN glycoconjugates will remain an impotant area for future KDNology. Advances in these areas will likely extend the current foundation upon which the studies herein were built. For a review of these methods, the reader should consult references [70–79] for a treatment of the analytical methods and references [80–87] for synthetic methods used to study KDN and KDN-containing glycoconjugates.

Future direction of KDNology

Expression of sialic acid residues on glycoconjugates is important in normal development and differentiation [88]. The temporal change in the levels of sialic acid at different stages during normal development and on different cell types is a highly regulated and dynamic process. Many sialoglycoconjugates are stage-specific antigens that can be re-expressed on cancer cell as oncofetal or oncodevelopmental antigens [89], of which α2,8-linked polyNeu5Ac epitope expressed on neural cell adhesion molecules is the one most extensively studied [90]. *N*-Glycolylneuraminic acid (Neu5Gc) that is not expressed in normal human cells has been reported to be expressed in gangliosides of colon cancer tissues [91], and in human tumor mucin [92]. Unusual sialyl structures have also been reported in some tumor cell lines, for examples α2,9-linked dimer of Neu5Ac in human ovarian teratocarcinoma cell PA-1, α2,8-linked polyNeu5Ac in MCF7 human breast cancer and RBL rat basophilic leukemia cells, and α2,9-linked polyNeu5Ac in murine neuroblastoma (clone NB41A3), but the functional significance of these glycotopes remains unsolved [93–95]. As an unusual type of sialic acid, KDN is a candidate sugar residue that occurs not only during normal development but whose aberrant expression may also underlie some metabolic disorders and malignant transformation. We have already described, for example, an increased level of free KDN in fetal cord red blood cells compared with maternal red blood cells, and the elevated expression of free KDN in ovarian tumors compared with normal ovarian tissues [29]. Elevated expression of free KDN was a feature of ovarian and breast cancers, yet it was not found in hepatic and colorectal tumors. More recently, we have shown the occurrence of KDN-glycoproteins in human ovarian teratocarcinoma cell PA-1, and have characterized them by biochemical and chemical methods. The mechanism of aberrant expression of KDN in human cells has been clarified partially and it appears to be closely related to the elevated intracellular level of mannose caused by enhanced supply of mannose. Further, our results have shown that enhanced levels of mannose also affect the metabolism of Neu5Ac, resulting in overexpression of membrane-bound sialoglycoproteins. Enhanced sialylation has been reported in some tumors [89], and we also observed that the levels of bound Neu5Ac in ovarian tumor tissues were >2-fold that of normal ovarian tissue [22]. When the biosynthetic pathway of Neu5Ac is compared with that of KDN, one of the key precursors for KDN, Man-6-P is replaced by ManNAc-6-P for Neu5Ac (see Scheme 1). A relevant pathway known for the formation of ManNAc-6-P in animal cells involves reactions catalyzed by a bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase. This enzyme is allosterically inhibited in a feedback mechanism by CMP-Neu5Ac [55]. However, if ManNAc is supplied by other means in which UDP-GlcNAc 2-epimerase is not involved, the feedback inhibition does not operate, resulting in the overexpression of Neu5Ac. An analogous pathway for UDP-Glc to Man-6-P has not been reported. Studying the expression of KDN in tumor cells is thus closely related to studying the mechanism and significance of elevated expression of Neu5Ac in some cancer. In contrast to Neu5Ac that occurs abundantly in both normal and tumor cells and tissues, the occurrence of KDN is rare and almost undetectable in normal adult human tissues. Thus, it is possible that KDN may serve as a sensitive and reliable marker for the presence of some human tumors. The study of specific KDN-containing glycoprotein(s) that appear in some type of human cancers may be a clue to solve the mechanism of tumorigenesis in some type of the cell.

As described in the introduction, an outstanding feature of KDN is in its biased mode of distribution in nature; KDN most abundantly occurs in ovarian secretions and skin mucus of lower vertebrates. KDN is also found in capsular polysaccharides of pathogenic bacteria. It is noted that in all of these cases, KDN was found in glycoconjugates that occurred outside of cells. If we compare the biosynthetic pathway of KDN with that of Neu5Ac, the supply of the precursor monosaccharide Man (for KDN) appears to be easier than that of ManNAc (for Neu5Ac) (see Scheme 1). However, as described above, no feedback mechanism is known in the biosynthetic pathway leading to KDN synthesis. This means that there is no known metabolic mechanism for regulating KDN synthesis. For the biosynthesis of KDN-glycoproteins that accumulate extracellularly, such as ovarian secretions and skin mucus, abundant supply of the precursor sugar may be preferential and the biosynthesis of KDN-glycoproteins that uses Man instead of ManNAc may have become through evolution the predominant pathway. On the other hand, for the biosynthesis of Neu5Ac-glycoproteins that play important roles in cell-cell communications, a strictly regulated

process could be most important to avoid overexpression, and thus Neu5Ac may be a preferable sialic acid component of cells and tissues. If this reasoning has validity, then, why does selection between KDN and Neu5Gc occur in ovarian secretions of fish and amphibian within close species? Similar selection is also found between KDN and KDO in cell envelope polysaccharide of phytopathogenic bacteria. These phenomena may be related to adaptation to environment and/or necessary selection for symbiosis. An approach for solving the problem may be to see how deaminated neuraminic acid (KDN) is different from *N*-acylneuraminic acids in molecular interaction and recognition.

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