Identification of Free Deaminated Sialic Acid (2-Keto-3-deoxy-D-glycero-b-galacto-nononic Acid) in Human Red Blood Cells and Its Elevated Expression in Fetal Cord Red Blood Cells and Ovarian Cancer Cells*

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Chemical studies have shown the occurrence of the deaminated sialic acid 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) in paired samples of blood obtained from mothers and newborns of healthy human individuals. Most of the KDN was found in red blood cells, although low levels were detected in mononuclear cells. No N-glycolylyeuranaminic acid was detected. Unexpectedly, nearly all of the KDN in fetal cord and matched maternal red blood cells was present as the free sugar and comparatively little occurred conjugated or as sialic acid 5'-KDN phosphate. The amount of free KDN in fetal newborn 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 ovariens, in ovarian tumors, 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. A positive correlation was found between an increase in the ratio of free KDN/N-acetylyneuraminic acid in ovarian adenocarcinomas and the stage of malignancy. This was particularly evident in tumor cells isolated from the ascites fluid. The central importance of these new findings is 2-fold. First, they show that free KDN is a minor but ubiquitous sialic acid in human red blood cells and that its elevated expression in red blood cells from fetal cord blood compared with maternal red blood cells may be developmentally related to blood cell formation during embryogenesis. Second, the enhanced expression of KDN in ovarian cancer cells suggests that this sialic acid, like the α2,8-linked polysialic acid glycophosphate, can be an oncofetal antigen in these tumors and thus could be an “early warning” signal for onset of disease and/or a marker for detection of recurrence of disease. These new findings highlight the importance of elucidating the role that KDN and KDN-containing glycoconjugates may play in normal development and malignancy.

Expression of sialic acid (Sia) (1) residues on glycoconjugates is of critical importance in normal development and differentiation because these sugar molecules appear to mediate a variety of specific biological functions (1, 2). N-Acetylyneuraminic acid (Neu5Ac) is the most common occurring Sia, and more than 40 naturally occurring derivatives have been described. The temporal appearance and disappearance of Sia at different stages during normal development and on different cell types is a highly regulated and dynamic process (3). It is also believed that Sia plays a central role in malignant transformation, because changes in the levels, type, or linkage of Sia in tumor cell glycoconjugates can affect the malignant potential of tumors (4). For example, some lymphoid tumors expressing N-glycolylyeuranaminic acid (Neu5Gc) are highly metastatic (4), and many sialylated glycoconjugates are stage-specific antigens that can be re-expressed on cancer cell as “oncofetal” or “oncodevelopmental” antigens (3). The α2,8-linked polysialylate Sia glycophosphate is one such oncodevelopmental, tumor-associated antigen. The cell surface expression of polysialylated neural cell adhesion molecules is positively correlated with increased malignancy in a number of human tumors, including Wilms tumor (5), human neuroblastomas, some of which are metastatic to bone and brain (6, 7), malignant lymphoma (8, 9), acute myeloid leukemia (10), and in head and neck malignancies (11). The importance of the polysialylate glycophosphate in normal and malignantly transformed human tissues was recently reviewed (12). The general involvement of carbohydrate chains in metastasis and prognosis of human carcinomas and the roles of tumor associated carbohydrate antigens in cell adhesion during tumor metastasis have also been recently summarized (13, 14).

In 1986, we discovered the occurrence of deaminated neuraminic acid (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; KDN; Structure 1) in fish eggs as a new class of Sia (15). Subsequent studies showed that KDN was expressed on cells ranging in evolutionary diversity from microbes to lower vertebrate species, in some cases as 9-O-acetyl-KDN (16), and recently in mammalian cells and tissues (Ref. 17 and references therein).

We now provide unambiguous evidence for the occurrence of KDN in normal human red blood cells and in normal and normal...
malignant ovarian tissues. An increased expression of free KDN in fetal cord red blood cells from newborns, compared with maternal red blood cells, and its elevation in ovarian tumors compared with normal ovarian tissues, are central new findings that highlight the importance of elucidating the role that KDN and KDN-containing glycoconjugates may play in normal development and malignancy.

**EXPERIMENTAL PROCEDURES**

*Collection of Human Blood Samples—*1 ml each of paired maternal and fetal cord blood was collected from full term parturients at the Tri-Service General Hospital, Taiwan in anticoagulant coated-tubes that had been treated with EDTA. All patients consented to the blood test. Maternal blood was collected at the beginning of labor and fetal cord blood was collected immediately after delivery before placenta separation. Plasma and red blood cells were separated by centrifugation at 12,000 rpm for 10 min. Adult blood (10 ml each) was collected from healthy female donors (age ranged 18–37) in heparinized, anticoagulant tubes.

*Preparation of Red Blood Cell Lysates and Ghost (Membrane) Fractions—*All procedures were carried out at 4 °C. Red blood cells were collected from whole blood by centrifugation at 2,500 rpm for 15 min and washed three times with 3 volumes of 0.85% NaCl, 5 mM Tris-HCl (pH 7.4). Washed red blood cells were lysed with 9 volumes of 10 mM Tris-HCl (pH 7.4) and centrifuged at 25,000 × g for 30 min. The supernatant was removed, and the pelletized ghosts were washed with 10 mM Tris-HCl (pH 7.4) until free of hemoglobin.

*Collection of Human Tissues and Cells—*All tissues and body fluids were obtained from biopsy or surgical dissection and stored at −80 °C until use. Approximately 5–50 mg of tissue or cells were used for each analysis. Asistles were collected by centrifugation of ascites at 12,000 rpm and washed with phosphate-buffered saline (pH 7.4).

*Quantitative Analysis of Sia, CMP-Sia, and Protein in Ethanol-soluble Fraction from Red Blood Cell Lysates—*Free Sia and CMP-Sia were separated from soluble glycoproteins in the red blood cell lysates by ethanol precipitation. To 2.0 ml of the lysate, corresponding to 0.2 ml of red blood cells, 8.0 ml of cold absolute ethanol were added. The mixture was kept on ice for about 1 h before centrifugation at 3,000 rpm for 15 min. This allowed separation of free Sia and CMP-Sia from the soluble glycoproteins that were precipitated by ethanol (18). The precipitate was washed three times with cold 80% ethanol, and the supernatant fractions were combined. The ethanol-soluble fraction was subjected to centrifugation and washed three times with cold 80% ethanol. The combined supernatant fractions were dried under vacuum at 30 °C, and the total amount of Sia was quantitatively determined as their DMB derivatives by HPLC analysis, as described below. The total amount of Sia in the 20P and 100P fractions were also quantitatively determined after mild acid hydrolysis, as described below.

*Sialic Acid and Protein Analysis—*The amount of Sia was quantitatively determined as their DMB derivatives by HPLC, as described previously (17, 19). For the analysis of bound Sia, samples were first hydrolyzed in 0.1 M HCl for 1.0 h at 80 °C. The HCl was removed by evaporation. In some samples, the hydrolysate was treated with a Bio-Rad AG-1 column before derivatization. The DMB derivatives were resolved on TSK-gel ODS-120T (Toosoh, Tokyo, Japan) or Micorsorb C18 (Rainin, Woburn, MA) columns on a Jasco series 900 or Hewlett-Packard series 1100 HPLC systems. The fluorescent detectors were set at 373 nm for excitation and 448 nm for emission. The columns (inner diameter, 250 × 4.6 cm) were eluted isocratically at room temperature with a mixture of methanol/acetonitrile/water (7:5:8). The amount of protein was estimated with the bicinchoninic acid kit (Pierce) using bovine serum albumin as a standard.

**RESULTS**

*Quantitative Determination of the Sialic Acid Levels in Fetal Cord and Maternal Red Blood Cells—*The amount of total KDN and Neu5Ac in red blood cells isolated from 14 matched pairs of fetal cord and maternal blood samples was quantitatively determined after mild acid hydrolysis by a highly sensitive fluorescence-assisted HPLC method (17, 19). As shown in Fig. 1, the mean values of total KDN in fetal cord red blood cells was approximately 2.4-fold higher than the levels in matched maternal red blood cells (1.4 ± 0.67 μg/ml versus 0.59 ± 0.23 μg/ml). Further, the relative KDN levels were determined to be on average 2.2 and 0.94 mol % of the Neu5Ac levels in fetal cord and maternal red blood cells, respectively. Thus, these results show a statistically significant higher level of KDN in umbilical cord red blood cells from neonates compared with their matched maternal red blood cells. This difference appears to be even more significant when compared with the small difference found between the levels of Neu5Ac in cord and maternal red blood cells, determined to be 69 ± 21 and 74 ± 20 μg/ml, respectively. It is interesting to note that the relative amount of KDN in human red blood cells is 3.6-fold higher than that in adult rat red blood cells (0.94 mol % versus 0.26 mol % of the total Sia).

*Elevated Levels of KDN in Fetal Cord and Maternal Blood Is Localized in the Red Blood Cells—*To determine whether the high level of KDN expression in cord and maternal red blood cells was restricted to these cells or was possible due to contamination of this fraction with other serum components, we analyzed both plasma and red blood cells fractions that were separated from cord and maternal blood by centrifugation. The results clearly showed that a major proportion of KDN was associated with both the cord and adult red blood cells (95 and 86%, respectively; Table 1). Essentially the same results were obtained when cord and maternal blood were separated by
An aliquot of each red blood cell sample was hydrolyzed in 200 μl of 0.1 M HCl for 1 h at 80 °C. The hydrolysate was passed through a small column of Bio-Rad AG-1 × 8 (formate form), and the sialic acid mixture that was eluted with 0.7 M formic acid was subjected to HPLC analysis after derivatization with DMB reagent as described (17, 19). RBC, red blood cells.

### Table 1
Quantitative determination of the levels of KDN and Neu5Ac in the plasma, cytosol, and membrane fractions of fetal cord and adult red blood cells.

| Sample | KDN (ng/ml) | Neu5Ac (μg/ml) |
|--------|-------------|---------------|
| Plasma | 34 ± 13     | 97 ± 16       |
| RBCs   | 610 ± 470   | 43 ± 16       |
| RBC supernatant | 740 ± 530 | 4.0 ± 2.2 |
| RBC membrane | 16 ± 4     | 27 ± 1.7     |

### FIG. 1. Differential determination of KDN (a) and Neu5Ac (b) in 14 matched pairs of fetal cord and maternal red blood cells. A 20-μl aliquot of each red blood cell sample was hydrolyzed in 200 μl of 0.1 M HCl for 1 h at 80 °C. The hydrolysate was passed through a small column of Bio-Rad AG-1 × 8 (formate form), and the sialic acid mixture that was eluted with 0.7 M formic acid was subjected to HPLC analysis after derivatization with DMB reagent as described (17, 19). RBC, red blood cells.

### Table I
Characterization of the chemical form of KDN and Neu5Ac present in the cytosol of fetal cord and maternal red blood cells.

- Determined by HPLC analysis of its DMB derivative in two broad peaks, which preceded the peak of free Sia.
- All KDN and Neu5Ac were eluted exclusively from the Q-Sepharose column equilibrated with 0.02 M Tris-HCl (pH 9.0) in the cold.
- The material that bound to the column was eluted with a linear gradient of NaCl.
- The eluted fractions were monitored for the presence of KDN and Neu5Ac by HPLC.

| Sample          | KDN (ng/ml) | Neu5Ac (μg/ml) |
|-----------------|-------------|---------------|
| Cord (n = 7)    |             |               |
| KDN             | 34 ± 13     | 97 ± 16       |
| Neu5Ac          | 22 ± 13     | 114 ± 5.0    |
| Adult (n = 6)   |             |               |
| KDN             | 610 ± 470   | 43 ± 16       |
| Neu5Ac          | 140 ± 80    | 54 ± 5.9     |
| RBC supernatant | 740 ± 530   | 4.0 ± 2.2     |
| RBC membrane    | 16 ± 4      | 27 ± 1.7     |

density gradient centrifugation in Ficoll-Paque (Amersham Pharmacia Biotech). Although a small proportion of KDN was associated with the mononuclear cell fraction, only negligible amounts were found in plasma, which may have been due to red blood cell lysis during fractionation. In this experiment, plasma contained about 80% of the total blood Neu5Ac. We also sought to determine the distribution of KDN and Neu5Ac in the cytosolic and membrane fractions isolated from fetal cord and adult red blood cell lysates. The results from these studies showed that about 98% of the KDN was in the cytosolic fraction in both cord and adult red blood cells. In marked contrast, about 90% of the Neu5Ac was found in the membrane fraction (Table I). On the basis of these results we conclude that KDN, unlike Neu5Ac, is uniquely localized to the cytosolic fraction in both fetal cord and adult red blood cells.

Nature of KDN and Neu5Ac Found in the Cytosol of Fetal Cord and Adult Red Blood Cells—To determine whether the high levels of KDN present in the supernatant fraction of red blood cells lysates was free or bound to soluble glycoproteins, ethanol and phenol were used separately to precipitate any soluble proteins. Essentially all of the KDN in the supernatant fractions derived from adult or cord red blood cell lysates was recovered in the ethanol-soluble fraction and the water-soluble fraction after phenol treatment, suggesting that KDN either existed as the free sugar, as CMP-KDN or associated with low molecular weight components. Unlike KDN, less than 10% of the total Neu5Ac found in red blood cells appeared in the ethanol-soluble fraction, and this amount was similar for cord and adult red blood cells.

Characterization of the Chemical Form of KDN and Neu5Ac Present in the Cytosol of Fetal Cord and Maternal Red Blood Cells—To determine the chemical nature of KDN and Neu5Ac that was present in the cytosol of cord and adult red blood cell lysates, the ethanol-soluble fractions were applied separately to Q-Sepharose columns equilibrated with 0.02 M Tris-HCl (pH 9.0) in the cold. The material that bound to the column was eluted with a linear gradient of NaCl. The eluted fractions were monitored for the presence of KDN and Neu5Ac by HPLC. All of the KDN eluted in the column flow-through fraction from both fetal and adult red blood cells, whereas 63% of the Neu5Ac in both fetal and adult red blood cells bound to the gel and was eluted with 0.1–0.25 M NaCl. The flow-through fraction was then applied to a Bio-Gel P-2 column and eluted with 0.05 M NaCl. The chemical nature of the eluted (free) Sia was shown to be exclusively free Neu5Ac (accounted for 37% of the total) and KDN as determined by HPLC analysis of its DMB derivative (Fig. 2). As shown in Fig. 2, KDN and Neu5Ac were eluted under discrete peaks that coincided with the peaks of authentic KDN and Neu5Ac, respectively. This separation thus provided an unambiguous method to quantitatively determine the amount and chemical form of each Sia derivative in the cytosol of fetal and adult red blood cells.

The Neu5Ac-containing fraction from maternal red blood cells that bound to Q-Sepharose and contained about two-thirds of the Neu5Ac was subsequently eluted from the Bio-Gel P-2 column in two broad peaks, which preceded the peak of free Neu5Ac. This elution profile would be that expected for Neu5Ac-containing small glycoproteins soluble in 80% ethanol. The nature of these Sia-containing structures was not further studied. No CMP-KDN or CMP-Neu5Ac was detected in the ethanol-soluble fraction from either fetal or adult red blood
cells, even when using sensitive and specific HPLC methods and separation methods that avoided hydrolysis of labile sugar nucleotide linkages (20, 21). The quantitative levels of free and bound KDN and Neu5Ac in cord and maternal red blood cells, determined after chromatographic separation of their DMB derivatives (Fig. 2), are summarized in Table II. These findings provide unambiguous evidence for the occurrence of free KDN in normal human red blood cells and its elevated expression in matched fetal cord red blood cells. On the basis of these new findings, we sought to determine whether free KDN was expressed in other normal human cells, e.g., ovarian tissues, and, if so, if this expression differed in ovarian cancer.

Identification and Quantitative Determination of KDN and Neu5Ac Levels in Normal Human and Malignant Ovarian Cancer Cells—A qualitative comparison of the amount of KDN and Neu5Ac in normal human ovaries, ovarian tumors, and in ascites cells isolated from ovarian cancer patients is shown in Table III. Based on these initial studies, our findings support the following important conclusions: (a) Normal human ovarian tissue expressed KDN in amounts comparable with the amount found in adult human red blood cells. (b) The level of total KDN expressed was elevated 2.6-fold in ascites cells obtained from ovarian cancer patients, while showing a 55% increase in the ovarian tumor cells. These ascites cells showed a 5-fold higher KDN/Neu5Ac ratio than normal ovarian cells (1.8 versus 0.36 mol/100 mol). However, the values of the total KDN/Neu5Ac ratio for normal and ovarian tumor cells were similar (0.36 versus 0.41 mol/100 mol), perhaps because the Neu5Ac levels are reported to be elevated in some ovarian tumors (22). (c) Like human red blood cells, the major proportion of KDN was found as the free sugar in both normal and ovarian tumor cells. Based on the mean values shown in Table III, 80 and 89% of the total KDN from normal and ovarian or ascites tumor cells, respectively, was found in the ethanol-soluble fraction. In contrast, the Neu5Ac levels in the ethanol-soluble fraction accounted for only 21, 6, and 10% of the total Sia in normal and ovarian tumor and ascites cells, respectively. Thus, this reciprocal difference in the percentage of distribution of KDN and Neu5Ac in the ethanol-soluble fraction between normal and ovarian tumor cells, which led to a 4.2-fold increase in the KDN/Neu5Ac ratio in ovarian tumor cells and a 77-fold increase in ascites tumor cells, is a distinguishing feature of KDN expression in ovarian cancer cells, even though the total mol % ratio of KDN/Neu5Ac showed a smaller, yet statistically significant change as noted above.

We determined the KDN/Neu5Ac ratio in the ethanol-soluble fraction prepared from different types and stages of ovarian tumors to test the hypothesis that this ratio might correlate with the type and stage of disease. As shown in Table IV, significantly higher KDN/Neu5Ac ratios occurred in all of the ovarian tumors and in the corresponding tumor cells isolated from the ascites fluid of each patient, compared with control normal ovarian tissue. Based on these initial findings, two observations are of particular relevance. First, of the ovarian tissues examined, a mature ovarian teratoma showed a 12-fold increase in the KDN/Neu5Ac ratio. Second, the most dramatic increase overall (22-fold) in this ratio was in ascites cells isolated from a patient with a stage IIIa adenocarcinoma (serous ovarian cyst). A patient with the same type of pathological tumor, but at stage I, showed only a 6.7-fold increase in the KDN/Neu5Ac ratio. These results suggest that a positive correlation exists between the level of free KDN within these ovarian adenocarcinomas and the extent of invasion or stage of malignancy. Although the number of patients in this initial study was too small to draw definitive conclusion regarding the
usefulness of the comparative value of free KDN as a potential diagnostic and/or prognostic marker for assessing the malignant state, we are encouraged that the high KDN/Neu5Ac ratios observed in ovarian tumors and in tumor cells recovered from the ascites fluid of these patients may represent a clinical and biochemical indicator of cell anomaly.

The present method that we have developed for identification and quantitative determination of three different types of Sia (KDN, Neu5Ac, and Neu5Gc) has now provided a facile, sensitive, and specific way to study the level and type of these Sia residues in normal and malignant cells. The key to our instrumental analysis (Fig. 3a) is an HPLC system equipped with a fluorescent detector, as described under “Experimental Procedures.” This method is capable of detecting as little as 1 pmol of KDN and can readily identify and quantitatively determine the amounts of KDN in normal ovarian tissue (Fig. 3b), ovarian tumor tissue (Fig. 3c), and ascites cells collected from the same patient (Fig. 3d). The data in Fig. 3 (b–d) also show that no Neu5Gc was detected in any of the ovarian tumor examined thus far, although this Sia was previously reported to be expressed in other types of human cancers and established tumor cell lines (23, 24).

DISCUSSION

The central importance of our new findings is 2-fold. First, elevated levels of free KDN in fetal cord red blood cells compared with matched maternal red blood cells is an intriguing observation that may be developmentally related to blood cell formation and the multi- or pluripotential stem cell formation during embryogenesis. To challenge this hypothesis will require a systematic study of KDN levels in different committed stem cells and other differentiated, end stage cells represented by red blood cells, including platelets, neutrophils, monocytes, basophils, B and T cells, and NK cells. It may also be informative to determine the free KDN/Neu5Ac ratios in different hematopoietic malignancies and in the myriad of hematological dyscrasias. Second, the enhanced level of free KDN in ovarian cancer cells compared with normal ovarian tissue leads us to hypothesize that this level could conceivably be used as an “early warning” signal or predictive marker for the detection of the potentially malignant phenotype in ovarian and perhaps other human cancers. This elevation was particularly striking in ascites cells from a patient with a stage III adenocarcinoma compared with a stage I tumor of the same type. Such a marker may also provide an adjunctive way to monitor for recurrence of tumor. It is therefore possible that free KDN levels, like surface expression of the α2,8-polySia glyctype, may be an oncofetal antigen for ovarian cancer, as polySia is for a number of human tumors (reviewed in Ref. 12).

These studies raise several important questions that remain to be answered. First, where does free KDN come from, i.e. how is it synthesized? Second, what regulates the intracellular level

| Pathological diagnosis | Tissue or cell examined | KDN/Neu5Ac mol/100 mol |
|------------------------|------------------------|------------------------|
| Ovarian mature teratoma (26 y) | Tumor ovary | 18 |
| Ovarian mucinous cyst adenocarcinoma (36 y) | Tumor ovary | 5.3 |
| Ovarian serous cyst adenocarcinoma, stage I (74 y) | Tumor ovary | 5.6 |
| Ovarian serous cyst adenocarcinoma, stage IIIa (25 y) | Tumor ovary | 5.1 |
| Normal (control) ovary | | 33 |

## Table IV

Quantitative comparison of the free KDN and Neu5Ac ratio in normal and ovarian tumor tissue and in ascites cells isolated from each patient

The level of free KDN and Neu5Ac was determined in the ethanol-soluble fraction isolated from the normal ovarian tissue and from the ovarian tumor and ascites of each patient, as described under “Experimental Procedures” and in the legend to Table III.

![Fig. 3. HPLC elution profiles of the sialic acids in the ethanol-soluble fractions prepared from normal and ovarian tumor patients.](image)
of KDN? And third, do increased intracellular levels of KDN regulate expression of any of the genes encoding for the enzymes leading to (a) KDN synthesis (KDN synthase); (b) activation (CMP-KDN synthetase); and/or (c) transfer (KDN transferase)? Answers to the latter two questions must await the cloning and detailed examination of the genes and enzymes responsible for KDN activation and transfer. However, our recent studies on the biosynthesis of KDN in trout testes, an organ rich in KDN (25, 26), have been instructive in addressing the origin of KDN (27, 28). These studies have shown that KDN is synthesized from mannose (Man) via the following three sequential reactions:

\[
\text{Man} + \text{ATP} \rightarrow \text{Man-6-P} + \text{ADP}
\]

\[
\text{Man-6-P} + \text{PEP} \rightarrow \text{KDN-9-P} + \text{Pi}
\]

\[
\text{KDN-9-P} \rightarrow \text{KDN} + \text{Pi}
\]

**Reactions 1–3**

Reaction 1, catalyzed by a hexokinase, is the 6-O-phosphorylation of Man to form Man-6-P. Reaction 2, catalyzed by KDN-9-P synthetase, condenses Man-6-P and P-enolpyruvate (PEP) to form KDN-9-P. Reaction 3, catalyzed by a phosphatase, is the dephosphorylation of KDN-9-P to yield free KDN. It is not known if a kinase specific for Man (Reaction 1) and a phosphatase specific for KDN-9-P (Reaction 3) may exist in tissues actively synthesizing KDN. It does appear, however, that the KDN-9-P synthetase (Reaction 2) is at least one enzyme that is uniquely specific in the KDN biosynthetic pathway. This conclusion is based on substrate competition experiments, which have shown that this enzyme is distinct from Neu5Ac 9-phosphate synthetase, the enzyme that catalyzes the condensation of N-acetyl-D-mannosamine-6-P and P-enolpyruvate to form Neu5Ac 9-phosphate, the immediate precursor to Neu5Ac (27, 28).

Man-6-P appears to be derived from the phosphorylation of Man (Reaction 1) and not from the conversion of d-fructose 6-phosphate to Man-6-P by phosphomannoisomerase. This conclusion is supported by our findings of free Man in trout ovary and testis, whereas the pool size of free Man-6-P was relatively small (27, 28). It was previously reported that the concentration of free Man in normal human serum is approximately 20–50 M (29). In the present study, the concentration of free Man in cord red blood cells was about 2 M, whereas in fetal cord red blood cells it was about 5 M. We have also shown that pig salivary glands actively involved in synthesis of Neu5Gc/Neu5Ac-containing glycoconjugates contain high levels of free KDN. Moreover, we have also recently obtained evidence that the levels of free and bound forms of KDN in cultured mammalian cell lines are increased when the cells are grown in the presence of extracellular Man, but not N-acetyl-D-mannosamine, the precursor of Neu5Ac. These findings taken together thus show a positive correlation between the intracellular levels of free Man and free and conjugated KDN and emphasize the importance of additional studies to elucidate the molecular mechanisms regulating KDN expression at both the gene and enzyme level.

Our present findings also indicate that the intracellular accumulation of free KDN does not appreciably increase the level of CMP-KDN, presumably because KDN is a poor substrate for CMP-Neu5Ac synthetase (30). Consequently, only a small proportion of the free KDN is activated to CMP-KDN, thus maintaining the relatively higher level of free KDN in these cells. Our results also suggest that the activity of UDP-GlcNAc 2-epimerase, a key enzyme regulating Neu5Ac synthesis, is not affected by the low, steady-state level of CMP-KDN, even though this sugar nucleotide may control its activity by feedback inhibition.

While the reason(s) for higher free KDN levels in fetal cord red blood cells and malignant human ovarian cancer cells remains unclear, further studies on the *de novo* synthesis, transport, storage, catabolism, and potential oncogenic nature of KDN in normal human cells and their malignant counterparts are required. Such studies should prove of critical importance to fully develop this aspect of the biochemistry, molecular biology, and glycopathology of KDN that has not been previously reported.

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\(^2\) S. Inoue, unpublished results.

\(^3\) T. Angata, unpublished results.