Sialic Acid as a Tumor Marker

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ABSTRACT

The term sialic acid is used to describe derivatives of neuraminic acid, where the amino group of neuraminic acid is substituted by either an acetyl or glycolyl group. The unique structural features of the molecule, which includes a negative charge owing to a carboxyl group, enables it to play a role in cellular functions, such as transport of positively charged compounds, cell-to-cell repulsion, influencing conformation of glycoproteins on cell membranes, and even masking antigenic determinants on receptor molecules. Focus on sialic acid as a tumor marker should be examined from the perspective of aberrant glycosylation in cancer cell membranes owing to activation of new glycosyl transferases that are characteristic of tumor cells, and the role played by sialic acid in tumor cell metastasis including increased capacity to adhere to vascular endothelium, and decreased capacity of cancer cells to be destroyed by host defence mechanisms. The high sensitivity of sialic acid as a tumor marker has been reported in a variety of cancerous conditions. Its specificity, however, is relatively low since there is also an increase in sialic acid-rich glycoproteins in inflammatory diseases. Sialic acid measurements, however, have value in monitoring cancer patients during treatment. A variety of methods are available for the measurement of both total and lipid associated sialic acids in serum or plasma. The newer high performance liquid chromatographic procedures can detect picogram levels of sialic acid and are relatively free of interferences seen with classical procedures.

Introduction

The term sialic acid is used to describe derivatives of neuraminic acid. There are more than 20 natural derivatives of neuraminic acid. In the structure of sialic acid depicted in figure 1, the amino group is substituted by an acetyl group, thus forming N-acetylneuraminic acid. Alternatively, the amino group could also be substituted by a glycolyl residue. The hydroxyl groups could either be methylated or esterified with acetyl, lactyl, phosphate, or sulfate groups. Sialic acids are present in high concentrations as components of glycoproteins, glycolipids such as gangliosides, or polysaccharides, and are usually located on the outer cell membrane linked to other sugars such as galactose or N-acetyl galactosamine by glycosidic bonds. While the usual location of sialic acids is at the terminal pos-
SIALIC ACID AS A TUMOR MARKER

**Figure 1.** Structure of sialic acid.

SIALIC ACID

\[
\begin{align*}
\text{COOH} \\
\text{C} = \text{O} \\
\text{H-C-H} \\
\text{H-C-OH} \\
\text{CH}_3\text{CNH-C-H} \\
\text{O} \\
\text{HO-C-H} \\
\text{H-C-OH} \\
\text{H-C-OH} \\
\text{CH}_2\text{OH}
\end{align*}
\]

The human red blood cell is studded with nearly 20 million molecules of sialic acid on the outer cell membrane which contributes to its electronegative charge (zeta potential), and by cell to cell repulsion prevents red blood cells from aggregating. Owing to its negative charge, sialic acid can bind positively charged molecules and thus play a role in the transport of such molecules. Since they are an essential component of cell-surface receptors for a wide range of endogenous (such as peptide hormones) and exogenous (bacteria and viruses) substances, their presence is both a blessing as well as a scourge. Thus, they have a role in the cellular actions of hormones, such as insulin, and also can modulate aminoacid transport in some cells. On the other hand, infection by bacteria or virus is solely dependent on the presence of sialic acid as a component of specific receptors for the microorganisms on the cell membrane.1

As antigenic determinants of glycoproteins or glycolipids, sialic acid molecules contribute to the specificity of blood group substances.

The negative charges on sialic acid have an influence on the conformation of glycoproteins in terms of their proper alignment in cell membranes, the expression of enzymatic activity of glycoprotein enzymes, and even resistance to proteolytic enzyme degradation. Indeed, the clustering of cell membrane glycoproteins owing partly to the repulsion of their oligosaccharide sialic acid residues, is important for cell rigidity, since the loss of sialic acid molecules can increase the deformability of cells.2

An intriguing role for sialic acid is in its ability to serve as biological masks by preventing ligands from recognizing receptors. Thus, a glycoprotein layer rich in sialic acid acts as an immune barrier between the mother and the fetus. Indeed, this masking effect is lost by the removal of terminal sialic acid residues from oligosaccharide chains since it leads to the exposure of a penultimate galactose residue which is then recognized and bound by naturally occurring antibodies, thus facilitating the removal of glycoprotein or the cell by the reticuloendothelial system.

**Relevance of Sialic Acid to Tumor**

The relevance of sialic acid to the tumor cell is apparent from the increased sialylation and sialyltransferase activity observed in many cancer cells.1 The aberrant glycosylation found in cancer cell membranes is presumably due to the activation of new glycosyl
transferases that are characteristic of tumor cells and are absent or present only in small quantities in normal cells. Thus, for instance, a relatively specific sialyl transferase is found to be present by as much as 2.5 to 11 times in greater amounts in transformed cells when compared to control cells.

Sialic Acid and Tumor Cell Metastasis

The ability of murine tumor cells to metastasize spontaneously from subcutaneous sites is apparently related to the total sialic acid content of cells in culture. Other factors that influence metastasis include the extent to which sialic acid molecules are exposed on the tumor cell surface and most pointedly to the extent of sialylation of galactosyl and N-acetyl galactosaminyl residues present on the cell surface oligosaccharide chains. Apparently, the tumor cells use its heavily sialylated surfaces as a mask to evade recognition by immune surveillance, and thus facilitate the metastatic spread. The increased amount of sialic acid on the tumor cell surface can, by increasing adhesiveness, contribute to the formation of larger tumor emboli. Metastatic spread is also facilitated by sialic acid molecules increasing the adherence of tumor cells to vascular endothelium at secondary sites of implantation and by increasing the ability to aggregate platelets.

Alteration of Carbohydrate Chains in Malignancy

During neoplastic transformation, the carbohydrate chains in glycolipids and glycoproteins are frequently altered. There is a close relationship between the expression of certain carbohydrate antigens and oncogenesis. In an elegant study examining the significance of the linkage of sialic acid residues in cancer-associated carbohydrate antigens, by using specific monoclonal antibodies, it was demonstrated that not all sialic acids are specific to cancer. Indeed, this study demonstrated that there are significant variations in the cancer specificity depending on the difference in linkage in sialic acid residues. Thus for pancreas, the 2–3 sialylation at the terminal galactose of the Lewis(a) (Le(a)) antigen appeared to be significantly increased in the cancer cell in contrast to the decrease in 2–6 sialylation at the penultimate N-acetylglucosamine during the course of development of tumor.

Indeed, the epitope of carbohydrate antigen CA19-9, well known as a tumor marker for pancreatic and gastric cancers, is sialylated lacto-N-fucopentaose II which is synonymous with the Lewis(a) or Le(a) antigen. Interestingly, an antigen that has a chemical structure isomeric to the Le(a) antigen is the sialylated Le(x) antigen, which is a tumor-associated marker for lung adenocarcinomas.

Entry of Sialic Acid in Circulation

Sialic acid bound to membrane glycoproteins and glycolipids apparently enters the circulation by either shedding or by cell lysis. Approximately 98 to 99.5 percent of total sialic acid found in serum or plasma is bound to glycoproteins. Only a very small fraction of sialic acid is bound to lipids which is mainly in the form of gangliosides.

Normal levels of total sialic acid in serum are approximately in the range of 51 to 84 mg/dl. In contrast, the contribution of the pure lipid fraction to the total sialic acid level is barely in the range of 0.4 to 0.9 mg/dl.

Methods for Determination of Sialic Acid

A variety of procedures have been used for the measurement of total sialic acid.
These can be broadly classified as colorimetric, fluorometric, enzymatic and highly sensitive high performance liquid chromatographic (HPLC) procedures.

**Colorimetric Procedures**

Two classical procedures have stood the test of time. One uses resorcinol and the other uses periodic and thiobarbituric acids. The resorcinol based assay uses heat and strong acid to hydrolyze glycosidic bonds. The released free sialic acids are reacted with resorcinol and copper ions to give a colored compound, which is extracted and measured at 580 nm. To overcome interference from sugars forming furfural and furfurol analogues, such as pentoses, and other interferents, such as glucuronic acid and 2-deoxyglucose which incidentally have an absorption maxima at 450 nm and a 2nd maxima at 580 nm, measurements are also taken at 450 nm. Sialic acid concentration is then calculated using simultaneous equations.\textsuperscript{9} It has been reported that the previous assay has a within-run coefficient of variation (CV) of approximately 8 percent.\textsuperscript{2}

The procedure described by Warren is typical of the periodic and thiobarbituric acid procedure, which measures only free sialic acid that is released after an initial hydrolysis step. In this procedure, formyl pyruvic acid formed as a result of periodic acid oxidation of free sialic acid is reacted with thiobarbituric acid to yield a red color which is measured at 549 nm. Interference owing to malonaldehyde, the oxidation product of 2-deoxyglucose, is corrected by taking measurements also at 532 nm.\textsuperscript{10} Hemolysis is reported to affect the sensitivity of the assay. The procedure is reported to have an intra-assay CV of approximately 3 percent.\textsuperscript{2}

The preliminary hydrolysis step required in the Warren Assay is eliminated in an assay where sialic acid is oxidized by periodic acid to form formaldehyde which is then reacted with MBTH (methyl-3-benzothiazolone-2-hydrazone) to give a color which is measured at 625 nm. This procedure has a sensitivity similar to the classical periodic and thiobarbituric acid procedures, and is subject to similar interferences.\textsuperscript{11}

**Fluorometric Procedures**

In a typical and more specific assay formaldehyde that is formed upon oxidation of free sialic acid by periodic acid is reacted with acetyl acetone. The yellow product is excited at 410 nm, and the resulting fluorescence is measured at 510 nm.\textsuperscript{12}

**Enzymatic Procedures**

Enzymatic assays are based on conversion of free sialic acids released by the enzyme neuraminidase to pyruvate and acetylmannosamine with the aid of the enzyme acetyl neuraminic acid pyruvate lyase or neuraminic acid (NANA) aldolase. The resulting pyruvate can be coupled to the lactate dehydrogenase NADH system to measure the oxidation of NADH to NAD at 340 nm. Alternatively, pyruvate can be coupled to pyruvate oxidase, flavine adenine dinucleotide (FAD), and thiamine pyrophosphate (TPP) to form hydrogen peroxide, which in turn is coupled to peroxidase in presence of 4-aminocoumarin and a toluidine derivative to form a red chromogen which is measured at 550 nm. In figure 2 are listed the reactions associated with the NANA-aldolase-pyruvate oxidase-peroxidase system,\textsuperscript{13} which requires only 20 μl of serum and has been adapted to a kit form. Since endogenous pyruvate can be a potential interferant, some investigators couple the alternative product N-acetylmannosamine formed by the action of the enzyme NANA-aldolase to suitable enzymes such as either N-acetyl-
mannotosamine dehydrogenase or acetyl glucosamine 2-epimerase-N-acetylhexosamine oxidase-peroxidase to measure either NADH at 340 nm or the chromogen resulting by coupling a quinone dye with peroxidase at 590 nm, respectively.\textsuperscript{14,15}

Enzymatic procedures although, not completely interference-free are precise, with some procedures achieving a within-run CV of approximately 1 percent, and are convenient especially when adapted to a kit form.\textsuperscript{16}

High Performance Liquid Chromatographic (HPLC) Procedures

The HPLC procedures provide the ultimate sensitivity. In one such procedure, sialic acid released from the sample by acid hydrolysis is converted to highly fluorescent derivatives by reacting with a fluorogenic agent for alpha-keto acids such as 1,2-diamino 4,5-methylene dioxybenzene in dilute sulfuric acid. The fluorescent derivatives are separated on an octadecyl (C18) bonded silica column using a reverse phase solvent system. The chromatographic step takes only 12 minutes allowing detection of levels as low as 25 femtomoles (f.mol) or 7.7 picograms (pg) of N-acetyl-neuraminic acid and 23 f.mol or 7.5 pg of N-glycolyl neuraminic acid, in an injection volume as small as 10 microliter. The procedure is capable of analyzing precisely sialic acids in a 5 µl of serum sample.\textsuperscript{17}

Procedures for Measurement of Lipid-bound Sialic Acid (LBSA)

The basis for the measurement of LBSA in serum is the increased levels of gangliosides seen in patients in various types of cancer. Apparently, these gangliosides are shed from the tumor cell surface; since they have a relatively long half-life compared to lipids lacking sialic acid, their accumulation in serum lends itself to its measurement.\textsuperscript{8} However, there is a difference in levels of LBSA depending on the procedure used for measurement. This is because some procedures measure predominantly glycoprotein-bound sialic acid, thus grossly overestimating the LBSA. The non-specificity of such procedures for the measurement of lipid associated sialic acid (LASA or LSA) is indicated by placing the abbreviation in quotation marks (“LASA” or “LSA”). Some procedures do measure lipid-bound or lipid associated sialic acid.\textsuperscript{18,19} Essentially, these procedures involve extraction of glycolipid-bound sialic acid with solvents such as chloroform-methanol-water or chloroform-methanol and the gangliosides are separated from other lipids either by phase partition or precipitated with a 0.1 percent solution of tri-potassium citrate. The isolated LASA fraction is measured by either the classical resorcinol or periodic-thiobarbituric acid methods.

The procedure that is widely used for
the measurement of LBSA or LASA or LSA, because of its rapidity, good precision, and low cost, actually measures glycoprotein-bound sialic acid predominantly. In this “LASA” or “LSA” procedure 45 to 50 μl of serum or plasma is mixed with 150 μl of cold distilled water, and extracted with 3 ml chloroform: methanol (2:1 vol/vol) at 4°C to 5°C. The aqueous phase containing the sialolipid fraction is precipitated with 1 percent phosphotungstic acid solution. After centrifugation and removal of the supernatant mixture, the precipitate suspended in distilled water is analyzed by the classical resorcinol-based method at 580 nm. The “LSA” analyzed by this method was subsequently reported to contain considerable amounts of glycoproteins containing sialic acid such as alpha-1 acid glycoprotein, antitrypsin, haptoglobin, anti-chymotrypsin and immunoglobulins.

Sialic Acid Measurements in Cancer

Both total and lipid-bound or “lipid-associated” sialic acid levels are increased in a variety of tumors. Representative studies are discussed.

### Lung Cancer

In a recent study on the usefulness of total and “lipid associated sialic acid” (“LASA”) in lung cancer, a total of 152 patients with primary lung cancer who had not undergone treatment and 107 patients with benign pulmonary disease were evaluated along side 207 normal subjects. Data obtained in the study are summarized in table I. From these data it is apparent that the mean concentration of total sialic acid (TSA) and “LASA” were significantly higher in lung cancer patients when compared to benign and normal controls. At the designated cut-off levels in serum for TSA and “LASA”, which were respectively 80 mg/dl and 20 mg/dl, the sensitivity or the percent of true positives for lung cancer was 86.5 percent for TSA and 77 percent for “LASA”. However, the specificity of TSA and “LASA” were low when compared using as negative control, the value obtained on benign pulmonary patients. (TSA and “LASA” had both a specificity of 44 percent).

### Head and Neck Cancer

In a very recent study 37 patients with carcinoma of hypopharynx and larynx

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<td>*<em>Total Sialic Acid and Lipid–associated Sialic Acid in Lung Cancer</em></td>
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Lipid-bound sialic acid (LBSA) levels were measured in these patients by a specific procedure. Ten healthy subjects and 12 non-cancer patients were also included as controls. The LBSA levels obtained on non-cancer and healthy subjects were similar and were clearly lower when compared to cancer patients. Pre-operative LBSA levels were significantly increased in 94.4 percent of cancer patients but dropped to somewhat lower levels within 1 month of tumor resection. The LBSA levels decreased steadily reaching normal levels within 6 to 24 months after surgery in patients with complete tumor resection and no recurrence of tumor within 2 years. However, in patients with recurrence of tumor or incomplete tumor resection LBSA levels became elevated again within 6 months of tumor resection. The true LBSA levels measured in this study perhaps reflect increased shedding or secretion of LBSA from tumor cell membrane which apparently contains much more lipid-bound sialic acid than the membrane of normal cells. Thus, this study demonstrates the usefulness of a true LBSA measurement in following patients with head and neck cancer.

Usefulness of TSA and “LASA” for Monitoring

In a typical study, four groups of cancer patients were evaluated. These groups included 69 patients with bladder cancer, 58 patients with lung cancer, 31 patients with cancer of the uterus, and 29 patients with breast cancer. In addition to TSA and “LASA” levels in sera, the carcinoembryonic antigen (CEA) levels were also measured. The sensitivity of the three assays prior to initiation of radiotherapy, in relation to established cut-off values given in parenthesis were: TSA 89.3 percent (80 mg/dl) “LASA” 88.8 percent (20 mg/dl), and CEA 26.8 percent (5 ng/ml). After completion of radiotherapy, the overall response to treatment as judged by percent of patients with a final serum level below the zero time value for each of these markers were: “LASA” 85.6 percent, TSA 81.3 percent, and CEA 65.8 percent. Thus, in this study the diagnostic sensitivity of “LASA” and TSA in terms of their ability to detect true positives was more than three times greater than CEA. For monitoring response to treatment, TSA and “LASA” were able to follow correctly 15 percent more patients with post treatment values below the zero time values when compared to CEA.

Sialic Acid Levels in Other Cancers

“LASA” levels have been reported to be useful in monitoring patients with malignant melanoma. In one study when tumor recurrence was correlated with elevated “LASA” levels, the increased level was found as early as 9.3 months (median value) prior to recurrence. Higher levels of TSA and “LASA” have been reported in leukemia patients compared to patients with anemia. The TSA levels were significantly higher in acute myeloid leukemia (AML) compared to chronic myeloid leukemia (CML) and acute lymphatic leukemia (ALL) patients. The “LASA” levels were significantly elevated in AML patients as compared to other leukemic patients. The sensitivity of sialic acid as a marker for leukemia is high with the sensitivity of “LASA” approaching 85 percent.

The TSA levels in patients with oral and maxillofacial malignancy were reported to be significantly higher in patients with Stage III and IV cancer, when compared to patients with Stage I and II cancer. During follow-up of response to treatment while TSA levels declined during remission of disease, they became elevated with recurrence and metastasis.
The utility of sialic acid measurements in colorectal cancer has been examined. In one study it was shown that although TSA and “LASA” measurements were not useful for detecting early-stage (Dukes A and B) colorectal cancer, TSA and TSA/Total protein ratio (mg/gm) were significantly elevated in each colorectal cancer subgroup compared to values obtained with normal subjects. In another study where 146 colorectal cancer patients were studied prior to surgical therapy, the TSA/total protein ratio (mg/gm) for colorectal cancer was 13.4 in contrast to 9.7 for normal controls and 12.1 for pathological controls. Thus, the TSA value normalized to total protein (TSA/TP) may have utility in detecting colorectal cancer and following patients on treatment.

Conclusion

Sialic acid measurements appear to have a high sensitivity for a wide range of tumors. However, the specificity of sialic acid measurements, especially the non-specific “LASA” measurements, is low since the latter is elevated in benign disorders particularly associated with inflammatory conditions. The non-specificity of the “LASA” measurement is not surprising since the test actually measures predominantly glycoprotein-bound sialic acid. Hence, in inflammatory conditions where the level of acute phase proteins such as alpha-1 acid glycoprotein are expect to be elevated, one can expect an apparent elevation of “LASA”.

Perhaps standardization and refinement of a truly specific lipid-bound sialic acid measurement could improve the diagnostic specificity of this measurement.

While some investigators have questioned the utility of TSA or “LASA” measurements in the early detection of cervical cancer or to even complement the clinical staging of tumor, the utility of sialic acid measurements for monitoring progress of therapy and the detection of recurrence in a variety of tumors has been amply demonstrated in literature.

References


