Serum Sialic Acid and Sialyltransferase as Monitors of Tumor Burden in Malignant Melanoma Patients

Hulbert K. B. Silver, Karim A. Karim, Elizabeth L. Archibald, and Fernando A. Salinas

ABSTRACT

This study examines two associated tumor markers in malignant melanoma, sialic acid and sialyltransferase (EC 2.4.99.1). Both markers were measured in the same sera from 66 malignant melanoma patients, 20 rheumatoid arthritis patients, and age- and sex-matched normal controls. Bound sialic acid was measured by the thiobarbituric acid method, and sialyltransferase activity was measured by cytidine 5'-monophosphate-N-[4-14C]acetylneuraminic acid incorporation in desialated fetuin. Serum values for both markers were higher among rheumatoid arthritis patients than among controls (p = < 0.0001). Melanoma patients were divided into three groups: Group I, 34 patients with no evidence of disease at the time of sampling; Group II, 13 patients with minimal tumor burden; and Group III, 19 patients with advanced metastatic disease. For sialic acid, there were significant differences between all group comparisons except the normals versus Group I. By contrast, for sialyltransferase only sera from Group III patients showed significant increased activity. Using the calculated upper limit of normal of 2.37 μmol/ml for sialic acid and 18.1 nmol/ml/hr for sialyltransferase, 2 (6%) Group I, 4 (31%) Group II, and 18 (95%) Group III patients had sialic acid elevations, while corresponding results for sialyltransferase were 0, 0, and 4 (42%). There was a direct relationship between sialic acid and sialyltransferase, especially for melanoma patients with established disease (p = 0.0002). Of 24 patients with objective evidence of changing tumor burden 19 (79%) showed corresponding alterations in sialic acid concentration, while 14 (58%) showed such changes in sialyltransferase. Sialic acid was directly related to recurrence rate in Group I patients, while sialyltransferase was not. When used as monitors of tumor burden either to detect recurrent disease or to follow response to treatment, tumor markers need not be tumor specific. However, most of the clinically validated markers are relatively tumor specific and thereby clinically restricted to a limited spectrum of tumor cell types. Tumor markers with relatively broad specificity need to be identified and evaluated for use in the many other forms of human cancer. Sialic acid (N-acetylneuraminic acid) is one such marker.

The rationale for evaluating sialic acid as a possible tumor marker arose from experimental findings that an increased density of this carbohydrate is frequently found at the malignant cell surface in both animal models and humans (18, 31). In tissue culture experiments, tumor-related sialylglycoproteins are readily detected free in spent medium and might therefore be detectable in the serum of cancer patients as well (1, 12). Similar observations have been made for sialyltransferase, the enzyme responsible for the linking of sialic acid to polysaccharide chains (2, 35). Although there are remarkably few detailed studies, serum elevations of either sialic acid or sialyltransferase activity have been independently reported in patients harboring a variety of neoplasms (2, 4, 6, 10, 11, 19, 21, 22). In addition to more detailed investigation, the relationship of these 2 markers in the same sera would be of interest.

The objectives of this study were to extend our previous findings on sialic acid in a different and larger group of malignant melanoma patients, to evaluate the use of sialyltransferase activity as a marker for tumor burden in malignant melanoma, and to investigate the relationship between sialic acid and sialyltransferase in the same individual patients’ sera.

MATERIALS AND METHODS

Serum Collection. Whole blood was collected in 10-ml glass vacutainer tubes, and the samples were allowed to clot at room temperature for 1 hr. After centrifugation at 500 × g for 10 min, the sera were removed, placed in polypropylene tubes, and stored at −70° until used. Prior to assay, the serum samples were allowed to thaw at room temperature.

Sialic Acid Assay. Sialic acid determinations were carried out essentially as described by Warren (34) but with the following modification in the volume of reagents used. A mixture of 1 ml of test serum and 1.9 ml of 0.1 N H2SO4 was incubated for 1 hr at 80°. After cooling in a 20° water bath for 5 min, a 100-μl aliquot of this hydrolysate was mixed with 50 μl of 0.2 M sodium periodate in 9 M phosphoric acid, and the resulting oxidation was allowed to proceed at 20° for 20 min. The reaction was then terminated by adding 0.5 ml of a mixture of 0.8 M sodium arsenite, 0.5 M sodium sulfate, and 0.1 N H2SO4.

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3 The abbreviations used are: CEA, carcinoembryonic antigen; CMP-[4-14C]-NANA, cytidine 5'-monophosphate-N-[4-14C]acetylneuraminic acid; BCG, Bacillus Calmette-Guérin.

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Next, 1.5 ml of 0.04 M thiobarbituric acid in 0.5 M sodium sulfate were added to the reaction mixture, and the whole mixture was placed in a boiling water bath for 15 min. The sample was subsequently cooled in a 20°C water bath for 10 min, followed by extraction with 2.15 ml of cyclohexanone. The absorbance of the cyclohexanone layer was determined at 549 and 532 nm by a Beckman Model 25 spectrophotometer. The absorbance of a series of standard solutions of sialic acid and deoxyribose was also determined with each batch of patient samples in order to calculate extinction coefficients. Serum standards were also included with each assay as internal controls. The calculation of extinction coefficients and sialic acid concentration was aided by a computer program allowing for minor contamination with interfering substances (34). The intra assay variation of 10 analyses (±2 S.D.) was 1.9% and, the interassay variation of 20 analyses was 10%.

**Enzyme Assay.** Sialyltransferase activity was measured by a modification of the sialic acid incorporation method, as described by Bosmann (3). CMP-[14C]NANA (specific activity, either 1.47 or 1.68 Ci/mol) was obtained from New England Nuclear, Boston, Mass. Fetuin (Sigma Chemical Co., St. Louis, Mo.) was used as an acceptor after the terminal sialic acid residues were removed by mild acid hydrolysis in 0.1 N H2SO4 at 80°C for 1 hr. Release of sialic acid as monitored by the above-described method indicated a 98% removal of the sialic acid residues. After neutralization with 0.1 N NaOH, the mixture was thoroughly dialyzed against distilled water at 4°C, followed by freeze-drying. The lyophilized desialated fetuin was then dissolved to a standard concentration of 10 mg/ml in distilled water, aliquoted in 1-ml fractions, and stored at −70°C until used.

The complete reaction mixture for assay of sialyltransferase was 25 μl test serum, 50 μl desialated fetuin solution, 12.5 μl of 0.1 M MnCl2, 6.25 μl of 1 M N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (supplied by Sigma) buffer (pH 6.5), 25 μl of CMP [14C]NANA containing 50 nmol or approximately 70,000 dpm, and 6.25 μl of 2% Triton X-100 (New England Nuclear, Boston, Mass.). After 30 min of incubation at 37°C, the reaction was terminated by the addition of 1% phosphotungstic acid in 0.1 N HCl, washed twice with 0.6 M trichloroacetic acid, and once with ethanol:diethyl ether (2:1, v/v), and the resulting precipitate was dissolved in 300 μl of Protosol solubilizer (New England Nuclear). The radioactivity of this solution was then determined by liquid scintillation in a Beckman LS-3100 system. Results were expressed as nmol sialic acid transferred per ml serum in 1 hr. The optimum amount of CMP-[14C]NANA for each reaction mixture was determined in preliminary experiments and was found to be between 48 and 51 nmol. The intras assay variation of 10 analyses (±2 S.D.) was 3.6%, and the interassay variation of 20 analyses was 19%.

**Stability Testing.** The stability of sera for both sialic acid and sialyltransferase activity was examined to evaluate any special precautions needed for serum collection or storage. Sera were tested for loss of activity serially for up to 10 cycles of freeze-thaw, bimonthly for 18 months while stored at −70°C, weekly for 4 weeks at 4°C, daily for 7 days at 20°C, and after incubation at 56°C for 15 min.

**Patients.** Sixty-six malignant melanoma patients with evaluable tumor burden were studied. The criteria for eligibility included histological confirmation of diagnosis, completion of prestudy evaluation including serum liver function studies (lactic acid dehydrogenase, glutamic oxaloacetic transaminase, alkaline phosphatase, and bilirubin), complete blood count, and chest X-ray. Additional laboratory investigation including radionuclide scans, ultrasound examinations, or specific X-ray procedures were performed when clinically indicated. All patients had measurable disease and could be grouped by estimated tumor burden as previously described (29). Group I patients consisted of 34 patients having no evidence of disease at the time of serum sampling. All known disease had been removed either by wide excision for primary disease alone in 24 cases or by additional lymphadenectomy for regional metastatic disease in 10 cases. At the time of serum sampling, 23 of the 34 Group I patients were receiving adjuvant immunotherapy consisting of 40 mg BCG (Connaught Laboratories, Willowdale, Ontario, Canada) administered by scarification. Group II included 13 patients who had relatively small tumor burden consisting of either primary melanoma, local recurrence, or intransit metastases estimated at less than 5 g. In these patients, disease was confined to measurable skin lesions, a feature of malignant melanoma that particularly well suits it to this type of analysis. Group III patients all had relatively advanced regional or distant metastatic disease clearly greater than a tumor burden of 5 g. Of the 19 patients in this group, 5 had regional disease only consisting of regional lymph node involvement with or without additional intransit metastases. In 1 patient, systemic spread was clinically confined to skin. The remaining 13 patients demonstrated major organ involvement alone or in combination including metastases to lung in 5 cases, liver in 6 cases, brain in 4 cases, and bone in 3 cases.

Sex- and age-matched normal control sera were selected from our serum collection for comparison with each patient group (Table 1). As a control for nonmalignant illness, single serum samples from 12 active and 8 inactive rheumatoid arthritis patients were kindly supplied by Dr. D. K. Ford, The Arthritis Centre, University of British Columbia. Diagnosis was determined by accepted American Rheumatism Association criteria (24). Disease activity was recorded by the attending rheumatologist at the time of serum sampling, and this clinical assessment was based on signs and symptoms of inflammatory arthritis, elevation of erythrocyte sedimentation rate, and rheumatoid factor detection.

After 1 year of study, 24 melanoma patients had developed objective evidence of increasing or decreasing tumor burden. The 14 patients with increasing tumor burden included 5 patients progressing from no evidence of disease to development of measurable local recurrence in 1 case, regional lymph node involvement in 2 cases, and major internal organ involvement in 2 cases. Advancing disease in an additional 9 patients with

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<th>Age and sex of malignant melanoma patients, rheumatoid arthritis patients, and normal controls</th>
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<td>Patients</td>
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<td>Normal control</td>
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established disease was characterized by an increase greater than 50% in the product of perpendicular diameters of measured lesions and/or the appearance of new lesions in the absence of evidence of response in any additional areas. The 10 patients with objective evidence of decreasing tumor burden included surgical excision of all known disease in 6 cases. In the remaining 4 patients, there was a greater than 50% reduction in the product of perpendicular diameters of measured lesions; 2 of these had been treated by intrallesional BCG immunotherapy, and 2 had been treated with dimethyl triazeno imidizole carboxamide. For patients undergoing chemotherapy treatment, serum samples were obtained at least 3 weeks following a treatment.

RESULTS

Stability tests determined that with routine laboratory precautions there would be no loss of either sialic acid or sialyltransferase activity during handling, storage, or assay. Although sialic acid levels were unaffected by any of the stability tests, enzyme activity did diminish with some manipulations. Repeated freezing and thawing for 10 times did not affect sialyltransferase activity, nor did storage at −70° for 18 months. However, after 4 weeks at 4°, there was a 45% reduction in enzyme activity; after 4 days at 20°, there was a 44% loss of activity; and all activity was lost after incubation at 56° for 15 min.

Compared with an equal number of age- and sex-matched normal sera (Chart 1), the rheumatoid arthritis sera had significantly higher sialic acid concentration (p < 0.0001, Mann-Whitney test) and higher sialyltransferase activity (p < 0.0001). There was a clear tendency for both markers to be more elevated in patients with active arthritis.

The relationship of sialic acid concentrations and sialyltransferase activity to tumor burden in malignant melanoma patients' sera is illustrated in Chart 2. Serum sialic acid levels were higher among melanoma patients than among the normal persons studied, and the levels tended to increase with increasing tumor burden. The same sera examined for sialyltransferase activity showed little difference between normal control sera and melanoma Groups I and II. Group III patients with relatively high tumor burden did tend to have higher serum sialyltransferase activity.

For sialic acid, there were significant differences between all group comparisons except the normal controls versus Group I, patients with no apparent residual cancer (Table 2). By contrast, for sialyltransferase only sera from Group III patients with advanced disease showed a statistically significant increase in sialyltransferase activity compared to other groups. In fact, Group I sera had less enzyme activity than did normal sera.

Results were also analyzed to determine how many values were outside the normal range. The upper limit of normal in this study (2 S.D. greater than mean of normal control sera) was 2.37 µmol/ml for sialic acid and 18.1 nmol/ml/hr for sialyltransferase (Table 2). Using these criteria, serum sialic acid elevations were seen in Groups I and II and in 18 of 19 patients in Group III. However, elevations of sialyltransferase
activity occurred in less than one-half of Group III patients and not at all in Groups I and II.

Linear regression analysis did demonstrate a significant relationship between sialic acid and sialyltransferase for patients with established malignant melanoma (Groups II and III) or rheumatoid arthritis (Charts 3 and 4). This relationship was not as significant for normal controls or melanoma patients with no evidence of neoplasm (Chart 5).

Results of serial sample testing in patients with objective change in tumor burden are shown in Charts 6 and 7. An increase in sialic acid with increasing tumor burden was seen in 13 of 14 patients. The result in 2 of the 13 patients was not significantly outside the interassay variation ($\sqrt{2} \times $ interassay variation) (23). Analysis of the same sera for sialyltransferase activity demonstrated increases in 12 of 14 patients, in 1 of the 12 the increase was not significant. All 10 sera from patients with decreasing tumor burden showed an associated decrease in serum sialic acid; 2 of these were not significant. The same sera showed decreases in sialyltransferase activity in 9 of the 10 patients. However, only 3 patients demonstrated significant decreases.

All patients have now been followed for at least 60 weeks since initial serum samples were obtained. During this interval, 9 patients developed recurrent malignant melanoma between 14 and 119 weeks following serum sampling (mean, 46 weeks). Recurrence was more frequent in patients with greater than median serum sialic acid values (7 of 17, 41%) compared to those with less than median concentrations (2 of 17, 12%). The 2 patients with sialic acid levels outside the normal range for this study (Table 2) are among those with recurrent disease. For sialyltransferase, the recurrence frequency for those with greater than median enzyme activity (5 of 17, 29%) was not remarkably different from those with less than the median
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of plasma glycoproteins in cancer, MacBeth and Bekesi (19) noted elevated sialic acid concentrations in serum from breast carcinoma patients. While no direct assessment was made of tumor burden and no statistical comparison was made, sialic acid concentrations appeared to be higher among patients with distant metastatic disease. Brozmanova and Skrovina (6), looking more directly at total serum sialic acid, reported statistically significant elevations among sarcoma patients compared with normals or patients with benign neoplasms. More recent investigations evolving from the study of glycoproteins at the malignant cell surface have looked more closely at the relationship of a variety of carbohydrates, their respective transferase enzymes, and malignant disease. We have reported a relationship between tumor burden and serum sialic acid concentration in malignant melanoma (29). In a serial sample study, Mrochek et al. (22) found a correlation between tumor burden and several protein-bound carbohydrates including sialic acid. Increased sialyltransferase activity has been reported in association with a variety of neoplasms; however, there has not been a more detailed analysis of site correlating tumor burden to enzyme activity (10, 11, 14, 21).

Unlike tumor antigen markers associated with a limited spectrum of tumors, increased sialyltransferase activity and associated sialylglycoprotein production appear to be a common feature of a variety of neoplastic cells (15, 18, 29, 31). As a result, relatively nonspecific markers such as sialic acid and sialyltransferase may have broad clinical application. The increases of both markers among the 20 rheumatoid arthritis sera suggest that at least part of the elevation in cancer patients’ sera may be explained by the elaboration of nonspe-

activity (4 of 17, 23%). Logistic regression analysis, as described by Cox (9), was used to examine the relationship of tumor marker values to time of recurrence. For sialic acid, there was a remarkable correlation ($\chi^2 = 6.695; p = 0.0001$); for sialyltransferase, there was not ($\chi^2 = 0.313; p = 0.6$). Sialic acid values could not be related to clinical features that might nonspecifically lead to sialic acid elevations. For example, serum levels were not remarkably different among those who received BCG compared with those who did not ($p = 0.88$, Student’s t test). Also, there was no correlation between sialic acid and time from surgery either among those who had recurrent melanoma ($p = 0.11$, linear regression analysis using Student’s t test) or those who did not ($p = 0.55$). In no patient was the serum sample obtained within 15 days of surgery, an interval when nonspecific acute phase reactant elevations would be most expected (14). There was also no apparent relationship between sialic acid concentration and anatomic level of invasion as described by Clark (8) ($p > 0.25$, F analysis of variance), optical micrometer measurement of invasion as described by Breslow (5) ($p = 0.3$, linear regression analysis using Student’s t test), or whether there was regional metastatic disease prior to complete surgical excision ($p = 0.77$, Student’s t test).

**DISCUSSION**

Elevations of total serum sialic acid have been repeatedly observed in cancer patients’ sera. As part of an investigation
cific acute-phase reactants such as α1-acid glycoprotein (4, 15, 17, 30). The relative contribution to both markers of acute-phase reactants has yet to be determined. It may be that only one of the group of human sialyltransferase enzymes is of tumor origin (35), while others are more closely associated with acute-phase reactants. The tumor-related sialyltransferase(s) might possibly be distinguished by using a more specific acceptor than desialylated fetuin or by assessing sialyltransferase isoenzymes. It is equally possible that sialic acid containing glycoproteins of tumor origin may be separable from acute-phase reactants. Others have reported complex procedures for the identification and separation of serum sialyglycoproteins of tumor origin (17). Preliminary experiments in our laboratory suggest that quantitation of serum sialyglycoprotein of tumor origin is possible through the relatively simple techniques of salt fractionation and radial immunodiffusion (28). The sensitivity for tumor burden of both sialyltransferase and sialic acid might well be improved through identification of the tumor-related component. However, the clinical significance of these findings has yet to be determined. In this study, we were primarily concerned with the relationship of serum sialic acid and sialyltransferase activity as currently evaluated (14, 29). It is important that the potential value of these markers as monitors of tumor burden need not be affected by the influence of acute-phase reactants. Some acute-phase reactants have been suggested for this purpose (32, 33).

The results of our study again demonstrate the highly significant relationship between serum sialic acid and tumor burden among malignant melanoma patients (29). Further, this study demonstrates a direct relationship between serum sialic acid and changing tumor burden in individual patients (Chart 6). In contrast with sialic acid, sialyltransferase activity was elevated only with relatively advanced disease (Chart 2). This suggests that the clinical application of the enzyme as a tumor marker may be restricted to patients with relatively advanced disease. In keeping with this relative insensitivity for small tumor burden, the direct relationship between sialic acid and sialyltransferase was most significant in patients with established disease (Chart 3).

In the serial study (Charts 6 and 7), the sialic acid correlated significantly with objective change of tumor burden in 19 of 24 (79%) patients, while this was true for sialyltransferase in 14 of the 24 (58%) patients. Changes in sialyltransferase tended to be less marked than for sialic acid, and the greater interassay variation of the enzyme assay decreased the significance of small changes.

Serum sialic acid levels were higher among Group I patients who later developed recurrent malignant melanoma. A similar relationship between tumor marker concentration and frequency of recurrence is seen with CEA and colon cancer patients (7). Although most sialic acid values were within the normal range for this study, it is possible that determinations could prove clinically important in evaluating prognosis, as has been reported for CEA (20). There was no significant relationship between sialic acid and clinical features that might be associated with nonspecific sialylglycoprotein production such as BCG use or proximity to date of surgery. This suggests that high normal values can be a direct result of occult recurrent neoplasm and is in keeping with our previously reported suggestion that serum sialic acid may serve as a rather sensitive correlate of small tumor burden (29). The absence of correlation between sialyltransferase and recurrence again indicates relative insensitivity of enzyme measurement for small tumor burden.

In comparing sialic acid and sialyltransferase assays, there are additional practical considerations. The procedure for the sialic acid assay is less complex than for sialyltransferase. Further, the sialic acid assay is more reliable, less expensive, requires less technical sophistication, does not use radioactive reagents, and would lend itself to automation. The clinical significance of sialic acid measurement needs to be evaluated for a variety of human neoplasms.

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