Isolation and Chemical Characterization of Cell Surface Sialoglycopeptide Fractions during Progression of Rat Ascites Hepatoma AS-30D

David F. Smith and Earl F. Walborg, Jr.

The University of Texas Graduate School of Biomedical Sciences at Houston, and Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77025

SUMMARY

In order to ascertain whether chemical alterations in cell surface glycopeptides may be associated with tumor progression, we isolated sialoglycopeptide fractions from the surface of AS-30D rat ascites hepatoma cells before and after tumor progression. During the 70th to 90th transplantation generation of the AS-30D tumor, a morphological change and an increase in tumor virulence were observed. The tumor morphology progressed from a predominance of very large cell clusters to a predominance of single cells, doublets, triplets, and smaller clusters. The neuraminidase-labile, cell surface sialic acid of the AS-30D tumor decreased 27% after morphological progression of the tumor. Sialoglycopeptide fractions were prepared by papain digestion of cell suspensions obtained from the 11th to 14th, 49th to 55th, and 116th transplantation generations. These fractions were partially purified by trichloroacetic acid precipitation, dialysis, and gel filtration on Sephadex G-50. Compositional analysis and ion-exchange chromatographic behavior of the sialoglycopeptide fractions were similar except for a marked decrease in their glucose content associated with increased passage of the tumor.

INTRODUCTION

Cancer has been characterized as a disorder of the social interactions of cells which evolves from chemical alteration at the cell periphery (1, 13, 20, 27, 34). The elucidation of the molecular basis of cell-to-cell interactions is dependent upon the determination of the chemical nature of the cell periphery. Altered oligosaccharide moieties, present at the cell periphery, appear to be involved in several properties of the cancer cell, namely, decreased intercellular adhesion (1, 11), altered antigenicity (17, 22), and altered agglutinability by certain plant lectins (2, 4, 9, 18, 19). Chemical differences in cell surface glycopeptides from normal and virally transformed cell lines have been demonstrated (7, 8, 25, 36).

One approach to investigating these cell surface oligosaccharide moieties has been the isolation and chemical characterization of glycopeptides released from the cell periphery by the action of proteolytic enzymes. Codington et al. (10) have demonstrated the removal of carbohydrate and protein material from TA3 mammary carcinoma ascites cells by the action of proteases. Langley and Ambrose (23) have isolated and partially characterized a sialoglycopeptide fraction obtained by digestion of Ehrlich ascites tumor cells with trypsin, while Walborg et al. (33) have investigated a glycopeptide fraction obtained by digesting Novikoff ascites tumor cells with papain. These tumor systems have been maintained by serial transplantation for years and are probably characterized by a high degree of progression from the original neoplastic state.

This paper describes the isolation and partial chemical characterization of sialoglycopeptide fractions isolated from the rat ascites hepatoma AS-30D (29) before and after tumor progression, characterized by a morphological alteration and an increase in virulence. We performed studies to ascertain whether chemical alterations in cell surface glycopeptides could be correlated with changes associated with tumor progression.

MATERIALS AND METHODS

Collection of Tumor Cells. The transplantable rat ascites hepatoma AS-30D (29) was maintained in 6- to 9-week-old Sprague-Dawley rats (A. R. Schmidt, Inc., Madison, Wis.). Animals were sacrificed by decapitation. For prevention of spontaneous agglutination of the tumor cells, the ascitic fluid was diluted by i.p. injection of 36 ml of cold 0.9% NaCl solution. The diluted tumorous ascitic fluid was withdrawn, and 25-ml portions of this suspension were distributed into 30-ml graduated Corning 8380 Kolmer centrifuge tubes. Since this tumor grows as clusters of cells, it was possible to sediment the cells at extremely low centrifugal force, 2 X g, for 2 to 5 min. In this manner, contaminating red blood cells...
were easily removed and cell damage prior to enzyme incubation was avoided. After removal of the supernatant fluid by aspiration, the cells were resuspended in 20 ml of Buffer 1 (33). The suspensions were then centrifuged again for 2 to 5 min at 2 X g. All washes of cells utilized 0.9% NaCl solution or buffer chilled to 4°C.

Neuraminidase Digestion. The conditions utilized for neuraminidase digestion at pH 5.5 of AS-30D tumor cell suspensions have been described (33). Neuraminidase digestion was also performed at pH 6.5 with the following buffer: 0.105 M NaCl, 4.8 mM KC1, 5.0 mM glucose, 0.91 mM Na2HPO4, and 4.6 mM CaCl2, adjusted to pH 6.5. Approximately 3 ml (2 X g for 2 to 5 min) of the twice-washed AS-30D tumor cells were incubated at 37°C in a total volume of 20 ml of the appropriate buffer containing 0.5 or 1.0 ml of neuraminidase solution (from Vibrio cholerae strain Z4; 500 units/ml; Schwarz/Mann, Orangeburg, N. Y.). At various times, aliquots were taken for the colorimetric analysis of the released sialic acid as described previously (33). Packed-cell volumes were determined by centrifugation of cells incubated without neuraminidase for 10 min at 250 X g.

Papain Digestion. Initial studies utilized conditions for papain digestion of the cell suspensions as described previously (33). However, a 10-fold decrease in papain concentration produced no change in the yield of sialoglycopeptide. Large-scale preparations of sialoglycopeptide were performed by incubation of the cells in a total volume of 140 ml containing 15 to 25 ml of packed cells and 0.8 ml of papain suspension. Incubations were performed at 37°C for 40 min.

Cell viability was determined by staining with trypan blue (14). An aliquot of cell suspension was mixed with an equal volume of a 0.1% solution of trypan blue (diamine blue 3B; Allied Chemical Corp., Morristown, N. J.) in Buffer 1 (33) at room temperature. After 10 min, dye permeability was determined by microscopic examination. The cell preparations utilized contained only 5 to 7% nonviable cells.

Since the AS-30D tumor grows as clusters of cells, the number of cells present per ml of packed cells (250 X g; 10 min) was estimated after dispersion of the cell clusters by incubation with papain. Cell counts on the single-cell suspensions were performed with a hemacytometer. One ml of packed cells contained 4.1 to 5.0 X 108 cells.

Preparation of C-SGP Fraction. The supernatant fluid obtained from papain-treated cells was collected and partially purified by precipitation with TCA, dialysis, and gel filtration on Sephadex G-50 as described previously (33). In some cases, the precipitation step was performed with 10% rather than 50% TCA.

Determination of the Composition of C-SGP. Sialoglycopeptide fractions (5 to 10 mg) were dried in a vacuum over P2O5 at 100°C in an Abderhalden-type vacuum-drying apparatus, and the dry material was weighed on a microanalytical balance to an accuracy of ±20 µg. This material was dissolved in deionized water, and aliquots were taken for analysis. Amino acid analyses were performed with the Beckman Model 120B amino acid analyzer following hydrolysis in constant boiling HCl for 20 hr at 110°C under N2. Separation and quantitation of hexosamines were accomplished with the use of the Beckman Model 120B amino acid analyzer, according to the method of Walborg et al. (31), following hydrolysis in 2 N HCl for 8 hr at 110°C under N2. Separation and quantitation of neutral sugars were performed by means of the ion-exchange chromatographic procedure of Walborg and Kondo (32), following hydrolysis in 1 N H2SO4 for 8 hr at 100°C. Sialic acid was measured by the method of Warren (35), following hydrolysis in 0.1 N H2SO4 at 80°C for 1 hr. NANA (obtained from Pierce Chemical Co., Rockford, Ill.) was used as a standard. Uronic acid, expressed as glucuronic acid, was quantitated by the colorimetric method of Bitter and Muir (5). In order to correct for nonspecific interference from other sugar components present, we, included a solution containing hexosamine, neutral sugar, and sialic acid in amounts equivalent to those present in the sialoglycopeptide fraction analyzed as a control. Total phosphorus was determined by the method of Bartlett (3). Total lipid was determined by gravimetric analysis with a chloroform : methanol (2:1, v/v) extraction.

Microscale Fractionation of C-SGP by DEAE-cellulose. DEAE-cellulose (Whatman, microgranular DE-32), obtained from H. Reeve Angel & Co., Inc., Clifton, N. J., was equilibrated with 0.002 M pyridine : acetic acid buffer, pH 5.3. The DEAE-cellulose was packed into columns of approximately 3 mm x 10 cm (250-ml bed volume). Samples of C-SGP (2 mg) were placed on the columns in 0.002 M pyridine : acetic acid buffer, pH 5.3, and were fractionated with successive elutions of 1.5 ml of 0.002, 0.01, 0.05, 0.25, 0.6, and 1.0 M pyridine : acetic acid buffers, pH 5.3, at a flow rate of 1 to 3 ml/hr. The concentrations of each buffer are designated by the final molarity of pyridine. All buffers were adjusted to pH 5.3 with acetic acid. The effluent at each step of the gradient was dried in a vacuum over NaOH and concentrated H2SO4. Each fraction was dissolved in 1.0 ml of deionized water, and aliquots were taken for analysis. After hydrolysis (2 N HCl; 100°C 8 hr) hexosamine was determined by the Elson-Morgan reaction as described by Gatt and Berman (15) with D-glucosamine : HC1 (Mann assayed grade; Schwarz/Mann, Orangeburg, N. Y.) as a standard. Sialic acid was determined by the method of Warren (35), following hydrolysis in 0.1 N H2SO4 at 80°C for 1 hr. Neutral sugar was determined by the method of Dubois et al. (12), with D-galactose as a standard.

In order to obtain sufficient material for analysis of the neutral sugar composition, two 2-mg samples were submitted to chromatography on DEAE-cellulose. The following fractions were pooled: the 0.002 M and 0.01 M fractions (A), the 0.05 M fraction (B), and the 0.25 to 1.0 M fractions (C). The fractions were transferred to 1.5 ml of concentrated H2SO4. Each fraction was dissolved in 1.0 ml of deionized water, and aliquots were taken for analysis. After hydrolysis (2 N HCl; 100°C 8 hr) hexosamine was determined by the method of Bartlett (3), with 3 M HCl and 0.5 M NaOH, followed by hydrolysis in deionized water at 100°C. The neutral sugar composition of each fraction was determined by the method of Dubois et al. (12), with D-galactose as a standard.
The sum of the volume inside the gel \( V_o \) and \( V_i \) was determined by incubating without enzyme, and papain was incubated in Buffer 3 (33) was determined by measuring the effluent volume of Blue Dextran by the method of Dubois et al. (12). The volume outside the gel \( V_0 \) was measured by the method of Warren (35). Neutral sugar was determined using the average of the 90- and 120-min points, we determined the neuraminidase-labile, cell surface sialic acid to be, in \( \mu \)mole of NANA/ml of cells, 0.40 ± 0.04 \( \pm 0.02 \) (4) Transplantation generation 49 to 52, respectively. At transplantation generations 107 to 109, this value decreased to 0.31 ± 0.02 \( \pm 0.03 \) (4) for transplantation generations 15 to 18 and 0.44 ± 0.01 for transplantation generations 107 to 109, respectively. At transplantation generations 107 to 109, this value decreased to 0.31 ± 0.02 \( \pm 0.02 \) (4) and no significant decrease in transplantability (80 to 95%).

**Determination of the Neuraminidase-labile, Cell Surface Sialic Acid.** The rates of release of sialic acid from cell suspensions obtained from various transplantation generations of the AS-30D tumor are shown in Table 1. The results are calculated as \( \mu \)moles of sialic acid, expressed as NANA, per ml of packed cells. Using the average of the 90- and 120-min points, we determined the neuraminidase-labile, cell surface sialic acid to be, in \( \mu \)mole of NANA/ml of cells, 0.40 ± 0.04 ± 0.01 for transplantation generations 15 to 18 and 49 to 52, respectively. At transplantation generations 107 to 109, this value decreased to 0.31 ± 0.02 \( \pm 0.03 \) \( \mu \)mole of NANA/ml of cells. The neuraminidase-labile, cell surface sialic acid was not altered by incubating cells in buffer that had been adjusted to pH 6.5 rather than 5.5 or by increasing the neuraminidase concentration from 12.5 to 25 units/ml.

**Isolation of C-SGP.** Since ascitic fluid contains glycoproteins which may be a source of glycopeptides (24), 3 washes of the cells were performed prior to enzyme digestion. Inclusion of an additional wash in the procedure did not reduce the amount of sialic acid released by papain, indicating that soluble glycoproteins had been removed. Cell lysis during papain digestion was minimal, as evidenced by the fact that we were able to centrifuge the cells at relatively low centrifugal forces following their incubation with papain. If cell lysis occurs during papain incubation, nucleic acids are released into solution, increasing the viscosity and making it difficult to centrifuge the cells at low centrifugal force.

Chart 1 shows a representative elution pattern obtained by gel filtration on Sephadex G-50 of the TCA-precipitated, triplets, and smaller cell clusters; however, specializations of intercellular contact persisted (37).

An increase in virulence of the tumor cells, as evidenced by a decrease in the median host survival time (29), was observed in transplantation generations 81 to 90. The median host survival time in transplantation generations 52 to 80 was 17 days, with 3% of the rats dying within 10 days after injection. Later transplantation generations exhibited a decrease in median host survival time. In transplantation generations 101 to 107, the median host survival time was 8 days, with 25% of the rats dying within 10 days after injection. The increase in virulence of the tumor cells necessitated a decrease in the injection inoculum from 10⁸ to 10⁷ cells per rat. This inoculum produced a median host survival time of 13 days, with 3% of the animals dying within 10 days after injection and no significant decrease in transplantability (80 to 95%).

**Table 1**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Transplantation generations 15-18 (av. ± S.D.)</th>
<th>Transplantation generations 49-52 (av. ± S.D.)</th>
<th>Transplantation generations 107-109 (av. ± S.D.)</th>
<th>Transplantation generation 123 (av.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.22 ± 0.03 (3)</td>
<td>0.31 ± 0.05 (3)</td>
<td>0.23 ± 0.01 (4)</td>
<td>0.27 (2)</td>
</tr>
<tr>
<td>30</td>
<td>0.33 ± 0.02 (3)</td>
<td>0.35 ± 0.02 (3)</td>
<td>0.26 ± 0.02 (4)</td>
<td>0.29 (2)</td>
</tr>
<tr>
<td>60</td>
<td>0.37 ± 0.02 (3)</td>
<td>0.43 ± 0.03 (3)</td>
<td>0.28 ± 0.02 (4)</td>
<td>0.29 (2)</td>
</tr>
<tr>
<td>90</td>
<td>0.40 ± 0.06 (3)</td>
<td>0.44 ± 0.02 (3)</td>
<td>0.30 ± 0.03 (4)</td>
<td>0.31 (2)</td>
</tr>
<tr>
<td>120</td>
<td>0.40 ± 0.02 (3)</td>
<td>0.41 ± 0.01 (3)</td>
<td>0.31 ± 0.02 (4)</td>
<td>0.31 (2)</td>
</tr>
</tbody>
</table>

- *Sialic acid is expressed as \( \mu \)moles of N-acetlyneuraminic acid.
- *Packed cell volumes were determined by centrifugation of cells incubated without neuraminidase for 10 min at 250 \( \times \) g.
Table 2
Chemical composition of the sialoglycopeptide fractions isolated from the 11th to 14th (C-SGP I), 49th to 55th (C-SGP II), and 116th (C-SGP III) transplantation generations of AS-30D ascites hepatoma cells

<table>
<thead>
<tr>
<th>Component</th>
<th>C-SGP I µmole/mg of C-SGP</th>
<th>Residue</th>
<th>C-SGP II µmole/mg of C-SGP</th>
<th>Residue</th>
<th>C-SGP III µmole/mg of C-SGP</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>13.9</td>
<td></td>
<td>21.5</td>
<td></td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.21</td>
<td>6.0</td>
<td>0.24</td>
<td>7.0</td>
<td>0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.9</td>
</tr>
<tr>
<td>Glucosamine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.40</td>
<td>8.1</td>
<td>0.58</td>
<td>11.7</td>
<td>0.73</td>
<td>14.8</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>0.11</td>
<td>2.3</td>
<td>0.12</td>
<td>2.5</td>
<td>0.14</td>
<td>2.9</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.18</td>
<td>1.6</td>
<td>0.23</td>
<td>3.8</td>
<td>0.26</td>
<td>4.3</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.11</td>
<td>2.9</td>
<td>0.11</td>
<td>1.6</td>
<td>0.13</td>
<td>1.8</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.44</td>
<td>7.1</td>
<td>0.53</td>
<td>8.6</td>
<td>0.54</td>
<td>8.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.40</td>
<td>22.7</td>
<td>0.48</td>
<td>7.7</td>
<td>0.14</td>
<td>2.3</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>&lt;0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td></td>
<td></td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>64.6</td>
<td></td>
<td>64.4</td>
<td></td>
<td>65.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as NANA.
<sup>b</sup> When C-SGP III was treated with 50% TCA and isolated as described above, this value was 0.24 µmole/mg.
<sup>c</sup> Expressed as N-acetylhexosamine.

Chart 2. Fractionation of C-SGP I, C-SGP II, and C-SGP III by ion-exchange chromatography on DEAE-cellulose. Samples were applied in 0.002 M pyridine:acetic acid buffer, pH 5.3, and fractionated with successive elutions of 1.5 ml of 0.002, 0.01, 0.05, 0.25, 0.6, and 1.0 M pyridine:acetic acid buffers, pH 5.3, at a flow rate of 1 to 3 ml/hr.

ether-extracted, dialyzed, concentrated sialoglycopeptide fraction isolated from the 11th transplantation generation. These samples represent the sialoglycopeptide fractions obtained from 10 to 20 ml of packed cells. Essentially the same elution pattern was obtained from later generations. Larger amounts of sialoglycopeptide, representing up to 100 ml of packed cells, were fractionated by gel filtration. The preparative scale columns were analyzed for UV absorbance and sialic acid (33). The sialic acid-containing fractions were pooled, lyophilized, and designated C-SGP. Digestion of AS-30D tumor cells with papain released 0.12 ± 0.02 S.D. µmole of NANA/ml of packed cells. The yield of C-SGP was 250 to 500 µg/ml cells.

C-SGP was isolated at the 11th to 14th (C-SGP I), 49th to 55th (C-SGP II), and 116th (C-SGP III) transplantation generations. No significant change in yield was observed when C-SGP was isolated from different transplantation generations.

Determination of the Composition of C-SGP Fractions. The peptide and carbohydrate compositions of the sialoglycopeptide fractions isolated from different transplantation generations of the AS-30D tumor are shown in Table 2. The amino acids present in the highest amounts were threonine, aspartic acid, glutamic acid, serine, proline, glycine, alanine, and valine. The molar ratio was 2:2:2:1.5:1:1:1 in C-SGP I and II and 4:2.5:2:3.5:1:1:1 in C-SGP III. The total weight recovery of C-SGP, based on compositional analysis, was approximately 64%.

C-SGP III was isolated with the use of 10 rather than 50% TCA in the precipitation step. A 40% increase in the sialic acid content of C-SGP III was observed when compared with C-SGP I and C-SGP II. When C-SGP III was dissolved in 50% TCA and reisolated according to the procedure described above, the sialic acid content was identical to that obtained for C-SGP II.

The UV spectrum of a solution of C-SGP I exhibited a maximum at 265 to 270 nm and a minimum at 245 nm. The absorbance of a 1% solution of C-SGP I, measured in a 1-cm light path at 260 nm, was 10.93 and, at 268 nm, was 11.48. Analysis of C-SGP I for total phosphorus revealed the presence of 0.16 µmole/mg. Extraction with chloroform : methanol of
Table 3

Carbohydrate composition of the fractions obtained by ion-exchange chromatography on DEAE-cellulose of C-SGP I, C-SGP II, and C-SGP III obtained from AS-30D ascites hepatoma cells

The following fractions were pooled for analysis: the material eluted in 0.002 M and 0.01 M (A), the material eluted in 0.05 M (B), and the material eluted in 0.25 to 1.0 M (C) pyridine : acetic acid buffer, pH 5.3 (Chart 2).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Neatural sugar (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NANA (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hexosamine&lt;sup&gt;b&lt;/sup&gt; : NANA</th>
<th>Mannose&lt;sup&gt;b&lt;/sup&gt; : galactose</th>
<th>Glucose&lt;sup&gt;b&lt;/sup&gt; : galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-SGP I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>27</td>
<td>5</td>
<td>6.4</td>
<td>0.7</td>
<td>19.8</td>
</tr>
<tr>
<td>B</td>
<td>19</td>
<td>13</td>
<td>3.9</td>
<td>0.4</td>
<td>5.4</td>
</tr>
<tr>
<td>C</td>
<td>53</td>
<td>82</td>
<td>3.1</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>C-SGP II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>25</td>
<td>11</td>
<td>8.2</td>
<td>0.5</td>
<td>2.2</td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>16</td>
<td>4.0</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>62</td>
<td>73</td>
<td>4.5</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>C-SGP III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>28</td>
<td>9</td>
<td>6.3</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>12</td>
<td>3.6</td>
<td>0.5</td>
<td>c</td>
</tr>
<tr>
<td>C</td>
<td>64</td>
<td>80</td>
<td>3.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of total recovered from column.
<sup>b</sup> Molar ratios.
<sup>c</sup> Glucose absent from this fraction.

C-SGP II resulted in the loss of only 2.9% of the weight. Analysis of C-SGP I for uronic acid indicated the presence of <0.10 μmole/mg.

Microscale Ion-Exchange Chromatography of C-SGP. The profiles of the stepwise elution of C-SGP I, C-SGP II, and C-SGP III from DEAE-cellulose are shown in Chart 2. The recovery of sialic acid from these columns was 87 to 104%. The quantitative analyses of the saccharide components of Fractions A, B, and C of C-SGP I, C-SGP II, C-SGP III are shown in Table 3. The average recovery of each monosaccharide was 86 to 102%. In all C-SGP fractions, the material eluted at the lowest ionic strength (Fraction A) contained only 5 to 10% of the total sialic acid, up to 28% of the neutral sugar, and was rich in glucose as indicated by the ratio, glucose : galactose. The sialoglycopeptide-containing components (Fractions B and C) were eluted at higher ionic strengths. The ratios, hexosamine : sialic acid and mannose : galactose, indicate that Fractions B and C have similar compositions with respect to these constituents. These fractions contained 89 to 95% of the total sialic acid and 80 to 90% of the total mannose, fucose, and galactose. The sialoglycopeptide-containing components (Fractions B and C) from C-SGP I contained 65% of the total glucose, those from C-SGP II contained 47% of the total glucose, and those from C-SGP III contained only 19% of the total glucose.

DISCUSSION

During early transplantation generations of the AS-30D tumor, morphological observations revealed the presence of large clusters of cells, with single cells, doublets, and triplets comprising only a minor component of the tumorous ascites (29). The cell clusters did not represent a nonspecific aggregation of cells, since specializations of intercellular contact (desmosomes, interdigitation, tight junctions, and related structures) were observed by electron microscopy (37). During the 70th to 90th transplantation generations, a change in morphology and an increase in tumor virulence were observed. Although the tumor morphology progressed from a predominance of very large cell clusters to a predominance of single cells, doublets, triplets, and smaller clusters, specializations of intercellular contact persisted in the smaller clusters (37). The loss of intercellular contact and the decreased incidence of tight junctions have been described as the only morphological features that distinguish hepatoma from normal liver plasma membranes (13). The observed decrease in size of the cell clusters reflects a further dedifferentiation or progression of the AS-30D hepatoma to a free cell tumor. The ability of certain tumors to grow as cell clusters characterized by the persistence of junctional specializations presumably reflects chemical differences at their cell peripheries. The morphological progression of the AS-30D tumor offered us the opportunity to ascertain whether alterations in the neuraminidase-labile, cell surface sialic acid or in the cell surface glycopeptides were correlated with this progression.

Although the neuraminidase-labile, cell surface sialic acid remained constant through the 52nd transplantation generation, a 27% decrease in this value was observed at the 107th to 109th transplantation generations. No further decrease in this value was observed at the 123rd transplantation generation. The term "neuraminidase-labile, cell surface sialic acid" was used, since these determinations were made on intact cells. The values obtained from the plateau at 90 to 120 min indicate the release of all sialic acid accessible to this enzyme. This decrease in neuraminidase labile, cell surface sialic acid was associated with the decreased size of tumor cell clusters. These data lend credence to the suggestion that sialic acid may play an important role in cellular adhesion (21).

Digestion of AS-30D tumor cells with papain represents a convenient means of preparing a cell surface sialoglycopeptide fraction in quantities sufficient for biochemical analysis. The procedure for isolation of C-SGP from AS-30D tumor cells is essentially that described by Walborg et al. (33). Investigations...
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of the acid lability of the sialic acid content of C-SGP III indicated that 10 rather than 50% TCA is preferable in the precipitation step. The C-SGP fractions that contain 30 to 40% of the neuraminidase-labile, cell surface sialic acid undoubtedly represent families of glycopeptides. The elution profiles obtained by gel filtration and ion-exchange chromatography show that the ratio of sialic acid : hexosamine : neutral sugar varied with effluent volume, indicating a multiplicity of components. Since amino acids and carbohydrates account for only 64% of the weight of the C-SGP fractions, the possibility of presence of other components is being pursued. Contamination with uronic acid-containing polysaccharides is minimal. The total phosphorus content of C-SGP II was found to be 0.16 µmole/mg. If this phosphorus were present as phospholipid and the average molecular weight of the phospholipids was assumed to be 770 (6), 10 to 12% of the C-SGP could be phospholipid in nature; however, extraction of C-SGP II with chloroform : methanol resulted in the loss of only 2.9% of the weight. Spectrophotometric analysis indicated that maximal contamination of C-SGP with nucleic acid was approximately 5%, a value more compatible with the phosphorus analysis.

The compositional analyses of C-SGP I, C-SGP II, and C-SGP III have revealed only minor differences in their peptide and carbohydrate compositions; however, a significant decrease in the glucose content of C-SGP was associated with continued passage of the tumor. Since control experiments (cells incubated without papain and papain incubated without cells, as shown in Chart I) indicated the presence in C-SGP of a neutral sugar-containing component that contained no sialic acid, the C-SGP fractions were submitted to chromatography on DEAE-cellulose. The material that eluted at low ionic strengths contained 25 to 28% of the neutral sugar and was low in sialic acid and rich in glucose. However, glycopeptides eluted from DEAE-cellulose with buffer of higher ionic strength also contained glucose. The decreased glucose content of C-SGP, associated with increased passage of the tumor, was distributed over all fractions of C-SGP resolved on DEAE-cellulose (Tables 2 and 3). Since the glucose content of C-SGP decreased significantly prior to the change in tumor morphology, this chemical alteration may not be associated with tumor progression. Although the neuraminidase-labile, cell surface sialic acid decreased 27%, no significant decrease in the sialic acid content of any C-SGP fraction was observed. These data indicate that this decrease in sialic acid was associated with cellular material not susceptible to removal by papain or that the decrease in sialic acid content of C-SGP was not observable by our method of isolation. Compositional analyses and ion-exchange chromatographic profiles of the cell surface glycopeptides revealed no other qualitative or quantitative differences that could be associated with tumor progression. However, these data do not preclude the occurrence of alterations in specific glycopeptide components of C-SGP. In order to detect chemical alterations of specific glycopeptides, it will be necessary to resolve C-SGP into its component glycopeptides. Such investigations with C-SGP III are in progress.

A comparison of the analytical data obtained for C-SGP from AS-30D tumor cells with that obtained by Walborg et al. (33) for C-SGP from Novikoff ascites tumor cells reveals several similarities. Neutral sugar is an important constituent of both C-SGP fractions. The C-SGP fractions obtained from AS-30D tumor cells were enriched in the same amino acids as were C-SGP fraction from Novikoff tumor cells (33) and the glycopeptides obtained from TA3 mammary carcinoma ascites cells (10).

The presence of neutral sugars, hexosamines, and sialic acid in isolated plasma membranes of normal rat liver and rat hepatoma 484 has been reported (13). Shimizu and Funakoshi (28) have reported the carbohydrate composition of lipid-free plasma membrane from rat ascites hepatoma 7974F. The carbohydrate composition of the lipid-free membrane is qualitatively similar to C-SGP from AS-30D tumor cells. The glucose content, relative to glucosamine, of C-SGP III and lipid-free membranes are the same, while C-SGP I, C-SGP II, and Novikoff C-SGP contain 4 to 15 times more glucose. Codington et al. (10) have also reported the presence of glucose in glycoprotein material liberated from TA3 cells by the action of protease. Miyajima et al. (26) reported the chemical composition of an acidic glycopeptide fraction isolated from plasma membrane structural protein of normal rat liver. Although neutral sugars are important constituents of these acidic glycopeptides, glucose is a relatively minor component.

Recently, a new tool has been introduced to demonstrate the chemical alterations of the cell periphery, i.e., the agglutinability of cells by the plant lectin wheat germ agglutinin (2, 4, 9). Cytoagglutination of AS-30D tumor cells by wheat germ agglutinin has been shown to be inhibited by C-SGP III (D. F. Smith and E. F. Walborg, Jr., unpublished results). Work is now in progress to isolate and chemically characterize the component glycopeptides of C-SGP III to determine whether these glycopeptides function as cell surface binding sites for wheat germ agglutinin.

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Isolation and Chemical Characterization of Cell Surface Sialoglycopeptide Fractions during Progression of Rat Ascites Hepatoma AS-30D

David F. Smith and Earl F. Walborg, Jr.


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