Surface Membrane Glycopeptides Which Coincide with Virus Transformation and Tumorigenesis

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Glycopeptides from the surface of clones of hamster embryo cells were examined at various intervals after infection with polyoma virus. Two types of transformed cells were examined: (i) clones that showed delayed transformation or an initially low tumorigenicity, and (ii) clones that were rapidly transformed showing an initially high tumorigenicity. The glycopeptides were removed from the cell surface by trypsin and, after Pronase digestion, were examined by filtration through Sephadex G-50. With delayed transformation, a specific group of glycopeptides was increasingly evident over an 85-day period as the cells showed phenotypic properties of transformation and the ability to form tumors. In the other series, all but one clone of hamster embryo cells showed rapid transformation after infection with polyoma virus. This clone was less tumorigenic and showed little of the specific glycopeptides. In all cases of delayed or rapid transformation examined, the specific group of glycopeptides increased proportionately to the ability of the cells to form tumors. All of the cells derived from progressively growing tumors formed by injection of these transformed hamster cells into adult animals showed an abundance of this group of glycopeptides. These results suggest that specific surface membrane glycopeptides accompany viral transformation and tumorigenesis.

In defining the role of surface membranes in virus transformation it is important to determine the sequence of events that lead to the expression of the malignant process. Specific changes from the normal in the glycopeptides of the surface membrane have been reported to accompany virus transformation (1, 2, 8, 9, 11). In cells transformed after chemical treatment this alteration has been shown to correlate with tumorigenesis (4). It is, therefore, of considerable interest to determine the point after viral infection when this alteration takes place and if it correlates with the ability of the cells to form tumors and other expressions of the transformed phenotype.

After infection of hamster embryo cells by polyoma virus, the expression of the transformed properties and malignancy may be rapid or delayed. That is, the clones of cells selected after infection that show an initially low tumorigenicity are designated as delayed whereas the cells showing an initially high tumorigenicity are designated as rapid. In delayed transformation the expression of the transformed properties increases gradually during cultivation, in vitro, until a level characteristic of transformed cells is reached about 4 to 5 months after infection (5). Transformed properties are defined as phenotypic expressions that accompany virus transformation of cells, such as saturation density, cloning efficiency, the ability to form colonies at 41%, and the ability to form tumors when injected into adult hamsters.

The current study has followed the changes in the glycoprotein composition of the surface membranes under conditions of delayed or rapid transformation of the hamster embryo cells by polyoma virus. In addition, the glycopeptides derived from tumor cells formed by the virus-transformed cells under both these conditions were examined. In all cases, when the cells had the ability to form progressively growing tumors, specific alterations in the membrane glycopeptides were seen.

MATERIALS AND METHODS

Cell cultures and transformation. Minced whole embryos from Syrian hamsters were used as a source of control cells. Hamster embryos, male or female, were separated, and cells from the individual embryos were infected with 200 PFU of large plaque polyoma virus (u.11) per cell (10). Transformed colonies were selected from X-irradiated rat embryo

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feeder cells or from agar as described (7). Rapid transformation designated those clones of cells that were selected within a week after infection with polyoma virus and at that time were of high tumorigenicity. For delayed transformation, hamster embryo cells with no separation of the sexes were infected with 200 PFU of polyoma virus (n.11), and colonies showing a morphology and growth pattern that was intermediate between normal and transformed were isolated from feeder layers. Portions of the cloned cells were frozen so that the cells that were of low tumorigenicity could be subsequently examined at successive times in culture. These cells of initially low tumorigenicity were designated by the number of days in culture after polyoma virus infection to denote the progression of the expression of the phenotypic properties of transformation, including increased ability to form tumors. Clone 3 is a polyoma-transformed cell line maintained in culture for several years.

All cells were cultured in Eagle medium containing a fourfold concentration of amino acids, vitamins, and 10% calf serum. The cells were subcultured twice weekly by dissociation with a solution of 0.25% trypsin (Difco, 1:300) and seeded at 2.5 x 10^6 cells per 50-mm Petri dish. The transformed lines established from all of the colonies isolated were positive for the polyoma-specific T antigen. No contamination with Mycoplasma was detected according to the methods of Chanock et al. (3). All of the cell types were examined in duplicate. In addition, more than one series was examined, and the results were always similar.

Measurement of transformed properties. At specified days after viral infection, the cloning efficiency and ability to form colonies at 41°C was examined in some cultures. The saturation density and the ability to form tumors in adult hamsters were examined in all cultures. These methods have been described for polyoma-transformed cells (7). To determine the tumor formation, the cells were suspended in 0.2 ml of Eagle medium and inoculated subcutaneously into 5- to 7-week-old hamsters. Twice a week up to 90 days the animals were examined for the development of palpable tumors. Progressively growing tumors were considered positive. The time required for the tumor to become palpable was taken as the latency period.

Culture of tumor cells. The tumors that were examined for surface glycopeptides were collected, trypsinized, and seeded at 10^4 cells per 50-mm Petri dish. At least 80% of the cells were viable. Forty-eight hours after seeding, the tumor cells were reseeded and treated similarly to the other cells examined.

Preparation of membrane glycopeptides. All of the cells examined were reseeded at 5 x 10^6 cells per 50-mm Petri dish (Falcon Co.) and cultured for 72 h in the presence of 5 μCi of L-[1-14C]fucose (50.8 mCi/mM) or 8 μCi of L-[3H]fucose (general label, 4.3 Ci/mM) for 72 h. The radioactive isotopes were obtained from New England Nuclear Corp. After this time the cells, in the logarithmic phase of growth, were washed five times with Tris-buffered saline, pH 7.5, and removed from the monolayer by trypsination: 1 mg of 3 x crystallized trypsin (Worthington Biochemicals Corp.) in 1 ml of Tris-buffered saline. After 5 min at room temperature an equivalent amount of soybean trypsin inhibitor ( purified, Worthington Biochemicals Corp.) was added, and the cells were centrifuged at 600 x g for 5 min. The supernatant solution containing the surface glycopeptides was centrifuged at 40,000 x g for 30 min and lyophilized. The material removed from the cell surface by this procedure will be referred to as "trypsinate" and always represented 20 to 30% of the total radioactivity of the cells.

Characterization of membrane glycopeptides. The trypsinates containing the radioactive glycopeptides were dissolved in water, and the fractions to be compared were combined and digested exhaustively with Pronase (Calbiochem). Separation of the Pronase-digested glycopeptides was by gel filtration on Sephadex G-50 fine. All details of these procedures have been described (1) as well as the processing of the fractions for radioactive counting (4). Further fractionation of the glycopeptides revealed that the peak areas separated on Sephadex G-50 represent a family of glycopeptides so that reference is made throughout the text to groups of glycopeptides rather than a single glycopeptide (M. C. Glick, Fed. Proc. 30:1044, 1971).

RESULTS

Delayed transformation. To obtain evidence for the correlation of surface membrane glycopeptides with the phenotypic expressions of transformation and the ability to form tumors in adult hamsters, these properties were examined in cloned cultures that showed delayed transformation. After polyoma virus infection and clone isolation, portions of the cloned cultures were frozen so that the clones that showed delayed transformation (low tumorigenicity) could be subsequently examined at successive intervals in culture. The surface membrane glycopeptides from these cells were analyzed by chromatography on Sephadex G-50 of the Pronase-digested radioactive trypsins. Representative results from one of these clones are shown in Fig. 1 and Table 1.

On day 28 (Fig. 1a) after viral infection, the distribution of the fucose-containing glycopeptides was only slightly different from that of the hamster embryo cells. These cells were of low tumorigenicity and showed a low-saturation density (Table 1). Thirty-nine days after virus infection (Fig. 1b), the glycopeptide profile from Sephadex G-50 showed that there was a definite appearance of another group of glycopeptides (fractions 20 to 30). By 64 days (Fig. 1c) these specific glycopeptides were even more abundant. The saturation density at day 39 was increased by twofold when compared with that found 28 days after infection, and tumors were formed in 100% of the animals by injection of
10^4 cells as compared with 10^6 cells on day 28 (Table 1). The pattern of the fucose-containing glycopeptides 85 days after viral infection (Fig. 1d) again showed the appearance of this group of glycopeptides, and after more than 100 days in culture (Fig. 1e), an abundance of this group of glycopeptides was seen (fractions 20 to 30). In Fig. 1e, a comparison is given with a polyoma-transformed cell line, clone 3, showing that even after a prolonged time in culture the surface glycopeptides that appear to be characteristic of virus-transformed cells were still expressed. The cells after 85 days in culture were highly tumorigenic, and the saturation density was also
TABLE 1. Tumor formation and saturation density at different times after infection in hamster clones with delayed transformation

<table>
<thead>
<tr>
<th>Days after infection*</th>
<th>Saturation density per plate × 10^6</th>
<th>Tumor incidence*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10^6</td>
</tr>
<tr>
<td>28</td>
<td>4/4</td>
<td>1/4 (61)</td>
</tr>
<tr>
<td>39</td>
<td>0/4</td>
<td>3/4 (36)</td>
</tr>
<tr>
<td>85</td>
<td>1/4 (61)</td>
<td>4/4 (25)</td>
</tr>
</tbody>
</table>

* Hamster embryo cells were infected with polyoma virus and maintained in culture for the stated number of days.
* Number of animals with tumors per number of animals inoculated. Numbers in parentheses indicate the latency period in days for palpable tumors.
* Number of cells inoculated per animal. Cells were inoculated subcutaneously into 5- to 7-week-old hamsters; the animals were observed for progressively growing tumors for 90 days.
* ND, Not determined.

Increased to levels that are considered characteristic of transformed cells (Table 1). These results show that a gradual shift in the distribution of fucose-containing glycopeptides from the cell surface is accompanied by the gradual increase in saturation density and the ability of the cells to form tumors.

Rapid transformation. The cell surface glycopeptides were examined in relation to the expression of malignancy and the phenotypic expression of transformed properties through the use of clones that were rapidly transformed. Independently transformed clones were selected from feeder layers and from soft agar within 1 week after infection with polyoma virus of three female and three male hamster embryos. These cells were of initially high tumorigenicity. Of this series, one clone of male hamster embryo cells (designated male 2) from the feeder layer formed tumors to a lesser extent than any of the other clones examined, including a culture from the same embryo selected from agar rather than the feeder layer (Table 2). All of the other clones showed more rapid tumor formation. The elution profile from Sephadex G-50 of the fucose-containing glycopeptides from the surface of the cells that were less tumorigenic (male 2) was more like that of the control hamster embryo cells and markedly different from those obtained from the cells that formed tumors to a high degree. Figure 2a shows the comparison of the glycopeptides from the surface membrane of male 2 cells with male 1 cells. The pattern of male 1 is representative of the other transformed cells examined. Repeated examination of male 2 gave similar results. Figure 2b shows the comparison of glycopeptides derived from the transformed embryo cells designated female 3, which were grown on feeder layers or on soft agar. All of the clones from soft agar were highly tumorigenic (Table 2) and showed a distribution of glycopeptides similar to that shown in Fig. 2b for the agar-derived clone.

In this series of clones from cells that were rapidly transformed, there was no consistent correlation with the phenotypic expressions of transformation that were examined and the ability of the cells to form tumors when injected into adult hamsters (Table 2). For example, male 2 cells, although less tumorigenic than the other cells, had a high-saturation density and

TABLE 2. Tumor formation and properties of viral transformation in rapidly transformed clones

<table>
<thead>
<tr>
<th>Cell type*</th>
<th>Saturation density per plate × 10^6</th>
<th>Cloning efficiency (%)</th>
<th>Colonies at 41 C (%)</th>
<th>Tumor incidence*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^6</td>
</tr>
<tr>
<td>Male 2</td>
<td>107</td>
<td>7</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Female 1</td>
<td>94</td>
<td>20</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>Female 2</td>
<td>90</td>
<td>23</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>Male 1 agar</td>
<td>85</td>
<td>32</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>Male 2 agar</td>
<td>95</td>
<td>43</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>Female 1 agar</td>
<td>88</td>
<td>34</td>
<td>56</td>
<td>10</td>
</tr>
<tr>
<td>Female 3 agar</td>
<td>94</td>
<td>34</td>
<td>70</td>
<td>2</td>
</tr>
</tbody>
</table>

* Cells derived from male or female hamster embryos were infected with polyoma virus.
* See legend to Table 1.
* Number of cells inoculated per animal.
* Cell line cultured for more than 2 years after transformation by polyoma virus. Data from previous experiments.
increased ability to form colonies at 41°C; however, the cloning efficiency in soft agar was low. The clones derived from agar, which were highly tumorigenic, formed a low percentage of colonies at 41°C but had a high-saturation density. In addition, these phenotypic properties (Table 2) did not correlate with the distribution of the surface glycopeptides.

**Surface glycopeptides from cells derived from tumors.** Cells derived from tumors formed by the transformed clones of the male and female hamster embryos were examined. In all cases, the Pronase-digested trypsinates of the tumor cells contained an abundance of the fucose-containing glycopeptides that were eluted from the gel in fractions 15 to 25. Figure 3a shows that the elution profile of the membrane glycopeptides derived from tumors of the less tumorigenic cells (Male 2) was similar to those obtained for the highly tumorigenic cells derived from the same embryo but cloned on agar (male 2 agar). Re-inoculation of these tumor cells derived from male 2 showed them to be highly malignant. The elution profile of the membrane glycopeptides from the tumors of the...
highly tumorigenic female 3 cells was also similar to the female 3 cells cloned on agar (Fig. 3b). Indeed, all of the cells that were examined from progressively growing tumors had an abundance of the membrane glycopeptides that eluted from the gel in fractions 15 to 25.

Very small tumors (13 by 7 mm) were formed at a low incidence when a large number of cells ($10^4$) were injected into the animals 28 days after viral infection (Table 1). At this time the distribution of the fucose-containing glycopeptides from the surface of these cells was similar to hamster embryo cells (Fig. 1a); therefore, the fucose-containing glycopeptides from the surface of the cells derived from these small tumors were examined. The distribution of the membrane glycopeptides when examined by gel filtration remained more similar to those of hamster embryo cells than those of the other tumor cells examined (cf. Fig. 4a and Fig. 3). Re-inoculation of these cells into adult hamsters increased the tumorigenicity 10-fold in some cases, although the tumors remained small (13 by 8 mm) and not all of the injected hamsters formed tumors. The appearance of the additional group of glycopeptides from these "2nd-cycle" tumor cells was somewhat more pronounced (fractions 25 to 30, Fig. 4b) than from the "1st-cycle" tumor (Fig. 4a). However, although eight tumors were examined, the marked shift in the glycopeptide distribution characteristic of the other tumor cells was never observed nor did these cells form tumors as consistently or as productively as the other cells examined.

**Correlation of tumorigenicity and specific surface glycopeptides.** Figure 5 shows a plot suggesting that as the tumorigenicity of the population increased a higher percentage of the total fucose-containing glycopeptides was distributed into a specific group, fractions 15 to 25 (Fig. 2 and 3). All of the polyoma-infected cells that were examined fell into this pattern.

To obtain the plot, the radioactive glycopeptides from fractions 15 to 25 (i.e., Fig. 2 and 3) were expressed as percentage of the total glycopeptides, fractions 15 to 35, and plotted against the tumorigenicity of the same cells. Tumorigenicity, defined on a highly arbitrary basis, was the number of cells that formed tumors in 100% of the animals within approximately 15 days. In cases where the cells were comparatively more or less tumorigenic than other cells at the same dilution, the number was moved by one half of the exponent. For example, in Table 2, male 1 and female 3 cells formed 100% tumors within approximately 15 days with $10^4$ cells; however, when all the dilutions were examined female 3 appeared more tumorigenic than male 1. Female 3 was scored between $10^4$ and $10^5$ cells, whereas male 1 was scored between $10^4$ and $10^5$ cells. In the same manner, male 3 was scored as $10^5$ cells. All of the cells examined as shown in Tables 1 and 2, with the exception of clone 3, are plotted in this manner as well as the other experiments in this series, all having similar data. Clone 3 was not included because the data on tumor formation was taken from previous experiments. The few number of cells needed to form tumors in adult hamsters correlated with the abundance of a specific group of glycopeptides.
The correlation coefficient showed 25, more characteristic in data fractions the total glycopeptides. Transformations of these transformed cells had surface glycopeptides similar to the nontransformed cells when examined by techniques described in this paper. In contrast, the cells derived from tumors of all these cells showed the appearance of the specific group of glycopeptides described here in all of the progressively growing tumor- and virus-transformed cells examined.

The clones of cells showing delayed transformation produced very small tumors at a low incidence when injected into adult hamsters 28 days after infection by polyoma virus. Examination of the chromosomes of the cells from these small tumors (13) showed that this population contained 80% diploid cells. Hence, these cells were more like those of the nontransformed than the transformed cells, suggesting as the reason for low tumorigenicity and the presence of only a small amount of the specific surface glycopeptides.

Clones selected from agar appeared to be more tumorigenic than those from feeder layers, and this was reflected in the glycopeptide composition of the surface membranes. No difference was detected in cloned cells selected from male or female embryos. In the clones selected for rapid transformation, there appeared to be no correlation of the glycopeptide pattern with the phenotypic properties of the transformed cells such as saturation density and growth on soft agar (Table 2).

Glycopeptides derived by similar techniques from baby hamster kidney cells transformed by Rous sarcoma virus have been further isolated and characterized. Chemical analyses of the glycopeptides indicated that the increased amount of radioactive fucose incorporated into these glycopeptides was representative of an increase in sialic acid as well as mannose and galactose (M. C. Glick, Fed. Proc. 30:1044, 1971). That is, glycopeptides containing more carbohydrate appear to be present after virus transformation. In examining the Rous-transformed cells, Warren et al. (9) described the
appearance of a sialyl transferase, which was present only in transformed cells. The appearance of specific sugar transferases perhaps accompanies virus transformation and could account for the changes described here for the cells transformed by polyoma virus.

Thus, it seems to be established that specific changes in surface membrane glycopeptides are associated with tumorigenesis. These glycopeptides appear also to be associated with virus transformation in all cases examined (2). They are not characteristic of cells transformed after treatment with a chemical carcinogen (4) or short-term cultures of lymphocytes transformed after antibody stimulation or by Concanavalin A (L. Stavy and M. C. Glick, unpublished data). However, all progressively growing tumors of any chemical- or virus-transformed cells that were examined showed an abundance of this group of glycopeptides. The exact chemical nature of these glycopeptides and the significance of the association with tumor formation is the subject of current investigations.

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LITERATURE CITED


