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The Surface Glycoproteins of a Mouse Melanoma Growing in Culture and as a Solid Tumor *in Vivo*[1]

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SUMMARY

B16 melanotic tumors in various organs of mice were labeled with either [^{14}C- or [^{3}H]L-fucose or D-glucosamine. Glycopeptides derived from the membrane glycoproteins of these tumors were compared with those of normal tissues by double-label elution patterns from Sephadex G-50 columns. A marked increase of sialic acid-rich, fucose-containing glycopeptides (Peak A) was found in the glycoproteins of the surface and internal membranes of melanotic cells. The glycopeptides from the melanoma cells could be reduced in size by treatment with neuraminidase.

Comparison of the glycopeptide patterns of melanoma cells grown in culture and in mice revealed a greater complexity in *in vivo* material. Virtually all of the glycopeptides from melanoma cells grown in culture were of the larger type (Peak A) that correlates well with the malignant state. Comparison of two lines of B16 melanoma cells with greatly differing abilities to form tumors in lung revealed no significant, reproducible differences in their glycopeptide patterns.

INTRODUCTION

Previous work has shown that the composition of glycopeptides derived from glycoproteins of the surface (2, 3, 11) and internal membranes (1) of malignant cells grown in culture differ in a consistent manner from the normal. The membrane glycoproteins of a wide variety of malignant cells bear carbohydrate groups that are especially rich in sialic acid (12, 15, 16). Increased levels of a sialyltransferase in the malignant cell may be responsible for the formation of the larger, sialic acid-containing, protein-bound carbohydrate groups (15, 16). These groups have been detected by chromatography on Sephadex G-50 columns. Glick *et al.* (7) have found that cells transformed by chemical carcinogen or virus and subsequently passaged in animals, retained, on culturing *in vitro*, more of these sialic acid-rich glycopeptides than controls. It has also been found that these glycopeptides increase in amount in mouse embryos at a time when invasive trophoblastic cells are forming and the embryo is embedding in the uterine wall (9). In this paper we report that solid melanotic tumors (B16) growing in various organs of mice show the same glycopeptide patterns as do malignant cells in tissue culture. This work has been briefly described previously (14, 18).

MATERIALS AND METHODS

The transplantable B16 melanoma was used in syngenic C57BL/6J mice. The tumor was adapted to monolayer growth *in vitro*. Cultures were maintained in glass bottles in Eagle's MEM,[2] supplemented with 10% fetal calf serum, vitamin solution, sodium pyruvate, nonessential amino acids, penicillin-streptomycin, and L-glutamine (Grand Island Biological Co., Grand Island, N. Y.). Cells were cultured at 37° in a humidified atmosphere containing 5% CO_2. Cell suspensions were prepared by removing monolayers with trypsin (0.25%)-Versene solution, centrifuging at 200 × *g* for 10 min, washing with MEM, and resuspending in MEM.

Three strains of B16 melanoma were used: (*a*) cloned directly from a s.c. implant; (*b*) cloned as a 1st passage from an experimental lung tumor (F_1); and (*c*) produced by repeated passages from culture to lung and back to culture (F_{10}). The F_1 and F_{10} strains were used because they yielded few and many tumors, respectively, as described by Fidler (5, 6). All strains produced essentially identical results.

Local tumors were produced by s.c. injections of 1.5 × 10^4 melanoma cells in a volume of 0.2 ml in the flank. Experimental tumors were produced in a variety of ways. Melanotic nodules of the lung were obtained after i.v. injections of 5 to 15 × 10^4 cells in a volume of 0.2 ml; the dose depended on the predetermined yield of tumors. Liver tumors were obtained from the same mice, and in other experiments they were produced by intraportal injections. Likewise, peritoneal tumors were found in the same mice and were also produced independently by i.p. injections.

The interval between tumor injection and sacrifice varied from 3 to 5 weeks depending on site of injection, tumor strain, and predetermined interval necessary for maximal tumor yield. Isotope injections were started 1 week before sacrifice. Each mouse received 3 injections at 2-day intervals and was sacrificed 1 day after the last injection.

The isotopes used for surface labeling were all obtained from New England Nuclear; these were L-[1-^{14}C]fucose, 50 mCi/mmole; L-[1,5,6-^{3}H]fucose, 5 Ci/mmole; D-[1-^{14}C]-

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[2] The abreviation used is: MEM, minimal essential medium.

glucosamine, 45 mCi/mmole; and D-[6-^{3}H]glucosamine, 5 Ci/mmole. Each injection consisted of 2.5 μCi of isotope in a volume of 0.2 ml. Tissues and tumors were pooled from 3 animals similarly labeled for processing. Approximately 20 animals were used in each of the 5 experiments carried out. Tumors were dissected as free as possible from surrounding tissues. Larger organs and tumor masses were tamped dry, weighed, cut into small pieces approximately 1 mm or less in diameter, and incubated for 20 min at 37° in Tris-buffered 0.9% NaCl solution with trypsin (1 mg/ml, 3 times crystallized; Worthington Biochemicals, Freehold, N. J.). On centrifugation at 500 × *g* for 20 min the supernatant ("trypsinate") and a pellet were harvested. Pellets consisting of largely intact cells, as viewed by the phase-contrast microscope, were resuspended in buffer. Each of these, either alone or in appropriate combination, was digested with Pronase (Calbiochem, Los Angeles, Calif.) (1 mg/ml) for 5 days in the presence of a small amount of toluene as previously described (2). Fresh Pronase was added each day. The double-label combinations of Pronase digests containing phenol red and dextran blue 2000 as markers were chromatographed on Sephadex G-50 fine columns (200 x 1.0 cm) as previously described (2). Eluates in approximately 90 to 100 fractions were counted for ^{14}C and ^{3}H in an Intertechnique Model SL300 liquid scintillation counter, and the data was processed and graphed entirely by computer. The computer was programmed so that the 2 highest peaks, usually the ones of greatest interest, were plotted with equal heights.

Some Pronase digests were treated with neuraminidase from *Vibrio cholerae* (Calbiochem) as previously described (15) or were hydrolyzed in 0.1 N H_2SO_4 at 80° for 1 hr to remove their sialic acids.

Melanoma cells were grown in Falcon plastic flasks (75 sq cm) for 72 hr in the presence of 10 μCi of isotopic sugars. The cells were washed with Tris-buffered saline treated with trypsin and the trypsinates were digested with Pronase as described above. Combinations of ^{3}H- and ^{14}C-labeled Pronase digests were chromatographed on Sephadex G-50 fine columns as described above and in previous publications (2). No differences in results have been found when trypsinates are combined and digested with Pronase or when digests are combined after treatment with Pronase.

RESULTS AND DISCUSSION

Melanoma Cells as Solid Tumors. It can be seen in Table 1 that there is no marked, consistent difference in the extent of isotopic labeling and in the fraction of incorporated, isotopic sugar released by trypsin from normal tissues and melanotic tumors. Similar results were obtained from all tissue sources with either L-fucose or D-glucosamine. The kidney appears to incorporate more isotope than other normal or tumor tissues, and the fraction of that released by trypsin is less. No significant differences were found in incorporation rates of isotope or in percentage of isotope released by trypsin between various organs of normal mice and those bearing tumors nor were differences found in these parameters in pulmonary nodules of high- and low-take lines of melanoma. Chromatography of undialyzed Pronase digests of trypsinates on Sephadex G-50 columns reveals that a relatively small fraction of the isotopic material released by trypsin is free, unused L-fucose or D-glucosamine. These could be detected as radioactive peaks in the tube 80 region of the eluate patterns presented below.

Since the normal control for melanoma is not available, it was only possible to compare the carbohydrate components of the membrane glycoproteins of melanoma with those of other normal tissues and tissue culture cells. When L-[^{3}H]fucose-labeled glycopeptides derived from melanotic tumors of the liver were cochromatographed with the corresponding ^{14}C-labeled material from a Rous virus-transformed hamster cell (C_{13}/B_4) grown in culture, a relative enrichment of glycopeptides was found in the melanoma that we had previously found to be increased in malignant cells (Chart 1). These glycopeptides elute from a column of Sephadex G-50 in the tube 20 to 28 region,

Table 1

Tissue	Precursor	cpm/mg wet wt	Counts released by trypsin (%)
Liver	L-[^{3}H]fucose	31	51
Kidney	L-[^{3}H]fucose	241	13
Spleen	L-[^{3}H]fucose	40	44
Lung	L-[^{3}H]fucose	49	40
Tumors			
Liver	L-[^{3}H]fucose	34	39
s.c.	L-[^{3}H]fucose	28	56
i.p.	L-[^{3}H]fucose	36	42
Liver	D-[^{3}H]glucosamine	77	44
Kidney	D-[^{3}H]glucosamine	138	24
Brain	D-[^{3}H]glucosamine	39	39
Lung	D-[^{3}H]glucosamine	71	47
Tumors			
Liver	D-[^{3}H]glucosamine	101	40
i.p.	D-[^{3}H]glucosamine	54	50
Lung	D-[^{3}H]glucosamine	110	28

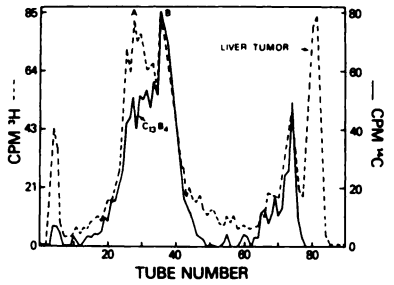

Chart 1. Elution profiles of radioactive glycopeptides from a column of Sephadex G-50 fine. Glycopeptides were prepared from cells grown in the presence of either L-[^{14}C or ^{3}H]fucose. Components from the surface of the cells removed with trypsin were exhaustively digested with Pronase as described previously (2, 3) (see text for details). ^{14}C-Labeled glycopeptides derived from Rous virus-transformed hamster cell (C_{13}/B_4) were cochromatographed with ^{3}H-labeled glycopeptides derived from the surface of melanoma cells (B16) in a solid tissue tumors in liver.

designated "Peak A." The glycopeptides eluting in this region are called "Peak A glycopeptides" as compared to those eluting in the peak following (tubes 35 to 40) which are called "Peak B glycopeptides." There is close correspondence between the peak A and B patterns of the melanoma and the virus-transformed cells.

We have shown previously that these glycopeptides also contain sialic acids. If the glycopeptides are treated with neuraminidase before chromatography, the peak A and B glycopeptides of transformed hamster cells, C_{13}/B_4, lose approximately 3 and 1 residues of sialic acid, respectively (unpublished), become smaller and thus elute later from the Sephadex G-50 column (5, 6). Chart 2 shows that when L-[^{14}C]fucose-labeled glycopeptides derived from melanotic tumors of the liver are incubated with neuraminidase they also lose residues of sialic acid and elute later. Essentially all the sialic acids of these glycopeptides are removed by neuraminidase under the conditions used as shown by colorimetric assay (13).

The elution patterns of glycopeptides from the surface of melanoma cells growing in the liver, lung, i.p., and s.c. have been compared with each other and with equivalent structures from normal liver, lung, spleen, and kidney. A prominent family of Peak A glycopeptides is derived from the melanotic tumor cells growing in various sites in the mouse, similar to those seen in Charts 1 and 2, while in the material derived from normal tissues a major peak is seen in the Peak B area, which corresponds approximately to that of the melanoma Peak B or is slightly to its right, *i.e.*, the glycopeptides from the surfaces of various types of cells in normal tissues are probably smaller. The elution patterns of glycopeptides from normal tissues display a hump in the Peak A area somewhat like control hamster cells in culture (BHK_{21}/C_{13}) (2, 3). In general, the elution patterns in the tube 20 to 50 region of glycopeptides derived from various normal tissues (liver, lung, spleen, kidney) are remarkably similar. Treatment of these glycopeptides with neuraminidase, however, does not alter the shapes or positions of the peaks of glycopeptides in the elution curves. On the other hand, the glycopeptides from all these control tissues (and melanoma) are known to contain sialic acid because they release sialic acid as shown by the thiobarbituric acid assay (13) and are reduced in size by gentle chemical hydrolysis (0.1 N H_2SO_4, 80° for 1 hr) (10). In our experience these hydrolytic conditions remove only sialic acid from the glycopeptides. The apparent resistance of sialic acids in normal tissues to release by neuraminidase is being investigated at the present time.

Double-label experiments have also been using D-[^{14}C, ^{3}H]glucosamine with the same results. The labeling patterns described above were not influenced by the site of injection of the malignant cells.

It has been shown previously that the increase in the relative abundance of Peak A glycopeptides in the surface glycoproteins of hamster cells transformed by Rous virus also takes place in the glycoproteins of the nuclear, endoplasmic reticular, mitochondrial, and lysosomal membranes, *i.e.*, the internal cellular membranes (1); the elution patterns of the labeled glycopeptides of all membranes are much the same. In this study it was also found that the elution profiles of the glycopeptides from the external membranes (trypsinate) and internal membranes (cell pellet) are similar (Chart 3). Comparisons of glycopeptides from internal and external cellular sites of liver, pulmonary, and s.c. tumors give the same results. Thus if there was contamination of surface glycopeptides by those from internal membrane glycoproteins the same results would be obtained. The conditions of trypsin treatment in these experiments were similar to those with hamster cells (1) in which it was clearly shown that the cell (pellet) and surface (trypsinate) fractions were separate.

Melanoma Cells in Culture. A double-label experiment was done on B16 melanoma cells growing in tissue culture. The glycopeptide elution pattern appears to be more simplified than that of the same cells growing as solid tumors in mice (Chart 4). The major peak appears to be Peak A; virtually no Peak B can be seen.

This simplified pattern in which Peak A glycopeptides predominate might reflect a possible lack of restraint on growth and metabolism of cells in culture as compared to cells in tissues. The formation of Peak A glycopeptides is

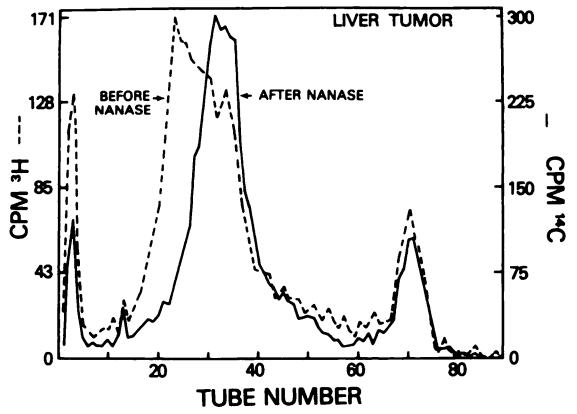

Chart 2. Elution profiles from a column of Sephadex G-50 fine of L-[^{14}C]fucose-labeled glycopeptides treated with neuraminidase (*NANASE*) derived from melanotic tumors of the liver cochromatographed with untreated ^{3}H-labeled glycopeptides from a similar source.

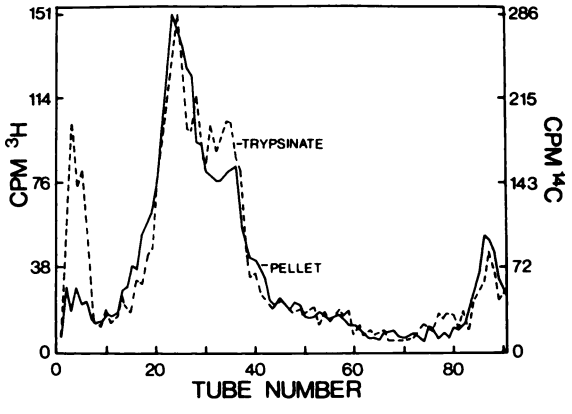

Chart 3. Double-label elution profile of glycopeptides from a column of Sephadex G-50 fine. Glycopeptides derived from the surface (*trypsinate*) of i.p. tumors labeled with L-[^{3}H]fucose, were cochromatographed with glycopeptides from the "internal" (*pellet*) membrane glycoproteins of i.p. tumors labeled with L-[^{14}C]fucose.

dependent on growth (4). Thus cells growing *in vivo* at a slower and less uniform rate may synthesize more Peak B glycopeptides relative to Peak A glycopeptides. The simplified pattern *in vitro* might also be a reflection of the less complex, relatively poor environment of the cell in culture. When treated with neuraminidase, sialic acid is removed from the glycopeptides and they elute later (Chart 4).

High- and Low-Take Lines of Melanoma Cells. Fidler (5, 6) demonstrated that an increased yield of experimental tumors could be obtained from a given number of B16 melanoma cells by repeated passages of lung tumors to *in vitro* culture and then back to lung. In this way, stable melanoma strains of high (F_{10}) and low (F_1) take were obtained. Double-label experiments were done on these high-(F_{10}) and low-(F_1) take lines to see whether a difference in glycopeptides derived from the glycoproteins of the cell surface might be associated with the efficiency of tumor formation. However, there appeared to be few, if any, real differences in the glycopeptide populations of the 2 lines of cells, labeled in culture or as solid tumors in animals. Thus Peak A glycopeptides, although correlated with tumorigenesis (8), are not in this case correlated with the extent to which melanoma cells form tumors in the lung (Charts 5 and 6). On the other hand, the comparisons made in these studies are relatively crude since they are based on a blind separation dependent on size and shape of glycopeptides. Real differences in sugar composition and their linkages would not be detected. Again the more complex glycopeptide pattern of cells growing as solid tumors should be noted.

GENERAL DISCUSSION

An increase in sialic acid-rich glycopeptides in the membrane glycoproteins of malignant cells in tissue culture (1-4, 7, 11, 15, 16) and in human leukemic cells (12) is well documented, and it would appear that the same increase is found in solid melanotic tumors in various sites in mice. In previous studies virus-transformed cells from a variety of animal species were compared with control, untransformed

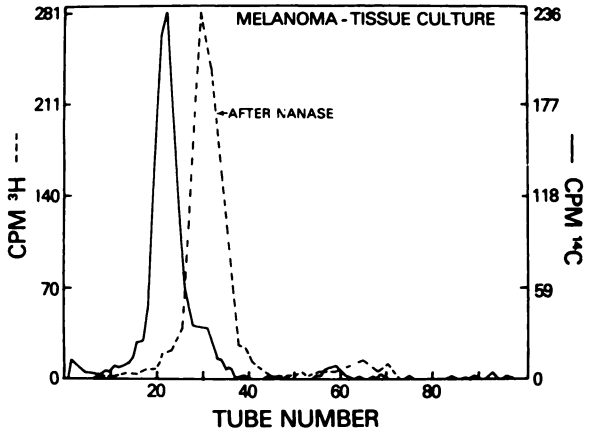

Chart 4. Double-label elution profiles of glycopeptides derived from B16 melanoma cells grown in culture *versus* the same glycopeptides treated with neuraminidase (*NANASE*) (see text for details).

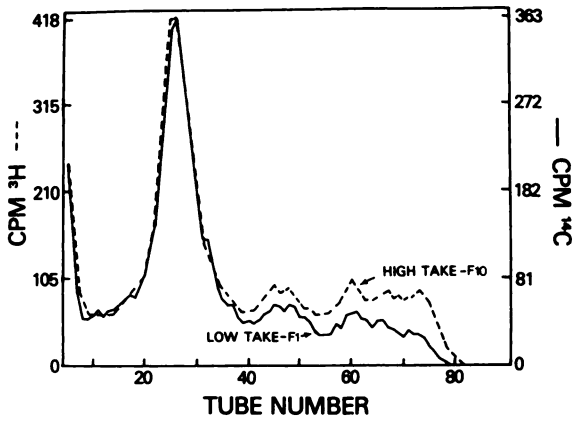

Chart 5. Double-label elution profiles of glycopeptides derived from F_1 (low-take) melanotic cells *versus* those from F_{10} (high-take) melanotic cells (5, 6). Cells were grown and labeled with L-[^{14}C, or ^{3}H]fucose in tissue culture (see text for details).

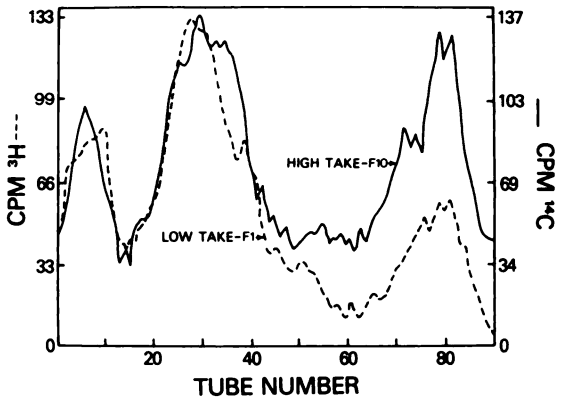

Chart 6. Double-label elution profile of glycopeptides from F_1 and F_{10} melanoma cells growing as solid pulmonary nodules (see legend to chart 5 and text for details).

cells. Unfortunately, normal controls for melanoma cells are not available and so we have used normal tissues from the same animals and virus-transformed hamster cells, C_{13}/B_4, which serve as a standard for comparison with malignant systems previously examined. These double-label comparative experiments lend support to the conclusion that the changes that occur in the carbohydrate component of both surface and internal membrane glycoproteins in cells in culture when they are transformed are also seen in solid melanotic tumors growing in animals. This conclusion is based upon the elution patterns of the melanoma glycopeptides in which a prominent Peak A is evident both *in vivo* and *in vitro*, and also on their susceptibility to neuraminidase.

In previous studies neuraminidase treatment of glycopeptides of control and malignant cells in culture eliminated differences in their size and therefore in their elution profiles (11, 12, 15, 16). While glycopeptides from melanoma cells were significantly reduced in size by neuraminidase treatment, this did not occur with the glycopeptides derived from normal tissue cells treated with enzyme. Glycopeptides from human chronic myelocytic leukemic cells also appear to be unchanged in chromatographic behavior by treatment with

neuraminidase (12). The elimination of size differences by neuraminidase does not mean that glycopeptides have been reduced to identical cores, which had differed only by their sialic acid content. The glycopeptides composition of the Peak A and B areas are complex and are unresolved by Sephadex G-50 (17). Furthermore, it is quite likely that the actual compositions of the Peak A and B glycopeptides of the melanoma differ from equivalent Peak A and B glycopeptides of transformed tissue culture cells although they cochromatograph on Sephadex G-50 columns.

It is doubtful that normal connective tissue cells contributed significantly to the glycopeptide elution patterns of the mouse melanotic tumor. Histological examination has shown that almost all cells in the solid tumor are malignant cells (I. Zeidman, unpublished data). Previous studies have shown that there is little Peak A glycopeptide derivable from the membrane glycoproteins of mouse fibroblasts in culture (3). The elution patterns of the glycopeptides derived from various types of cells (including fibroblasts) of normal liver, lung, spleen, and kidney clearly resemble that of the control fibroblast growing in tissue culture. Although the complexity of the populations of glycopeptides derived from tissues containing a variety of cell types is very great, this complexity is not reflected in the elution pattern, which is based only upon the size and shape of glycopeptides.

The results of this study show that an increase in sialic acid-rich, fucose-containing oligosaccharides bound to internal and surface membrane glycoproteins occurring in malignant cells in tissue culture also is found in solid melanotic tumors *in vivo*.

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