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Appearance of Altered Cell-surface Fucosyl Glycopeptides in Concomitance with Chromosomal Alterations in the Gross Virus-infected Pre-leukemic Thymus of the Rat*

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Abstract—*The appearance of a class of fast-eluting cell-surface glycopeptides that are encountered almost exclusively in malignant and certain pre-malignant cells was monitored in the course of leukemogenesis in the thymus of rats injected at birth with Gross leukemia virus. The altered glycopeptides appeared as early as* 15 days *after virus injection, when the animals were still clinically healthy and no histological signs of the disease were present in the thymus. Their amount was further increased at* 30 days, *and reached a maximum in the fully developed lymphoma. The development of this early phenotypic marker of malignancy appeared to be concomitant with that of chromosomal anomalies in the thymus. Since these anomalies are non-random, the existence of a causal relationship between the glycopeptide change and the loss of specific chromosomes might be hypothesized.*

INTRODUCTION

THE DETECTION of early events in the process of neoplastic transformation has important implications. It may help in understanding the basic mechanisms of carcinogenesis and, from a more practical point of view, it may be of assistance in the early diagnosis of human cancers.

Recently it has been shown [1] that the early stages of Gross virus-mediated leukemogenesis in the rat are characterized by the appearance of widespread chromosomal anomalies in the thymus. These anomalies are already detectable as early as 7 days after the injection of the virus in the newborn animal, and increase progressively thereafter. Thirty days after virus injection, when the animal is still clinically healthy, the chromosomal pattern in the thymus is virtually superimposable on that observed in the fully developed thymic lymphoma. In particular, chromosomes 1, 2, 9, 12, 18–20, and X in females and Y in males appear to be preferentially lost.

The purpose of the present investigation was to determine whether these genotypic alterations are accompanied by phenotypic changes at the cell surface, generally accepted to be of major importance with respect to malignant behavior of cells [2–5]. In particular, we have focused our attention on the family of fucose-containing glycopeptides that have shown to be consistently altered in tumor cells (reviewed in [6–8]), the alteration being based on more complex, branched carbohydrate structures, in many cases enriched for terminal sialic acid residues [9–11]. This particular change in glycopeptide structure was demonstrated in human-derived malignant tumors [12–14], as well as in peripheral blood cells of patients with hematopoietic dysplasia just before the onset of leukemia, or in patients in complete remission but imminent to the onset of a relapse ([15] and unpublished observations). Moreover, this particular glycopeptide change appears early in the process of neoplastic transformation of lymphoid cells, as indicated by

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investigations with human B lymphocytes infected *in vitro* with the Epstein–Barr virus [16].

In view of the above-mentioned data, it seemed appropriate to look at this alteration of lymphoid cell-surface glycopeptides as a possible early marker in the course of leukemogenesis induced by the Gross virus in the rat. Simultaneously we have tried to establish a possible correlation between the observed chromosomal changes [1] and the indicated modifications of the cell-surface phenotype.

MATERIALS AND METHODS

Virus preparation and tumor induction

A rat-adapted strain of the Gross leukemia virus, originally obtained from Dr. L. Gross as a generous gift, was subsequently prepared from lymphomatous tissues as previously described [1]. Tumors were induced by injecting approximately 0.5 ml of virus suspension intraperitoneally in 2- to 6-day-old rats of the inbred strain F344 (Charles River Breeding Laboratories, Inc., Wilmington, MA). A lymphoblastic lymphoma involving thymus and spleen developed within 3–5 months, and its presence could be recognized by clinical manifestations such as loss of weight, nasal bleeding and ruffled fur [1]. Animals were sacrificed at this stage or at 4, 15 and 30 days respectively after virus injection. Untreated control rats were killed at comparable time intervals.

The percentage of immature thymocytes in the different Gross virus-infected and control thymocytes was determined by means of fluorescein-conjugated peanut lectin (FITC-PNA) [17, 18]. PNA was purified according to Irlé [19] and conjugated to FITC according to Clark and Sheppard [20]. Thymocytes (3×10^6) were suspended in 0.1 ml HEPES-buffered salt solution (HBSS) containing 0.1% sodium azide and incubated with FITC-PNA (0.1 ml of a solution containing 350 μg/ml) for 20 min at 4°C. After 3 washings at 4°C in HBSS containing sodium azide, a drop of cell suspension was put on a clean slide and observed by microscopy with transmitted fluorescent light.

Cytogenetics

Chromosomal analysis was performed as previously described [1] on aliquots of the specimens that were also used for the preparation of fucosyl glycopeptides, i.e. thymic lymphomas, as well as pre-leukemic and normal thymi of various ages. For each sample 50 mitoses were counted directly under the microscope and the count confirmed on photographs.

Fucosyl glycopeptides

Cell-surface fucosyl glycopeptides were prepared essentially as described previously [15]. Briefly, thymus-derived tumors as well as pre-leukemic and normal thymi (one thymus for each glycopeptide investigation) were finely minced with scissors in RPMI 1640 medium with 10% fetal calf serum (Eurobio, Paris, France). Single-cell suspensions were obtained by mild pipetting of minced tissue through a Pasteur pipet several times. Residual tissue fragments were discarded after settling to the bottom of the tube. The single-cell suspension was washed and re-suspended in the above-mentioned growth medium to a cell density of $1\text{–}2\times10^7$ cells/ml. Changes in the fucosyl glycopeptides of the cell membrane were monitored by differentially labeling control and test cells containing $1\text{–}2\times10^7$ cells/ml with 10–20 μCi/ml of L-[1-^{3}H]-fucose (2.8 Ci/mmol; New England Nuclear, Boston, MA) or 5–10 μCi/ml of L-[1-^{14}C]-fucose (60 Ci/mol, The Radiochemical Centre, Amersham, U.K.) for 20 hr at 37°C in a humidified 5% CO_2 incubator. The radioactive ^{3}H- and ^{14}C-label was exchanged between test and control cells to investigate its possible effect on the gel-filtration profile. Supernatant-free cells were washed twice with HEPES-buffered Hank's salt solution and glycoprotein was digested from the cell surface by a very mild pronase digestion (0.02% pronase, B-grade, Calbiochem, San Diego, CA) in the same buffer for 30 min at 37°C in a slowly shaking waterbath. The supernatant was further digested with pronase as described earlier [15] and concentrated. Samples were desalted by gel filtration–centrifugation on Biogel P2. The desalted glycopeptides of cells under investigation were combined with the differentially labeled control glycopeptides and co-chromatographed on a 1.5×100 cm column containing Biogel P10, 200–400 mesh, and Sephadex G50 superfine (2:1) as described earlier [15, 21].

Thymidine incorporation

The proportion of cells engaged in DNA synthesis in the tissues under investigation was determined following the method of Priest [22], with minor modifications. Samples of 10^7 cells were incubated for 2 hr at 37°C in growth medium containing 10 μCi of [^{3}H]-thymidine, (20–30 Ci/mmol, The Radiochemical Centre, Amersham). After the incubation period the cells were recovered by centrifugation, fixed for 30 min with methanol:acetic acid (3:1 v/v) and washed 4 times with the same fixative. A drop of the cell suspension was spread on a clean, wet slide and air-dried. The slides were coated with NTB-2 Kodak emulsion, dried and exposed for at least 7

days, after which they were developed, fixed and stained for 15 min with 5% Giemsa in McIlvaine buffer, pH 6.0. The proportion of labeled cells was determined by scoring approximately 500 cells for each sample.

RESULTS

As described previously [1], a lymphoblastic lymphoma involving thymus, spleen and lymph nodes develops in approximately 75% of the animals injected at birth with the Gross virus, after a latency of 3–5 months. The animals that had not yet developed a tumor when killed appeared clinically healthy, with thymus and spleen appearing normal for their age. At 30 days after virus injection the histological appearance of the thymus was either normal or showed at most a modest increase of the medullary tissue, with corresponding thinning of the cortex.

The suspended cells obtained from normal, pre-malignant and thymic lymphoma looked intact even after 20 hr incubation in RPMI 1640 or McCoy 5A modified medium with 10% fetal calf serum. Viability as detected by trypan blue exclusion showed viabilities ranging from 70 to 95%.

The percentage of immature thymocytes in the different Gross virus-infected and control thymocytes as determined by means of FITC-PNA was very similar, and varied from 70 to 79% (mean 73%). The cells from the established lymphoma had slightly higher values (mean 81%), while the intensity of fluorescence in the latter cells was enhanced compared with the other cell preparations. These values correspond with those found in normal mouse and human thymocytes [17, 18], ranging from 60 to 80%. Chromosomal analysis of normal and virus-transformed thymus cells and the concomitant appearance of fast-eluting fucosyl glycopeptides are reported in Table 1. In the control thymi (from untreated rats of either 4 or 30 days of age) the proportion of aneuploid mitoses was on average 5%. A similar value was observed in the thymi of rats killed at 7 days of age, 4 days after virus inoculation. The proportion of aneuploid mitoses increased to 26.6% at 15 days post-virus injection and to 40.0% at 30 days. In the lymphomas the percentage of aneuploid mitoses was 43.7%. These results agree completely with those previously reported [1]. Fucosyl glycopeptides of pre-leukemic and leukemic thymi were compared with those of differentially labeled control thymi of similar age. Figure 1(A) (fraction 40–50) shows the existence of the so-called 'fast-eluting glycopeptides' as obtained from the cell surface of tumor cells. At day 4 (Fig. 1D) no change in the glycopeptide elution pattern was observed. A first but minute change was observed on day 15 after virus inoculation, as shown in Fig. 1(C) (fraction 40–50). This alteration was predominant in the 30-day pre-leukemic thymus (Fig. 1B, fraction 35–45) and became maximal in the lymphoma (Fig. 1A). The change in the fucosyl glycopeptide pattern is expressed as the number of fractions eluting in front of the control as used earlier [12], and the results are summarized in Table 1.

To investigate possible growth-induced changes of the glycopeptides, thymocytes obtained from rats of 4, 15, 30 and 90 days of age and leukemic thymocytes were labeled with [^{3}H]-thymidine. At the same time we investigated the effective fucose incorporation, defined as the fucose label which remains in the macromolecules after pronase

Table 1. Proportion of aneuploid mitoses and the relative appearance of fast-eluting glycopeptides (G.P.) in the thymus of control and virus-injected rats and in lymphomas

Source of cells	Relative appearance* of fast eluting G.P.	aneuploid mitoses (% of total)
Normal thymus:		
4 days	0 (3)	6.0 ± 4.3 (9)
30 days	0 (2)	5.2 ± 4.1 (5)
Thymus of virus-injected rats after:		
4 days	0 (2)	4.0 ± 3.6 (8)
15 days	+/0 (4)	26.6 ± 8.1 (6)
30 days	+ (4)	40.0 ± 8.9 (6)
Lymphomas	++ (4)	43.7 ± 9.1 (2)

Figures in parentheses denote the number of experiments performed.

*Change in the fucosyl glycopeptide profile on gel-filtration columns expressed as the number of fractions eluting in front of the control in Fig. 1, as used previously [12].

Table 2. Fucose incorporation and proportion of cells active in DNA synthesis in the thymus of control rats of different ages and in lymphomas

Source of cells	Effective fucose incorporation* (dpm/10^6 cells)	S-phase cells (% of total)
Normal thymus:		
4 days	2523 ± 200 (3)	20 ± 6 (5)
15 days	1308 ± 190 (4)	13 ± 3 (5)
30 days	321 ± 67 (4)	13 ± 4 (5)
90 days	276 ± 52 (3)	8 ± 2 (5)
Lymphoma	356 ± 68 (3)	22 ± 10 (4)

Figures in parentheses denote the number of experiments performed.
*Radioactive-labeled fucose metabolically incorporated in macromolecules excluded from Biogel P2 after pronase digestion.

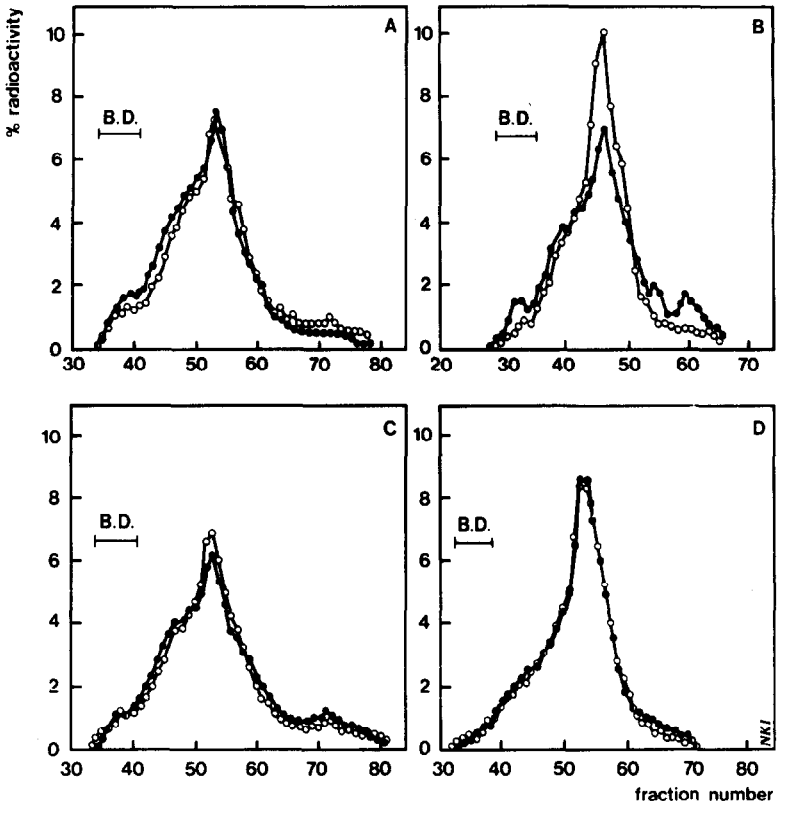

Fig. 1. Gel-filtration profiles of surface glycopeptides from Gross virus-infected (•) and uninfected control (○) thymocytes of comparable age. The Gross virus-infected cells were incubated with [^{3}H]-fucose and the control cells with [^{14}C]-fucose. The resulting glycopeptides were digested with pronase and subsequently co-chromatographed on a gel-filtration column. The charts A, B, C and D represent the surface glycopeptide distribution patterns of cells from a lymphoma and from thymi taken 30, 15 and 4 days respectively after Gross virus injection. BD, blue dextran-2000.

digestion and desalting on Biogel P2. Table 2 shows that the 4-day thymus contained approximately 20% S-phase cells, this number decreasing rapidly with age. In the lymphomas the proportion of S-phase cells varied widely, with a mean value of 22%, comparable to that of the 4-day control thymus. The effective fucose incorporation was 8-fold higher in the thymocytes of the 4-day animal than in the 30-day control or the leukemic thymocytes (Table 2). In this respect the pre-leukemic thymocytes behaved similarly to the control thymocytes of comparable age (data not shown). Exchange of radioactive ^{3}H- and ^{14}C-label between test and control cells had no effect on the elution profile. To investigate whether the differences as observed between animals of different ages could influence the glycopeptide distribution on gel filtration, we compared

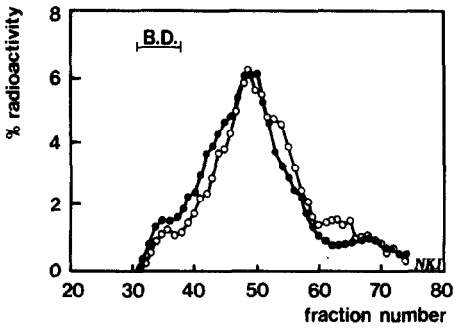

Fig. 2. Gel-filtration profile of surface glycopeptides from a thymic lymphoma (•) and from control thymocytes obtained from a 4-day-old animal (○) having a comparable proportion of S-phase cells. For explanation see legend to Fig. 1.

glycopeptides of cells with a comparable percentage of S-phase cells, i.e. the 4-day control with the lymphoma cells, as shown in Fig. 2. From this result it is apparent that the observed glycopeptide change became even more pronounced (cf. Fig. 1A).

DISCUSSION

Glycopeptide changes in the form of 'fast-eluting glycopeptides' on the cell surface of malignant transformed cells have been recognized in a variety of cell systems. This alteration seems to be firmly connected with the tumorigenic character of cells [6, 8, 23–26] and can be dissected from immortalization and non-tumorigenic transformation [16, 24, 27, 28]. It is of considerable importance to know whether the generation of these fast-eluting glycopeptides is an epiphenomenon of an already existing tumor or is directly involved in the early steps of tumor formation. In the latter case a rapid appearance of these altered surface glycopeptides shortly after carcinogenic induction is to be expected, whereas a delayed appearance would indicate its secondary involvement. The rat thymus system offers the possibility of accurately following with time the leukemic development and the subsequent alterations in surface glycopeptides. After tumor induction by Gross leukemia virus, the very early steps of leukemogenesis can be monitored by the appearance of conspicuous chromosomal anomalies in otherwise normal (morphological and histological) thymi [1].

Exposition of the fast-eluting glycopeptides on the cell surface first became noticeable on day 15 after Gross virus inoculation and became maximal in the established tumor. The time course of the appearance of fast-eluting glycopeptides correlated well with that of the chromosomal anomalies. The glycopeptide profile, however, was altered to a minor extent as compared with that found in mouse or human leukemia cells [12, 26]. Nevertheless, this small change appeared to be very reproducible and the elution profile was insensitive to exchange of radioactive labeled fucose.The reason for such a small shift is not known. A possible explanation is that only part of the leukemic thymocytes were malignantly transformed. Some tumors tend to increase their fast-eluting glycopeptides considerably after becoming transplantable [29].

On the other hand, considerable differences were noted in the effective fucose incorporation and in the percentage of S-phase cells in control thymi of different ages being maximal in the 4-day thymi. The percentage of S-phase cells in the 4-day control thymi was comparable to that found in the lymphomas. Nevertheless, the glycopeptides of the 4-day thymocytes appeared to elute more slowly on gel filtration than those from 15-day or older thymocytes. From this result it is deducted that growth *per se* is not instrumental in the generation of fast-eluting glycopeptides, in agreement with our earlier findings on exponentially growing T-lymphocytes [16] and the results from De Leij *et al.* [27] on rat kidney cells transformed by specific non-oncogenic fragments of adenovirus type 12 DNA.

In summary, the present experiments show that the emergence of fast-eluting fucosyl glycopeptides on the cell surface may be considered as an early phenotypic marker in the course of Gross virus-induced leukemogenesis in the rat, being already present at the time when chromosomal aberrations become apparent. At that time the animals are still clinically healthy, possessing histologically normal thymi. Although much more investigation has to be performed to establish a primary involvement of changed surface glycopeptides in the process of oncogenesis, it is clear from the present study that these changes at least meet one basic requirement: the swift recruitment of fast-eluting fucosyl glycopeptides on the cell surface after oncogenic induction. A similar swift appearance of fast-eluting glycopeptides seems to occur on human B lymphocytes infected by Epstein–Barr virus *in vitro* [16] and on peripheral blood leucocytes of patients with a developing leukemia ([15] and unpublished results). Morphological and histological examination of the peripheral blood leucocytes of these pre-leukemic patients revealed no deviation from normal leucocytes. Nevertheless, the fast-eluting glycopeptides were already present on the surface of these leucocytes, thus predicting to a high degree of certainty the onset of leukemia [15, 30].

Recent investigations in our laboratory on differentiation of leukemic cells *in vitro* have indicated that despite the gained morphological

and functional normal capacities, these matured cells still express fast-eluting glycopeptides on their surface [31]. This finding could possibly explain the presence of changed surface glycopeptides on peripheral blood leucocytes of preleukemic patients. Although this human cell system is less defined in the sense that the time of leukemic induction is unknown, it also points to an apparent swift appearance of fast-eluting glycopeptides on the cell surface and is for that matter consistent with the present finding.

It has been shown previously that the above-mentioned chromosomal alterations have some specificity in that chromosomes 1, 2, 9, 12, 18–20, and X in females and Y in males are preferentially lost [1]. We are led by the present findings to postulate that one or more of the preferentially lost chromosomes may play a role in the expression of the observed change in glycopeptides, that in turn could be instrumental in the loss of ordered growth of the affected thymus cells. To further substantiate a direct relationship between loss of chromosomes and surface glycopeptide change, thymocytes should be separated by means of a fluorescence-activated cell sorter (FACS) by exploiting the small changes in DNA content of the committed cells.

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