Cell surface glycolipids of transformed NIH 3T3 cells transfected with DNAs of human bladder and lung carcinomas

Shigeru Tsuchiya* and Sen-itiroh Hakomori

Program of Biochemical Oncology, Fred Hutchinson Cancer Research Center, Departments of Pathobiology, Microbiology and Immunology, University of Washington, 1124 Columbia Street, Seattle, WA 98104, USA

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Neutral glycolipids and gangliosides of NIH 3T3 cells oncogenically transformed by transfection of DNAs from human lung carcinoma (Lx-1) and human bladder carcinoma (Ej) have been investigated. The chemical quantity and the degree of cell surface exposure of gangliosylceramide (Gg₃) were greatly enhanced in NIH 3T3 cells transformed by transfection of DNAs of either Lx-1 or Ej carcinoma cells. An identical but more conspicuous change in cell surface exposure of Gg₃ was observed in BALB/c 3T3 cells transformed by murine sarcoma virus Kirsten strain, but the same glycolipid was absent in the original Lx-1 or Ej human carcinomas. The mechanism that defines the chemical quantity and the organization of glycolipids is controlled by multiple factors. These include not only the quantity but also the organization of glycosyl transferases and hydrolases in membranes. This also involves membrane dynamics regulated through a cytoskeletal-membrane conjunction which may determine the degree of glycolipid exposure at the cell surface. The similarity of the chemical and organizational change of a single glycolipid, Gg₃, between 3T3 transformants by Kirsten murine sarcoma virus and those by transfection of human cancer DNAs may indicate a common biochemical basis triggered by activation of the oncogene.

Key words: gangliosylceramide (Gg₃)/cell surface exposure/monoclonal antibody/carcinoma DNAs/transfected 3T3 cells

Introduction

Of many chemical changes detected at the surface of cells oncogenically transformed by tumor viruses and chemical carcinogens, the change of glycolipid composition, metabolism and organization is one of the most common (Hakomori, 1981; Brady and Fishman, 1974). Since glycolipid composition and metabolism vary extensively from one type of cell to another, the pattern of change associated with oncogenic transformation also varies extensively. Nevertheless, there are a few common changes which have been observed in a number of transformed cells, i.e., incomplete synthesis, neosynthesis, and changes in organization (Hakomori, 1981; Brady and Fishman, 1974; Hakomori and Kannagi, 1983; Gahmberg and Hakomori, 1975). Recently, cellular genes capable of inducing oncogenic transformation have been identified by transfection of DNAs of both normal and neoplastic cells (Krontiris and Cooper, 1981; Shih *et al.*, 1981). NIH 3T3 mouse fibroblasts have been found to be transformed by transfection of DNAs isolated from a

number of human cancer cell lines. The DNA sequences in some tumors that are capable of inducing oncogenic transformation in NIH 3T3 cells have been identified as homologues of the well-established transforming genes of retroviruses (McCoy *et al.*, 1983; Murray *et al.*, 1981; Der and Cooper, 1983), although others are not related to retrovirus transforming genes (Bishop, 1983). The activated transforming gene of the human bladder carcinoma cell lines Ej and T24 and that of the lung carcinoma cell line Lx-1 are the cellular homologues of the transforming genes of Harvey (*ras*^H) and Kirsten (*ras*^K) sarcoma virus, respectively (Der *et al.*, 1982; Ellis *et al.*, 1981; Santos *et al.*, 1982). The viral *ras*^H and *ras*^K genes encode closely related proteins with a relative mol. wt. of 21 000, designated p21. Thus far, the same *ras* gene class has been observed to be responsible in transfected-transformed NIH 3T3 cells. Since we and others previously observed a remarkable change of glycolipid synthesis in 3T3 cells transformed by murine sarcoma virus (Brady and Fishman, 1974; Rosenfelder *et al.*, 1977; Lockney *et al.*, 1982), it was of interest to examine the change of glycolipids in transformed-transfected NIH 3T3 cells in comparison with those in murine sarcoma virus transformants. The chemical level and the cryptic organization of a single glycolipid species, gangliosylceramide (Gg₃), is greatly enhanced in transformed-transfected NIH 3T3 cells in a similar fashion to the same molecule in 3T3 cells transformed by murine sarcoma virus.

Results

Glycolipid composition of NIH 3T3 cells transfected-transformed by human cancer DNA and those transformed by murine sarcoma virus

The chemical quantity of Gg₃ in NIH 3T3 cells oncogenically transformed by transfection of DNAs of human bladder carcinoma Ej and lung carcinoma Lx-1 was found to be significantly higher than in parent NIH 3T3 cells (double spots 4 in lanes B and D as compared with lane A, Plate A, Figure 1). An accumulation of Gg₃ was also found in mouse 3T3 cells transformed by murine sarcoma virus Kirsten strain (3T3 KiMuSV) (double spots 4 in Lane F, Plate A, Figure 1), supporting the results of previous work (Rosenfelder *et al.*, 1977). The enhanced synthesis of Gg₃ in 3T3 KiMuSV cells has been previously demonstrated, and Gg₃ is regarded as a tumor-associated antigen (Rosenfelder *et al.*, 1977). In contrast, human bladder carcinoma Ej and lung carcinoma Lx-1 did not contain Gg₃. A large quantity of double bands (one band with a slightly higher mobility than Gg₃ and the other with the same mobility as the upper band of Gg₃) was found in Ej tumor cells (doublet band 3 in lane C, Figure 1), and was identified as Gb₃. The presence of a high concentration of Gg₃ in transfected-transformed 3T3 cells as well as in 3T3 KiMuSV cells was confirmed by strong immunostaining with anti-Gg₃ monoclonal antibody (lanes B and D in Figure 2 for transfected-transformed 3T3 cells; lane F in Figure 2 for 3T3 KiMuSV cells). A large quantity of glycolipid in human blad-

*To whom reprint request should be sent.

Fig. 1. Glycolipid pattern of NIH 3T3 cells and that of cells transfected-transformed by human cancer DNAs. **Plate A**, neutral glycolipid fraction. **Lane A**, NIH 3T3 cells; **lane B**, NIH 3T3 cells transfected-transformed by DNAs of human bladder carcinoma Ej; **lane C**, human bladder carcinoma Ej; **lane D**, NIH 3T3 cells transfected-transformed by DNAs of human lung carcinoma Lx-1; **lane E**, human lung carcinoma Lx-1; **lane F**, BALB/c 3T3 cells transformed by murine sarcoma virus Kirsten strain. **Lanes R₁** and **R₂**, Folch's upper and lower phase glycolipids of human blood group O erythrocytes, respectively. Bands identified by numbers are as follows: 1, ceramide monohexoside; 2, ceramide dihexoside (lactosylceramide); 3, globotriaosylceramide (Gb₃); 4, gangliotriaosylceramide (Gg₃); 5, globoside; 6, H₁ glycolipid; 7, H₂ glycolipid; 8, H₃ glycolipid. **Plate B**, ganglioside fraction. **Lanes A–E**, gangliosides prepared from the same cells as **lanes A–E** of **Panel A**. 1, GM₃; 2, GM₂; 3, GM₁; 4, GD_{1a}. A solution of glycolipid derived from an equal amount of cells (5 mg of cellular protein) is applied in each lane on t.l.c. analysis; i.e., glycolipid fraction prepared from $3-6 \times 10^8$ cells (90–180 mg protein) was dissolved in 90–180 μ l of chloroform-methanol and 5 μ l aliquots were used for analysis.

der carcinoma Ej, which appeared as a double band in the location of Gg₃ (band 3 in lane C, Plate A, Figure 1), was identified as Gb₃ because of its high reactivity with a monoclonal antibody to Gb₃ on t.l.c. immunostaining (lane I; reference Gb₃ in lane L, Figure 2).

Surface exposure of Gg₃ in various transformed 3T3 cells as above

The degree of Gg₃ exposure at the cell surface, determined by galactose oxidase and tritiated borohydride, was found to be significantly enhanced in 3T3 cells transformed by human DNA from bladder carcinoma Ej and lung carcinoma Lx-1 as well as in 3T3 KiMuSV cells. The degree of exposure was much higher in 3T3 KiMuSV cells (lane D, Figure 3) than in intact human cancer DNA transfected-transformed 3T3 cells (lanes B and C, Figure 3). Nevertheless, transfected-transformed 3T3 cells showed a greater exposure than intact non-transfected NIH 3T3 cells (lane A, Figure 3). The degree of exposure was also clearly different in EDTA-detached cells, as the label in Gg₃ was clearer than in cells without EDTA treatment (lanes F and G, Figure 3). A clearly greater exposure of Gg₃ in transfected-transformed 3T3 cells (lanes F and G, Figure 3) than in control NIH 3T3 cells (lane E, Figure 3) was observed.

The ganglioside pattern of NIH 3T3 cells used for the transfection experiment lacked GM₁ (lane A, Plate B, Figure 1), indicating that the cells were partially transformed, since both BALB/c and Swiss 3T3 cells were characterized by the

presence of a large quantity of GM₁. No essential changes of ganglioside pattern were observed after oncogenic transformation by transfection of human DNAs (lanes B and D, Plate 2, Figure 1). No changes in immunostaining by monoclonal antibodies directed to NeuAc α 2 $\rightarrow$ 6Gal or NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 3Gal α 1 $\rightarrow$ 4Gal structures were observed in the ganglioside fraction.

Discussion

Dramatic changes in glycolipid composition, metabolism and organization have been observed in many tumor cells and may reflect a developmental arrest or a functional alteration of cell membranes such as aberrant cell social behavior or loss of cell growth control (Hakomori, 1981; Hakomori and Kannagi, 1983). Similar types of changes were found in oncogenic transformants irrespective of the kind of oncogenic viruses or chemical carcinogens. On the other hand, different types of glycolipid changes were observed depending on the host cell, although the cells were transformed by the same oncogenic virus (Hakomori *et al.*, 1968; Yogeewaran *et al.*, 1972; Fishman *et al.*, 1974). A blocked synthesis of GM₃ was found in BHK transformants caused by polyoma virus, SV40 virus, as well as Rous sarcoma virus (Hakomori *et al.*, 1968), whereas mouse fibroblast 3T3 showed a blocked synthesis of higher gangliosides by polyoma, SV40, and murine sarcoma virus (Yogeewaran *et al.*, 1972; Fishman *et al.*, 1974). GM₃ synthesis in chick embryonic fibroblast transformed by Rous

Fig. 2. Immunostaining pattern of Gb₃ and Gg₃ of NIH 3T3 cells and transfected-transformed derivatives. Immunostaining patterns of anti-Gg₃ monoclonal antibody (2D4) are shown in lanes A–F and those of anti-Gb₃ monoclonal antibody (38-13) are shown in lanes G–L. Lanes A and G, non-transfected NIH 3T3 cells; lanes B and H, transfected-transformed 3T3 cells by DNAs of human bladder carcinoma Ej; lanes C and I, human bladder carcinoma Ej; lanes D and J, NIH 3T3 cells transfected-transformed by DNAs of human lung carcinoma Lx-1; lanes E and K, human lung carcinoma Lx-1; lane F, BALB/c 3T3 cells transformed by murine sarcoma virus Kirsten strain; lane L, reference globotriaosylceramide. Arrows indicate the bands of Gg₃. The same amount of glycolipid fraction as was used for chemical analysis (see legend for Figure 1) was applied on t.l.c.

sarcoma virus correlates well with the level of transforming gene (*src*) activity. The synthesis of GM₃ in chick embryonic fibroblasts infected with temperature-sensitive mutants of Rous sarcoma virus is blocked at the permissive temperature and unblocked at the non-permissive temperature (Hakomori *et al.*, 1977). Similarly, GM₃ synthesis in BHK cells transformed by polyoma virus correlates with the polyoma transforming gene (Gahmberg *et al.*, 1974). Transformation-dependent glycolipid changes may reflect the enzyme systems and their organization pre-assembled in host cells. Thus, the same oncogenic virus, and hence the same transforming gene, could induce different changes in glycolipid synthesis depending on the host cell.

Recent developments in demonstrating transforming activity of human tumor DNAs provides an interesting model to examine whether glycolipid changes observed previously in various transformants by oncogenic viruses are also found in transformants induced by human tumor DNAs. So far, a successful transformation has been observed only in NIH 3T3 cells transfected with various human DNAs. The model used in this experiment was established by Cooper and associates using cells transfected with human bladder carcinoma Ej and lung carcinoma Lx-1. The transfected genes from Ej and Lx-1 were identified as *ras*^H (identical to Harvey sarcoma virus oncogene) and *ras*^K (Kirsten sarcoma virus oncogene), respectively (McCoy *et al.*, 1983; Murray *et al.*, 1981; Der and Cooper, 1983). The gene products were identified as p21,

Fig. 3. Fluorogram of cell-surface labeled glycolipids separated on t.l.c. Lanes A–D, glycolipids directly labeled on intact cells; lanes E–G, glycolipids of cells surface-labeled after detachment by EDTA. Lanes A and E, non-transfected NIH 3T3 cells; lanes B and F, NIH 3T3 cells transfected-transformed by DNAs of human bladder carcinoma Ej; lanes C and G, NIH 3T3 cells transfected-transformed by DNAs of human lung carcinoma Lx-1; lane D, BALB/c 3T3 cells transformed by murine sarcoma virus Kirsten strain. Glycolipid fraction derived from an equal amount of surface-labeled cells was used for t.l.c. autoradiogram. The amount of glycolipid fraction applied in each t.l.c. lane was equivalent to 0.5 mg protein for A–D, and 0.8 mg protein for E–G.

whose function is yet unidentified (Der and Cooper, 1983). It is clear, however, that a series of consequent events is followed by a cascade mechanism which is probably triggered by synthesis of this protein, although any correlation of the function of p21 and expression of multiple phenotypes of transformed cells, i.e., loss or disorganization of actin cables, disappearance of fibronectin, enhanced sugar transport, and Gg₃ changes, is totally unknown. Enhanced cell surface exposure of Gg₃ could be caused by a change of membrane dynamics involving disorganization of cytoskeletal-membrane conjunction. Interestingly, Gg₃ is absent in both human cancer cell lines Ej and Lx-1. This is, however, not unusual, since the same transforming gene may affect different phenotype changes depending on the assembly and the organization of genes directing glycosylation. It is possible that these human carcinoma cell lines are defective in the organization of the genes which arouse Gg₃ accumulation, whereas the genes of 3T3 cells are organized in such a way that accumulation as well as exposure of Gg₃ is ensured. Recently, cell surface glycopeptides were found to be altered to 'highly branched' at the core carbohydrate structure in transformed NIH 3T3 cells transfected by DNAs of neuroblastoma cells (Glick *et al.*, 1983). Thus, transfected-transformed cells are generally characterized by typical changes established for glycoproteins as well (Warren *et al.*, 1978).

The change of Gg₃ described in this paper and by others

(Rosenfelder *et al.*, 1977; Young and Hakomori, 1981) may be instrumental and important in establishing various membrane phenotypes for transformed cells. In mouse lymphoma LK5178, Gg₃ was also found to accumulate at the cell surface, and may have some role in function of the transferrin receptor (Y.Okada and S.Hakomori, unpublished observation). Further studies on the functional role of Gg₃ changes as correlated to tumor cell phenotype are in progress.

Materials and methods

Cells and cell culture

NIH 3T3 cells and their transformants isolated after transfection with human bladder carcinoma Ej and lung carcinoma Lx-1 are the same as previously described (Krontiris and Cooper, 1981; Der *et al.*, 1982) and are hereby designated N/Ej and N/Lx-1, respectively. All these cell lines, including the original Ej and Lx-1 carcinomas, were kindly donated by Dr. Geoffrey M. Cooper of Sidney Farber Cancer Institute, Harvard Medical School, Boston, MA. Cells were grown in Dulbecco-modified Eagle's medium supplemented with 10% fetal calf serum in a humidified 5% CO₂ atmosphere.

Glycolipid analysis and cell surface labeling

Cells were harvested with rubber or by EDTA solution in phosphate-buffered saline and were extracted in chloroform-methanol (2:1, v/v), and the glycolipids were separated from other lipid classes by the acetylation procedure (Saito and Hakomori, 1971). The neutral and acidic (ganglioside) fractions were separated by DEAE-Sephadex chromatography (Yu and Ledeen, 1972). In some cases, glycolipids were separated into polar and non-polar fractions by Folch's partition (Folch-Pi *et al.*, 1951) before further fractionation. Glycolipids were analyzed on high performance t.l.c. with orcinol and resorcinol spray. The amount of glycolipid applied on t.l.c. analysis was equalized based on cell number and cell protein (see legend for Figures 1–3). Cells were surface labeled with galactose oxidase and tritiated borohydride according to the method previously described (Gahmberg and Hakomori, 1973). To confirm the identification of glycolipids, immunostaining was performed on high performance t.l.c. by the procedure described by Magnani *et al.* (1980) and modified by Kannagi *et al.* (1982). Monoclonal antibodies directed to Gg₃ (IgM 2D4; Young *et al.*, 1979), nLc₄ or nLc₆ (IgM IB2; Young *et al.*, 1981), Gb₃ (rat IgM 38-13; Nudelman *et al.*, 1983), a new monoclonal antibody directed to NeuAcα2–6Gal (IgG2b IB9; S.Hakomori, C.M.Patterson, E.Nudelman and K.Sekiguchi, unpublished data) and to NeuAcα2–3Galβ1–3GalNAcβ1–3Galα1–4Gal (813, IgM; R.Kannagi, S.Hakomori, D.Solter and B.B.Knowles, unpublished data) were used for immunostaining.

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