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EFFECTS OF N-ACETYLGLUCOSAMINE ON CELL SURFACE GLYCOPROTEIN AND CELL BEHAVIOR OF GLUCOSAMINE REQUIRING MUTANT DERIVED FROM CHINESE HAMSTER LUNG CELLS*)

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ABSTRACT

We have reported preliminary biological properties of mutant, G72-8, derived from Chinese hamster lung cells (Onoda, 1980[1]): the mutant is characterized by low adhesion to substratum, round shape, increase in spontaneous aggregation, and decrease in growth rate and these altered properties restored to normal by addition of GlcNAc. In this study, when L-fucose was used as radioactive precursor, the rate of L-fucose incorporation into macromolecules was markedly stimulated by addition of GlcNAc to the mutant. Lactoperoxidase-catalyzed iodiation of the cell surface shows that the high-molecular weight cell surface protein detected in the parent cells was reduced on the mutant and restored reversibly to normal by addition of GlcNAc. A random migration of the mutant cells also was reverted to the directional moving of the parent cells by addition of GlcNAc. Our results suggest that the carbohydrate moieties of cell surface proteins has an important role in biological cell behavior, including in adhesion and directionality in cell locomotion.

INTRODUCTION

Malignant transformation of animal cells has been associated with several changes, such as biochemical changes in cell surface components (Hynes, 1973[2]; Vaheri and Ruoslahti, 1974[3]; Wickus and Robbins, 1973[4]; Duksin and Bornstein, 1977[5]), a decrease in adhessiveness (Shields and Pollock, 1974[6]), an increased lectin agglutinability (Burger and Goldberg, 1967[7]; Inbar and Sacks, 1969[8]), and cell shape changes (Porter, et al., 1974[9]; Pollack, et al., 1975[10]). All of these changes may be directly or indirectly related to modification of the cell surface membrane. Recently, Pouyssegur. et al. 1977[11] reported that feeding with GlcNAc to a mutant isolated from nontransformed fibroblast, Balb 3T3 restored the synthesis of the carbohydrate portion of the glycoproteins to normal, and this chemical reversion was accompanied by complete restoration of the altered biological properties: flatter shape, increase in cell-to-substratum adhesion, decrease in the number of microvilli, decrease in ag-

*) 小野田哲夫，西上一義：チャイニーズハムスター肺細胞由来グルコサミン要求変異株の細胞表面糖たんぱく及び細胞行動におけるN-アセチルグルコサミンの影響

glutinability by Con A, and recovery of directional movility.

In this communication we have attempted to evaluate the role of surface glycoprotein in attachment to the substratum by exploiting the availability of GlcN-requiring mutant clones of Chinese hamster lung cells (line CHL-36) isolated by method of Kao and Puck, 1969[12]. We report here that surface glycoprotein synthesis in the mutant recovered normally after GlcNAc feeding, and the biochemical reversion by GlcNAc is accompanied by restration of the altered biological properties: flatter shape, increase in cell adhesion, and recovery of directional motility.

MATERIALS AND METHODS

Cell cultures

Chinese hamster lung cells (CHL-36) and the derivative mutant cells (G72-8) were cultured in Eagle's minimal essential medium with 10% fetal bovine serum (Microbiological Assoc., Bethesda Maryland), penicilin (100 units/m*l*) and Kanamycin (60 μg/m*l*) at 37°C in a CO_2 incubator. The mutant cells were routinely cultured in the medium containing with 400 μg/m*l* N-acetylglucosamine (GlcNAc). Unless otnerwise stated, the mutant cells usually were starved in the medium without GlcNAc for 2 days prior to the experiments. All cells were carried out in 15×60 mm plastic tissue culture dishes (Nunc, Denmark). Cell counts were performed in duplicate with a hemocytometer.

Cell motility

A migration of the cells in Nunc plastic flask (40 m*l*/vol) was monitored with a phase contrast inverted microscope coupled to a Nikon 16 mm camera. The cells were maintained in a controlled thermobox at 37°C. Photographs were taken at 5 min intervals, starting 2-3 days after plating as sparse cells. Cell migration was analysed by print the film and making the location of the nucleous of each of the single cells every 15 min. Contact and mitotic cells were excluded from the analysis.

Incorporation of radioactive substances

For analysis of the radioactivity in the acid-insoluble fractions, duplicates of washed labeled cell suspension were chilled on ice and to them was added cold trichloroacetic acid (TCA) to give a final concentration of 5% (W/V). They were left for at least 1 hr on ice, and the precipitated macromolecules were then collected on millipore filters and washed with two 5 m*l* of cold TCA solution. The filters were dried and transferred to scintillation vials, and 16 m*l* of scintillation fluid (100 m*l* of methanol, 20 m*l* of ethylenglycol, 60 g of naphthalene, 4 g of PPO, and 0.2 g of POPOP to 1 liter of p-dioxane) was added to each vial. The radioactivity was determined with a Packard liquid scintillation spectrometer (Model 3320). Aliquots of the acid-soluble pools were also measured for the radioactivities.

Labeling procedure of fluorescamine

Labeling procedube was carried out according to the method described by Hawkes and Bartholomew (1977)[13] with slight modifications. Monolayer cultures of the mutant and parent cells were labeled 2-3 days after seeding. After removal of the culture medium the cells were washed with warm phosphate-buffer saline and borate buffer (0.2 M H_3BO_4, pH 9.0). A solution of fluorescamine (Hoffman-La Roche, Lnc.) in acetone was added to borate buffer to a final concentration of 0.5% acetone and 500 μg of florescamine per m*l* and immediately applied to the cells. After 30 sec the cells were washed 3 times with warm borate buffer and solubilized in 2% SDS.

Analytical procedures

Emission of fluorescent compounds was monitored at 470 nm with the exciting wavelength set at 390 nm. Protein concentrations were determined by the method of Lowry. et al., 1951[14].

Radioiodination of cell surface

Isotopic labelling procedure was carried out according to the method described by Pouyssegur and Pastan (1976)[15]. Cells for iodination were prepared by growing them in 100 mm plastic dish (Nunc, Denmark) to subconfluent state. They were incubated in a 5% CO_2 incubator at 37°C for 3 days. The medium was removed, cells were washed three times with phosphate buffer saline (PBS) (37°C), then 2 m*l* of PBS containing glucose (5 mM) and $Na^{125}I$ (400 μCi) was added. The reaction was started by the addition of lactoperoxidase (20 μg/m*l*) and glucose oxidase (0.1 unit/m*l*) and stopped after 10 min at room temperature by washing the cells once with

PBI (NaCl in PBS was replaced by NaI at the same molarity, 0.15 M) and twice with PBS. All washing media contained 2 mM phenylmethyl-sulfonylfluoride to inhibit proteases. The cells were solubilized with 2% SDS in 0.5 m*l* of phosphate buffer, 10 mM, pH 7.0, containing 2 mM protease inhibitor, and were scraped from the dish with a rubber policeman. Then cell extracts were boiled 3 min before being reduced with 0.1 M dithiothreitol. SDS gel electrophoresis was performed in 7.5% polyacrylamide slab gels

RESULTS

Incorporation of ^{3}H-fucose and ^{3}H-GlcN into macromolecules

In preliminary study (Onoda, et al., 1980), we indicated that the mutant G72-8 had a defect in adhesion to substratum. One of simplest hypothes for the adhesion defect is that an alternation of cell surface components have influence on cell adhesiveness. To investigate this possibility, we examined the incorporation of fucose into macromolecules. As shown in Table 1, an evidence that cell surface glycoprotein synthesis was recovered after GlcNAc feeding was obtained measuring the incorporation of fucose into macromolecules. Approximately equal amounts of radioactivities from ^{3}H-fucose were found in acid-soluble fractions of the mutant grown with or without GlcNAc. It was incorporated into macromolecules at a lower rate in the mutant, whereas the rate was stimulated about 2.5 times when the mutant was grown with GlcNAc. The incorporation of ^{3}H-GlcN into macromolecules was still low, comparing with the parent cells, in spite of feeding with GlcNAc (Fig. 1).

Labeling pattern of cell surface proteins

To evaluate the configuration of the cell surface, ^{125}I-labeled external proteins of the mutant and parent cells were examined by sodium dodecylsulfate/polyacrylamide slab gel electrophoresis (Fig. 2). Figure 2 shows autoradiograph of 7.5% slab gel on which several samples were run in parallel. Some differrences between the mutant and parent cells arose also in the study of externally exposed protein by the lactoperoxidase-catalysed radiodination. A high molecular weight protein (approx. 210, 000) present in the parent cells was hardly detected on electrophoregrams of the mutant cells. However, feeding with GlcNAc restored the iodination pattern of the mutant to that of the

Table 1. Incorporation of ^{3}H-fucose and ^{3}H-GlcN into acid-soluble and acid-insoluble fractions of parent and mutant cells

Addition to the growth medium	Incubated with	Time of Incubation (hr)	cpm/10^{6} cells ($\times 10^{-2}$)			
			Mutant		Parent	
			acid-soluble	acid-insoluble	acid-soluble	acid-insoluble
None	^{3}H-fusoce	3	7.3	0.9	12.6	4.0
GlcNAc		3	7.1	1.8	NT	NT
None		6	11.9	1.8	22.2	8.3
GlcNAc		6	11.1	4.6	NT	NT
None	^{3}H-GlcN	6	21.0	7.7	101.1	29.0
GlcNAc		6	39.2	10.0	NT	NT
None		12	29.9	17.5	181.5	54.3
GlcNAc		12	59.0	20.4	NT	NT

Parent cells (3×10^{5} cells/60 mm dish) and mutant cells (5×10^{5} cells/60 mm dish) were incubated in the medium with or without GlcNAc (400 μg/m*l*) for 3 days. Then, the cell cultures were gently washed with the fresh medium, and the reaction was started by addition of either D-[1-^{3}H]GlcN (5 μCi/m*l*) or L-[1-^{3}H] fucose (2.5 μCi/m*l*) to 2 m*l* of the medium. After a given time interval, the media were removed and the cultures were washed three times with 5 m*l* of cold phosphate-buffered saline (PBS). The cells were then scraped off the dish in cold PBS and the radioactivities in the acid-soluble and acid-insoluble fractions were measured.

* GLcNAc; 400 μg/m*l*. ** NT; Not tested.

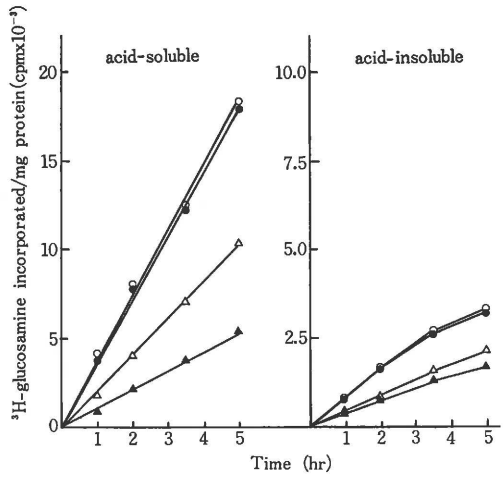

Fig. 1. Rates of ^{3}H-GlcN uptake and incorporation into acid-insoluble material of parent and mutant cells. Cells were sparsely planted, GlcNAc (400 μg /ml) added 24 hr later, and the experiment performed 1 day later. Then the cells were washed with serum free medium and the reaction was started by addition of ^{3}H-GlcN (5 μCi/ml) in 2 ml of medium. Triangle; the mutant cells incubated in the absence (▲) or presence (△) of GlcNAc. Circle; the parent cells incubated in the absence (●) or presence (○) of GlcNAc.

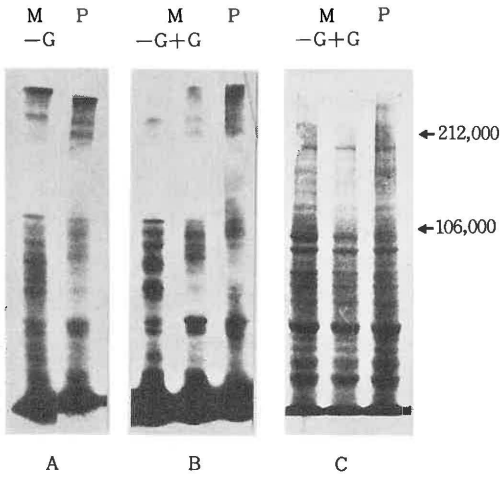

Fig. 2. Iodination of the mutant and parent cells. (A) and (B), Autoradiograms of 7.5% sodium dodecyl-sulfate polyacrylamide slab gel electrophoresis of whole cells labeled with Na^{125}I. (A) and (B) represent autoradiograms resulted from different experiments. (C), 7.5% sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of whole cell extracts stained with Coomassive blue.

parent cells. Furthermore, it was noticed that the mutant cells displayed more highly labeled peaks in relative low molecular weight range of approx. 70,000–100,000 than those of the mutant fed with GlcNAc and parent cells. On the other hand, the electrophoresis profiles of the whole cell proteins stained with Coomassive blue were essentially the same at both the cell types.

Surface alteration detected in the mutant cells by fluorescamine labeling

The mutant and parent cells were cultured in the medium with or without GlcNAc, and labelled with fluorescamine at various times after plating. Changes in the surface fluorescence were monitored during growth of cells under each cultured conditions. In the mutant cells, cultures without GlcNAc were generally 2- to 3-fold more fluorescent than those grown with GlcNAc (Fig. 3). The marked alterations in surface fluorescence were observed at 36 hr after plating. By addition of GlcNAc to the cell cultures, the fluorescence values were restored to those of the parent cells.

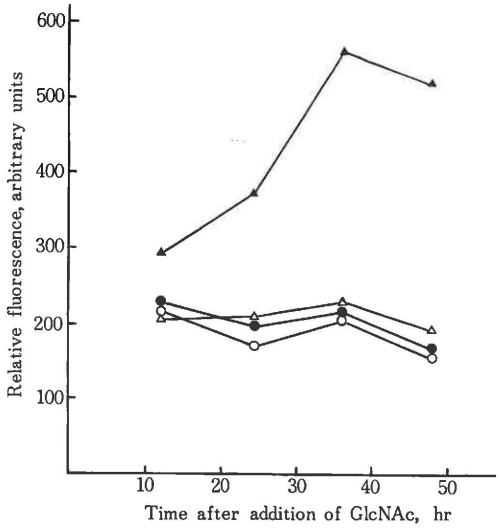

Fig. 3. Fluorescence of growing cells labeled at various times after addition of GlcNAc. The parent and mutant cells were cultured in medium with or without GlcNAc (400 μg/ml). Monolayers were labeled with fluorescamine at various times thereafter. Triangle; the mutant cells incubated in the absence (▲) or presence (△) of GlcNAc. Circle; the parent cells incubated in the absence (●) or presence (○) of GlcNAc. Fluorescence was determined as described in Materials and Methods.

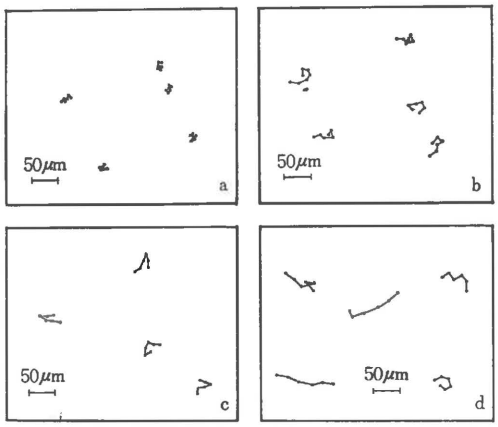

Fig. 4. Migration of the mutant and parent cells in Nunc plastic dishes. (a) the mutant cells; (b) the mutant cells grown with GlcNAc (400 μg/m*l*); (c) the mutant cells cultured in GlcNAc-free medium for 3 days and thereafter fed with GlcNAc (400 μg/m*l*) for 24 hr; (d) the parent cells. Each tracing represents the locomotion of a cell, recorded every 15 min.

The exact value of the fluorescence varied from one experiment to the next, but qualitatively similar results were obtained in all experiments. The parent cells did not demonstrate any of these alterations upon the each cultured condition.

Cell locomotion

A difference of cell motility may reflect actual changes in the composition or comformation of cell surface structures. We also investigated this property in the mutant and parent cells. Figure 4 showed tracing of the locomotion of sparse cells for 2 hr period. The moving tracks of the mutant was much less than that of the parent cells. When the mutant was grown in the presence of GlcNAc, its migrating pattern was closed to that of the parent cells.

DISCUSSION

In previous study (Onoda, 1980[1]); Onoda, et al., 1980[16], we reported that a glucosamine-requiring mutant (G72-8), derived from Chinese hamster lung cells (CHL-36) showed following characteristics: a decreased adhesiveness, an increased spotaneous aggregation, morphological alteration (round up), and reduced growth rate, while these altered biological properties were restored to normal by addition of GlcNAc. We also noticed that the mutant cells, which has a low adhessiveness to the substratum, has a random locomotion. This random migration was reverted to the directional moving of the parent cells by addition of GlcNAc. These observations seemed to accord with results obtained by Pouyssegur, et al., 1977[11]. Recently, Sanford, et al., 1979[17] reported that non-neoplastic clones of mouse fibroblasts tended to maintain the same direction of locomotion while the neoplastic cells did not. A migration pattern of the mutant cells may be resemble to a neoplastic cells. These findings raised the possibility that carbohydrate portion of cell surface components may be related to the altered biological properties. In the present study we examined the effects of GlcNAc on glycoproteins at cell surface of the mutant. From the lactoperoxidase-catalyzed iodination of cell surface proteins, we find a more extensive alteration of the cell surface proteins: iodination of the high molecular weight (approx. 210, 000) protein is markedly reduced and this band is recoveried by addition of GlcNAc. Current evidence strongly suggests that a high molecular weight (220,000 dalton) glycoprotein variously known as LETS protein, fibroblast surface antigen, or fibronectin can have an important role in mediating cell-substratum adhesion and in maintaining the flattern morphology of cultured fibroblasts (Yamada, et al., 1975[18]; Ali, et al., 1977[19]). A prominent finding in transformed cells has been the absence or marked reduction of a high molecular weight cell surface glycoprotein (Hynes, 1976[20]). Our experiments with the iodination indicate that high molecular weight protein we have detected probably is consistent with the LETS protein. In addition, when equal amounts of cell protein were applied to gels, bands of relative low molecular weight (approx. 70, 000–100, 000) protein range is labeled more strongly in the mutant than parent cells. Fluorescamine also can label surface components of cells grown as monolayers in culture (Parry and Hawkes, 1978[21]). The fluorescence of bound fluorescamine on mutant cells at presence of GlcNAc was found to be about 1/3 that of cells cultured at noaddition of GlcNAc. The results obtained using fluorescamine seem to

probe ^{125}I labeled bands of relative low molecular weight proteins. Some major iodinated bands lined up with those stained with Coomassive blue. Since many of proteins stained with Coomassive blue are probably intracellular, it may be possible also that for the mutant ^{125}I labels not surface components, but some intracelluar components. Alternatively, it has been postulated that most, if not all, of their glycoproteins are abnormal in respect with their oligosaccharide side chains and may take the form of modified proteins which easily react with these agents. However, little is known about the function of the carrohydrate moieties in cell surface membrane. Such cell lines with defective cell surface carbohydrates should be very useful for the studies of membrane structure and to evaluate the role of cell surface properties and carbohydrates in biological cell behaviors.

We have indicated in this study that the carbohydrate portion or glycoproteins of cellular surface components has an important role in a number of cellular properties of the mutant.

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