BIOSYNTHESIS OF PROTEINS, NUCLEIC ACIDS AND GLYCOSPHINGOLIPIDS BY SYNCHRONIZED KB CELLS

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Summary

The biosynthesis of glycosphingolipids and various types of proteins and nucleic acids at specific periods of the cell cycle was studied by using synchronized KB cells. Maximum incorporation of radioactive galactose, leucine and thymidine into several proteins and nucleic acids occurred as has been reported previously (6,11). Maximum incorporation of D-1[^{14}C] galactose into glycosphingolipids was observed during the M and G-1 phases. There was a 5 fold increase in the levels of gangliosides and combined neutral glycosphingolipids during the M and G-1 phases. Thus, regulated biosynthesis of glycosphingolipids and macromolecules might be important in the cyclic expression of some of the functional properties which are characteristic of these compounds.

Introduction

Synchronized cells have been used in the study of various biochemical events of mammalian cells in tissue culture (1-3). It has been demonstrated, for example, that normal cells exhibit density-dependent inhibition of growth (3) while neoplastic cells do not exhibit such a phenomenon. Parallel with this finding is the proposal that binding sites for plant agglutinins are exposed only during the mitotic phase in normal cells, whereas such sites are permanently exposed in neoplastic cells (4).

Recently, Hakomori and Kijimoto (5) demonstrated an increase in both the level and rate of synthesis of Forssman hapten glycosphingolipid in normal NIL hamster cells, and a significant decrease of this activity in contact-inhibited NIL hamster cells. Polyoma virus transformed NIL hamster cells did not exhibit such a phenomenon upon attaining high cell density.

Because of the above findings, and the possibility of the involvement of glycosphingolipids in cell regulatory processes, we have investigated the biosynthesis of glycosphingolipids and various macromolecules during discrete

phases in the growth cycle of synchronized KB cells. Hopefully, information from such studies might provide insight into relationships between various cellular control mechanisms and temporal changes of these compounds.

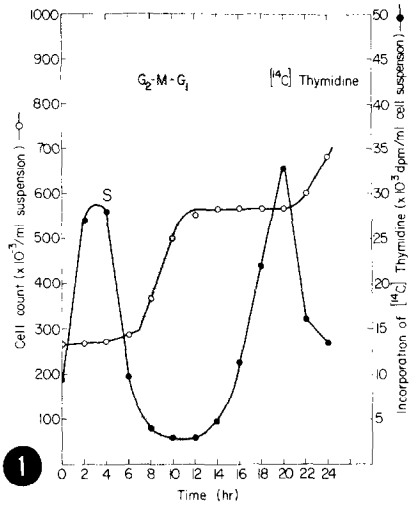
Materials and Methods

A culture of 2,250 ml of synchronized KB cells was prepared by a double thymidine block procedure (6). The time when the second thymidine block was terminated was considered as "zero" time (t_0). Triplicate aliquots of the cell suspension were withdrawn, mixed with trypan blue, and cell counts were performed using a hemocytometer.

Suitable aliquots of the synchronized cell suspensions were withdrawn at 2 hr time intervals for a period of 24 hr; each sample was pulse labeled for 1 hr at 37°C with constant stirring in a medium containing 1 μCi of D-1[^{14}C]galactose (45-55 mCi/mmole), L-U[^{14}C]leucine (270 mCi/mmole), and 2[^{14}C]thymidine (50 mCi/mmole) (New England Nuclear). The reaction was terminated by adding 20 vol of ice-cold 0.1M phosphate buffer-isotonic saline (pH 7.4), and was followed by centrifugation at 480 xg for 5 min. The cells were washed twice and then saved for appropriate analyses.

2[^{14}C]Thymidine-labeled cell pellets were mixed with 5 ml of ice cold 5% CCl_3COOH and vortexed for 30 sec. After 30 min at room temperature the cell suspensions were centrifuged as indicated above. The pellet was extracted once more with 5% CCl_3COOH and centrifuged. One ml of 1 N NaOH was added to the acid precipitable material and the contents solubilized overnight. Suitable aliquots of the solubilized samples were withdrawn and radioactivity was determined in a Beckman LS-50 liquid scintillation spectrometer.

D-1[^{14}C]galactose and L-U[^{14}C]leucine labeled cell pellets were extracted 3 times with 10% CCl_3COOH. The acid insoluble material from D-1[^{14}C]galactose labeled cell pellets was extracted 3 times with 5 vol of chloroform-methanol (2:1, by volume) and centrifuged. The pellets were air dried and solubilized overnight in one ml of 1 N NaOH; radioactivity was measured subsequently as described above.



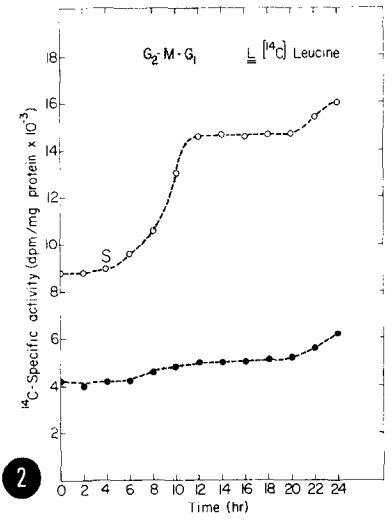
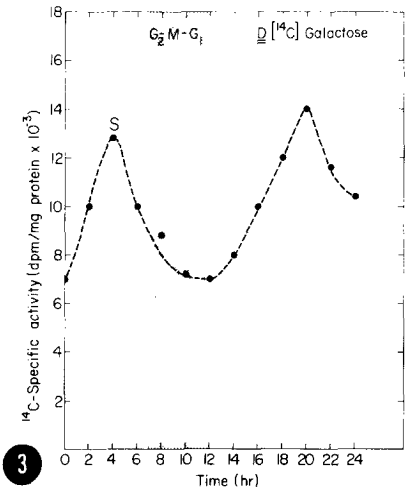


Fig. 1. Incorporation of 2[^{14}C]thymidine by KB cells synchronized with double thymidine (2mM) block. A culture of 2,250 ml of synchronized KB cells was prepared by the method of Bello (6). At indicated intervals 2 ml samples from the culture were removed and pulse labeled with 1 μCi of 2[^{14}C]thymidine for 1 hr. After the incubation period the cell pellets were extracted twice with 5 ml of ice cold 5% TCA, and specific activity determined (—●—). Three 0.5 ml aliquots of the cell suspension were withdrawn, and cell counts (—o—) made at the indicated times. The shaded zone represents the G_2-M-G_1 phases.

Fig. 2. Incorporation of L-U[^{14}C]leucine into TCA soluble (—●—) and insoluble fractions (—o—) of KB cells synchronized with double thymidine (2mM) block. At indicated time intervals 2 ml samples were withdrawn and incubated for 1 hr with 1 μCi of L-U[^{14}C]leucine. Following the incubation period cells were extracted three times with 10% TCA, and specific activity determined.

Glycosphingolipids were isolated from another series of D-1[^{14}C]galactose labeled cell pellets and control samples collected from 400 ml of unlabeled cell suspensions withdrawn at t_0, 4, 8, 10 and 12 hr intervals according to Suzuki (7). Radioactivity in the nondialyzable fraction from upper phase lipids was determined after 48 hr of dialysis against ice cold water. The lower phase lipids were dried under a stream of nitrogen and radioactivity measured.

Gangliosides in the unlabeled upper phase lipid samples were quantitated by the procedure of Svennerholm (8). Neutral glycosphingolipids derived from the lower phase lipids of Folch extracts of unlabeled cell samples were isolated and quantitated by the procedures described elsewhere (9). Protein was determined by the method of Lowry *et al.* (10) using bovine serum albumin as the standard.



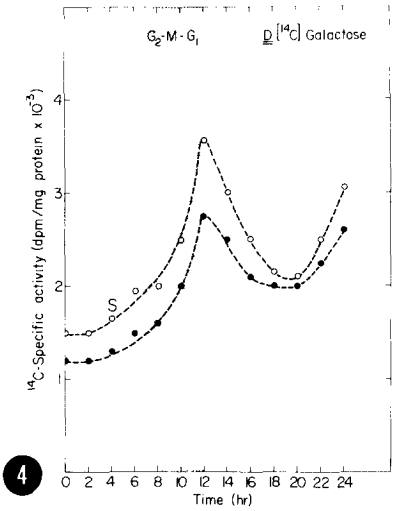


Fig. 3. Incorporation of D-[^{14}C]galactose into TCA insoluble, chloroform-methanol (2:1, by vol) extracted pellets (—●—) of KB cells synchronized with double thymidine (2mM) block. At indicated intervals 5 ml samples were removed and incubated for 1 hr with 2.5 μCi of D-1[^{14}C]galactose. Specific activity was determined as described in Materials and Methods.

Fig. 4. Incorporation of D-1[^{14}C]galactose into upper phase (—●—) and lower phase (—○—) lipid fractions of KB cells synchronized with double thymidine (2mM) block. At indicated intervals 10 ml samples were withdrawn and incubated with 5 μCi of D-1[^{14}C]galactose. Glycosphingolipids were isolated from the labeled cell pellets and specific activities determined as described.

Results

In Fig. 1, the rate of 2[^{14}C]thymidine incorporation is compared with total cell count over two cycles of synchronized KB cells. Immediately after removal of the second thymidine block, cells entered the S phase as indicated by increased incorporation of 2[^{14}C]thymidine. This period was maintained for approximately 7 hrs. Subsequently, the cells started to divide rapidly and the cell count was almost doubled during the period between 6-12 hrs. The second S phase began after 12 hr of removal of cells from the thymidine block. Cell counts remained unchanged throughout the S phases of the cell cycle.

Figs. 2-4 show specific activities of proteins, glycoproteins and glycosphingolipids at various phases of the cell cycle. Incorporation of L-U[^{14}C]leucine into acid insoluble protein (Fig. 2) paralleled the pattern

observed for total cell count (Fig. 1). Maximum incorporation of this precursor occurred during the M and G_1 phase, as compared with the finding that the rate of incorporation of L-U[^{14}C]leucine into acid soluble fraction was relatively unchanged throughout the cell cycle.

Maximum incorporation of D-1[^{14}C]galactose into acid insoluble material after chloroform methanol (2:1, by vol) washing (glycoprotein) occurred at approximately 4 hr after the cells were released from the second thymidine block. The peak was observed late in the S phase of the cell cycle and was reproducible from one cell cycle to another (see Figs. 1 and 3).

Maximum specific activities of both the upper phase and lower phase lipids occurred during the M and G_1 phases after incubation with D-1[^{14}C]galactose Later in the cell cycle the incorporation of labeled galactose into these compounds dropped to approximately the same level as that observed during the S phase.

The concentrations of purified gangliosides and neutral glycosphingolipids recovered from cells at t_0, S, G_2, M and G_1 phases of the cell cycle are given in Table 1. The maximum increase in the level of these glycosphingolipids occurred during the M and G_1 phases, resulting in an overall increase in glycosphingolipid content which was approximately five-fold within 10-12 hrs following release of cells from the second thymidine block.

Discussion

The results presented in this communication confirm previous reports that several kinds of macromolecules are synthesized at discrete phases of the cell cycle of synchronized mammalian cells (1-3). We can now add that the same is true of glycosphingolipids, which are synthesized mainly during the M and G_1 phases.

Although the synthesis of protein and glycoprotein was evident to some extent throughout the cell cycle, maximum incorporation of D-1[^{14}C]galactose into glycoprotein occurred late in the S phase. These results are consistent with those reported for synchronized HeLa cells (11), and synchronized

Table I

Glycosphingolipid Levels of Human KB Cells During the Different Stages of the Cell Cycle*

Time (hrs)	Total Gangliosides**	Total Neutral Glycosphingolipids***
t_o	0.58	0.90
4 (S phase)	0.63	1.05
8 (G_2 phase)	0.90	1.54
10 (M phase)	2.38	5.26
12 (G_1 phase)	2.72	5.54

*Human KB cells were synchronized by the method of Bello (6). At $0(t_o)$, 4, 8, 10, and 12 hr 400 ml of cell suspension were withdrawn (these cells were derived from the same batch of synchronised cells as utilized for the radioactivity incorporation studies) and pelletized. Glycosphingolipids were subsequently isolated and analyzed as described (9). The data represent averages of duplicate experiments.

**Specific activity is expressed as nmoles lipid bound sialic acid per mg protein.

***Specific activity is expressed as nmoles sphingosine per mg protein.

mouse L cells (12). These investigators found that maximum incorporation of D-[^{14}C]glucosamine and L[^{14}C]fucose into glycoprotein occurred late in the S phase. However, our results do not agree with similar studies with KB cells (13), in which it was observed that maximum incorporation of D-[^{14}C]-glucosamine occurred during the M phase. At present the reason for this discrepancy is not clear.

The most dramatic result obtained in the current investigation was the finding that the highest rate of incorporation of D-l[^{14}C]galactose into glycosphingolipids occurred during the M and G_1 phases. The qualitative pattern obtained by studying the rate of incorporation of labeled galactose into these lipids was further evaluated by quantitative analysis of the actual levels of gangliosides and combined neutral glycosphingolipids. The data obtained from

these analyses are compatible with the above finding that maximum glycosphingolipid synthesis occurred during the M and G_1 phases.

Our results suggest that most cellular material is probably synthesized in small amounts throughout the cell cycle, but it is clear that the synthesis of specific macromolecules is carefully regulated and occurs at maximum rates during specific cell phases. The messages for glycoprotein synthesis are perhaps translated very early in the cell cycle. The finding that glycosphingolipid synthesis occurs late in the cell cycle might be related to the observation that most of the glycolipid glycosyl transferases are glycoproteins, and hence might be synthesized preferentially at a time prior to functioning in glycosyl transfer reactions. Synthesis of some kinds of glycosphingolipid in the remaining stages of the cell cycle could be explained on the basis of the presence of preexisting enzymes in small amounts.

These results also support the hypothesis that glycosphingolipids are incorporated during final stages of cell membrane biosynthesis. Perhaps the cyclic expression of glycosphingolipid biosynthesis will provide an experimental approach for the evaluation of the cellular function of this class of lipids.

Acknowledgements

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