Studies on the stimulation of dolichol-mediated glycosylation by dexamethasone in HeLa cells

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The effect of the addition of 1 μ M-dexamethasone on the temporal sequence of the glycosylation process has been studied in HeLa S3G cells. In the presence of delipidized serum, dexamethasone caused an increase in 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and an accelerated synthesis of dolichols. These events were followed by an increase in the transfer of mannose from GDP-mannose to mannosyl-CoA. An increase in the rate of synthesis of lipid-linked oligosaccharides and a stimulation of glycosylation were observed in cells grown in the presence of delipidized serum in the culture medium. The data are consistent with the view that cellular syntheses of lipids and glycoproteins are co-ordinately controlled.

In the past few years compelling evidence has accumulated in the literature indicating that cell proliferation is closely linked to the synthesis of cholesterol (Chen *et al.*, 1978; Maltese *et al.*, 1980). As the cells reach confluency the synthesis of cholesterol diminished with concomitant decrease in the activity of HMG-CoA reductase (EC 1.1.1.34), the enzyme that under most circumstances is considered to be rate-limiting (Chen, 1981). HMG-CoA reductase has been subjected to a thorough investigation from the point of view of its importance in regulating the synthesis of mevalonate, the precursor for cholesterol and dolichols (James & Kandutsch, 1979; Keller *et al.*, 1979). Although disagreement does exist, data from different laboratories indicate that these two biosynthetic pathways share common intermediates at one or more rate-limiting site(s), while maintaining a large degree of regulatory independence (Mills & Adamany, 1978; James & Kandutsch, 1979, 1980; Tavares *et al.*, 1981). Extensive studies of Lennarz and his co-workers (Hanover & Lennarz, 1981) have shown that the concentration of cellular dolichyl phosphate can regulate protein glycosylation.

In the course of our studies of the action of glucocorticoids on cell-surface properties we observed that when HeLa cells were grown in the presence of glucocorticoids, cellular cholesterol synthesis was decreased concomitantly with a stimulation in the synthesis of dolichols and enhanced formation of

mannosyl-CoA (Melnikovych *et al.*, 1976; Ramachandran *et al.*, 1978, 1981). It was also seen that some of these glucocorticoid-induced biochemical changes may be related to cell-cycle (Cavenee & Melnikovych, 1978). In another study Baumann *et al.* (1980) showed that in dexamethasone-treated hepatoma tissue culture cells, the synthesis of glycosylated proteins was double that of controls.

Glucocorticoids are known to affect growth of cells in culture. These steroids reduce the growth of certain cell types such as those of lymphoid origin (Munck & Leung, 1978), whereas the growth of epithelioid cells is not significantly affected (Thompson & Lippman, 1974). If one examines the several biochemical changes taking place when the cells cease to grow, the most affected processes are the synthesis of cholesterol (as mentioned above) and the glycosylation of cell-surface glycopeptides (Muramatsu *et al.*, 1973; Ceccarini *et al.*, 1975; Sasak *et al.*, 1982).

The work embodied in this study demonstrates that lipid synthesis and the glycosylation of proteins are co-ordinately controlled during cell replication, suggesting how glucocorticoids could bring about changes in cell-surface properties leading to important biological consequences in the cell growth pattern.

Experimental

Materials and methods

All radiolabelled compounds, including [2- 14 C]-acetic acid (sp. radioactivity 60 Ci/mol). GDP-

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

[U-¹⁴C]mannose (sp. radioactivity 192 Ci/mol), [³H]glucosamine (sp. radioactivity 20 Ci/mmol), DL-3-hydroxy-3-[3-¹⁴C]methylglutaryl-CoA (sp. radioactivity 51.3 Ci/mol) and [5-³H]mevalonolactone (sp. radioactivity 7800 Ci/mol), were obtained from New England Nuclear Corporation. Dolichol, dolichyl phosphate and dexamethasone (Sigma Chemical Co., St. Louis, MO, U.S.A.), calf serum (Sterile Systems Inc., Logan, UT, U.S.A.) and plastic tissue-culture flasks (Corning, Corning, NY, U.S.A.) were obtained from the designated sources.

Cell culture

HeLa S3G cells were cultivated as described previously (Ramachandran *et al.*, 1978). The culture medium was Eagle's minimum essential medium (MEM; Gibco), containing 200 µg of streptomycin sulphate/ml and 200 units of penicillin/ml and also supplemented with 10% calf serum. The cultures were routinely split 6–8-fold twice weekly by using trypsin to detach cells from the surface.

Cells were seeded either in T flasks [(12–15) × 10⁶ cells/100 ml of medium] or in 60 mm dishes [(1–2) × 10⁶ cells/5 ml of medium] containing MEM supplemented with serum. The cells were grown for 18 h and then switched to either serum-free or delipidized serum containing medium. Dexamethasone (1 µM) was added at this time as an ethanolic solution. More details on the experimental design are given in the legends to the Tables and Figures.

Preparation of delipidized serum

Calf serum was delipidized by extraction with butan-1-ol/di-isopropyl ether (2:3, v/v) by the method of Cham & Knowles (1976). The delipidized serum was dialysed for 48 h at 4°C against 0.85% NaCl to remove any residual organic solvents. No detectable amount of cholesterol was present in these preparations.

Incorporation of [6-³H]glucosamine into lipid-linked oligosaccharide fraction

In these experiments cells were set up in 2% serum-containing MEM at different cell densities. After the cells were attached to the dishes (18 h) the medium was replaced and the cells were switched to the desired condition. At this time dexamethasone was added to one set of the dishes. After a 24 h incubation, the cells were pulsed with 2 µCi of [³H]glucosamine/ml (equivalent to 2 µM) and harvested 1 h, 2 h and 3 h later. The cells were suspended in 1.5 ml of 0.85% NaCl and homogenized. To the homogenate, 7.5 ml of chloroform/methanol (3:2, v/v) was added and mixed. The tubes were allowed to stay for 1 h at ambient temperature. After centrifugation at 1000 g for 10 min, the upper and lower phases were separated from the interphase. The interphase was washed once with 2 ml of water.

After drying the material under N₂, the residue was extracted three times with 4 ml portions of chloroform/methanol/water (10:10:3, by vol.). The combined extract was evaporated to dryness and the radioactivity was measured. This procedure is known to extract essentially all the lipid-linked oligosaccharides (Spiro *et al.*, 1976; Akamatsu & Hasegawa, 1980).

Synthesis of mannanlipid in vitro

The method of assay of mannanlipid synthesis was as described previously (Ramachandran *et al.*, 1981), except that microsomes were used as the enzyme source. The standard assay system contained 250 µg of microsomal protein, 10 mM-MnCl₂, 2.5 mM-AMP, 16 µg of dolichyl phosphate, 0.04% Triton X-100, 3 µM-GDP-[¹⁴C]mannose, all in a final volume of 200 µl of 50 mM-Tris/HCl, pH 7.1. The methods for extraction of lipid and measurement of the radioactivity were as described by Ramachandran *et al.* (1981).

Synthesis of dolichol

The procedure for measurement of the incorporation of [¹⁴C]acetate into dolichol was based on the method described by Keller *et al.* (1979) adapted in this laboratory (Ramachandran *et al.*, 1981). The method of isolation involved DEAE-cellulose column chromatography followed by high-pressure-liquid-chromatographic separation.

Details of other methods involved in the present study were published previously (Ramachandran *et al.*, 1980).

Results

Fig. 1 shows the time course of the effect of dexamethasone on HMG-CoA reductase activity in HeLa cells. The enzyme activity rose sharply within 6 h after switching the cells to the medium containing 10% delipidized serum. After reaching a peak at 6 h, the activity started to fall off and within 24 h returned to the original level. The pattern of change in enzyme activity was similar when these cells were switched to serum-free medium, although the enzyme activity reached a peak only after 16 h and the rise was much slower (Cavenee & Melnykovych, 1979). When dexamethasone (1 µM) was added at the time of addition of delipidized serum, the rise in enzyme activity was delayed, and reached a peak at around 12 h. The elevated level of enzyme activity was maintained for up to 24 h as a result of dexamethasone treatment. Routinely, these cells could be maintained for longer periods (up to 48 h) in the presence of delipidized serum compared with serum-free medium (36 h) as reported previously (Johnston *et al.*, 1979).

Fig. 1. Effect of addition of dexamethasone on HMG-CoA reductase activity

HeLa S3G cells were seeded on 60 mm plastic dishes containing minimum essential medium (MEM) supplemented with 10% calf serum. After 18 h the medium was replaced with MEM containing 10% delipidized calf serum in the presence or absence of $1\mu\text{M}$ -dexamethasone. The cells were harvested at regular intervals and the cell homogenates were used for the enzyme assay. The incubation system consisted of $100\mu\text{g}$ of cellular protein, $0.5\mu\text{mol}$ of NADP^+ , $1.5\mu\text{mol}$ of dithiothreitol, $4\mu\text{mol}$ of glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase and 10nmol of $[^{14}\text{C}]\text{HMG-CoA}$, all in a total volume of $220\mu\text{l}$. The reaction was stopped by the addition of $25\mu\text{l}$ of 6M-HCl . Subsequently, $0.1\mu\text{Ci}$ of $[5\text{-}^3\text{H}]\text{mevalonolactone}$ was added and the mevalonate formed in the reaction was lactonized. The mevalonolactone was separated on Dowex AG-1 (X8; formate form) resin. The recovery of the product was calculated from the $^{14}\text{C}/^3\text{H}$ ratio. Details of the incubation conditions are as described by Ramachandran *et al.* (1980). Values are means $\pm$ S.D. of three separate experiments.

Fig. 2. Time course of the effect of dexamethasone on the incorporation of acetate into dolichols

HeLa cells were grown in Blake bottles containing 100 ml of MEM supplemented with 10% calf serum. After 18 h the cells were switched to MEM containing 10% delipidized serum in the presence or absence of $1\mu\text{M}$ -dexamethasone. The cells were harvested at regular intervals by trypsin treatment. The cells were then resuspended in 5 ml of MEM containing 10% delipidized serum and transferred to a spinner flask. $[2\text{-}^{14}\text{C}]\text{Acetate}$ ($20\mu\text{Ci}$) was added to each flask and the cells were incubated at 37°C for 2 h. At the end of incubation the cells were pelleted and treated with chloroform/methanol (2:1, v/v). Dolichols were extracted by the method of Keller *et al.* (1979). Values are means $\pm$ S.D. of triplicate assays.

Fig. 2 represents the effect of dexamethasone on the synthesis of dolichols from acetate. As reported previously (Ramachandran *et al.*, 1981), dolichol synthesis was elevated to a significant degree in the presence of dexamethasone. The difference between the control and dexamethasone-treated cells was maintained even 24 h after the addition of dexamethasone.

A comparison of Figs. 1 and 2 indicates that even after HMG-CoA reductase activity began to decrease, the rate of synthesis of dolichols remained constant. The difference between control and dexamethasone-treated cells was evident at all time points examined. It is obvious that the onset

of the increase in HMG-CoA reductase and the elevation in dolichol synthesis were simultaneous (Figs. 1 and 2).

We have seen previously (Ramachandran *et al.*, 1981) that the mannosyl transfer from GDP-mannose to mannolipid was elevated when dexamethasone was present in the culture medium. Further studies indicated that the cells had to be in contact with the steroid at least for 12 h for the effect to be clearly evident. This prompted us to examine the time course of the changes in mannosyl formation. In these experiments (Fig. 3) the growing cells were switched to 10% delipidized serum-containing medium in the presence or absence of dexamethasone. The microsomal fractions obtained from cells harvested at regular intervals were used as the enzyme source. The assay was carried out both in the presence and absence of exogenous dolichyl phosphate. No apparent effect of dexamethasone on mannolipid formation was evident until 12 h after the addition of the steroid. The

mannosyl transfer increased slowly afterwards both in control and dexamethasone-treated cells through 36 h of incubation. Beyond this time point the enzyme activity started to decline. Throughout the experimental period, dexamethasone-treated cells

Fig. 3. Effect of dexamethasone on mannolipid synthesis by HeLa cells

The experimental design was exactly as given in the legend to Fig. 2. The cells were harvested by scraping and homogenized in 50 mM-Tris/HCl (pH 7.1) buffer and microsomes were isolated. The assay system contained 250 μ g of microsomal proteins, 10 mM-MnCl₂, 2.5 mM-AMP, 0.04% Triton X-100 (○ and ●) or 16 μ g of dolichyl phosphate (Δ and ▲) and 3 μ M-GDP-[U-¹⁴C]mannose (273 c.p.m./pmol), all in a final volume of 200 μ l. Details of incubation, lipid extraction and measurements of radioactivity were exactly as described previously (Ramachandran *et al.*, 1981). Each point represents the mean of triplicate determinations.

showed a higher activity. The effect of dexamethasone was very marked when the measurements were made in the presence of dolichyl phosphate. After an initial decline, the mannosyl transfer increased at 12 h after the addition of delipidized serum-containing medium. A sharp rise in the reaction rate was observed 18 h after the addition of dexamethasone. The data in Fig. 3 also suggest that removal of lipid components from the medium caused an increase in the mannosyl-transfer reaction only after a lag of at least 12 h.

To elucidate whether the change in mannosyl transfer observed in these cells as a result of the removal of lipids from the medium has any relation to cell growth, further experiments were carried out. In these studies cells were seeded at various densities in medium supplemented with 10% serum. After the cells were attached (18 h later) the cells were switched to the designated conditions. After 24 h the cells were used for measurement of mannosyl-transfer activity. Results presented in Table 1 show that mannolipid synthesis decreased slightly under all serum conditions as the cell inoculum was increased. This occurred both in the presence and in the absence of dolichyl phosphate. Also shown in Table 1, replacement of whole serum with delipidized serum had a stimulatory effect on mannolipid formation, particularly when assayed in the presence of dolichyl phosphate. This difference was observed under serum-free conditions as well, although to a lesser extent, presumably because of differences in the growth rate. Addition of dexamethasone stimulated mannolipid formation at all serum levels.

Another line of evidence indicating a relationship between cell growth and the glycosylation pathway is provided in Table 2. Since dolichol-associated oligosaccharides must be formed before glycosyl-

Table 1. Incorporation of GDP-mannose into dolichyl phosphate mannose under various culture conditions. Cells were seeded at three different densities (sparse, 1.0×10^6 cells/dish; medium 1.5×10^6 cells/dish; confluent, 2.5×10^6 cells/dish) in 5 ml of MEM containing 10% serum. After 18 h, the cells were switched to the respective conditions in the presence or absence of 1 μ M-dexamethasone. The cells were harvested 24 h later and the cell homogenates were used for the mannosyl transfer assay both in the presence and absence of dolichyl phosphate. Values are the averages of triplicate determinations.

Serum (%)	Dexamethasone	Addition of dolichyl phosphate (16 μ g) to the assay	10 ⁻² × ¹⁴ C-Labelled mannolipid (c.p.m./mg of protein)		
			Sparse	Medium	Confluent
10	—	—	20.45	14.64	14.42
10	—	+	222.64	164.15	162.24
10	+	—	27.87	25.38	21.94
10 (delipidized)	—	—	23.20	18.25	16.50
10 (delipidized)	—	+	392.75	402.89	357.41
10 (delipidized)	+	—	26.89	19.63	19.87
None	—	—	21.76	19.63	16.74
None	—	+	258.75	249.65	240.16
None	+	—	22.56	21.94	19.46

Table 2. *Effect of growth conditions on lipid-linked oligosaccharide synthesis*

HeLa cells were seeded in MEM containing 2% serum. After 18 h, they were switched to the designated serum conditions. The cells were pulsed with [^3H]glucosamine (2 $\mu\text{Ci}/\text{ml}$) and harvested after 1 h, 2 h and 3 h. The lipid-linked oligosaccharides were isolated by the method described in the text. Each value represents the average of triplicate determinations.

$10^{-6} \times$ Inoculum density (cells/dish)	Serum (%)	Dexamethasone	Time of incubation with [^3H]glucosamine (h)	$10^{-2} \times$ Lipid-linked oligosaccharide synthesis (c.p.m./mg of protein)		
				1	2	3
2.5	2	—	...	35.20	60.14	109.02
1	2	—		101.45	141.67	322.68
1.75	None	—		28.48	55.69	109.98
1.75	2	—		30.63	64.07	112.14
1.75	2	1 μM		40.08	75.86	194.82
1.75	2 (delipidized)	—		49.48	96.53	148.60

ation takes place, we examined the effect of various growth conditions on the synthesis of these intermediates with [^3H]glucosamine as the precursor. The growing cells were pulsed with [^3H]glucosamine and 1, 2 and 3 h later the cells were harvested and the radioactivity associated with the lipid-linked oligosaccharides was measured. As seen in Table 2, sparse cultures showed a higher rate of incorporation of glucosamine when compared with confluent cultures. Again, replacement of whole serum with delipidized serum and growth in the presence of dexamethasone enhanced lipid-linked oligosaccharide synthesis.

The washing procedure employed in the present study for the isolation of lipid-linked oligosaccharides was adopted from the original method of Spiro *et al.* (1976). Although with this procedure, the incorporation of label into the protein fraction can also be measured (Akamatsu & Hasegawa, 1980), much variation in the protein associated radioactivity was noticed. However, this problem could be solved by precipitation of the protein with trichloroacetic acid before washing. By using such a procedure, a 2-fold stimulation in the incorporation of label from [^3H]glucosamine into cellular protein was observed in dexamethasone-treated cells (results not shown).

Discussion

In the present paper we have demonstrated several lines of evidence substantiating the stimulatory effects of dexamethasone on the glycosylation pathway in HeLa S3G cells. The results presented show that (a) dexamethasone stimulates the synthesis of dolichols and dolichol-linked mannose and (b) lipid-linked oligosaccharide assembly is stimulated both by the removal of lipid components from the growth medium and by decreasing the cell

density. It is presumed that these findings have important physiological significance with respect to the cell-surface modulation by glucocorticoids and their fundamental role in growth regulation. The data also indicate a direct correlation between the lipid metabolism and the glycosylation pathway in these cells.

Dexamethasone appeared to enhance the activity of HMG-CoA reductase and the synthesis of dolichol simultaneously. Although HMG-CoA reductase activity declined after 12 h of dexamethasone addition, dolichol synthesis remained at a high level for longer periods. Work from several laboratories has demonstrated the existence of an interrelationship between sterol and dolichol synthetic pathways (James & Kandutsch, 1979; Keller *et al.*, 1979; Tavares *et al.*, 1981; White *et al.*, 1981). In our system, HMG-CoA reductase activity does not conform to the general parallel relation with sterol synthesis. Instead, the regulation is at the HMG-CoA synthase level (Ramachandran *et al.*, 1978). Considering that the flux of mevalonate through the pathway of dolichol synthesis is far below that of the sterol synthetic pathway, a broad range of fluctuation in HMG-CoA reductase need not necessarily be reflected in the dolichol level.

It is apparent that the elevation in mannosyl formation due to the addition of dexamethasone requires a lag period longer than that required either for dolichols (Fig. 2) or for the inhibition of cholesterol synthesis (Melnykovych *et al.*, 1976). This probably would suggest that the increase in the mannosyl transfer reaction cannot be attributed solely to the fluctuation in dolichol levels. Moreover, the increase cannot be abolished with exogenously added dolichyl phosphate. A possibility one should consider is that this increase is cell-cycle-related since dexamethasone is known to perturb the cell cycle (Cavenee & Melnykovych, 1978).

The observation that sparse cultures synthesized mannolipid at a higher rate than confluent cultures suggests that this process may be related to cell growth. Early work of Atkinson's group using human diploid cells is suggestive of this possibility (Ceccarini *et al.*, 1975; Muramatsu *et al.*, 1976). Furthermore, it appears that the mannosyl transfer reaction is linked to the lipid environment of the culture medium, since delipidization of the serum in the medium stimulates mannolipid formation.

Recent studies of Hemming and his co-workers (Tavares *et al.*, 1981) are of particular interest in this context. They demonstrated that when rats are fed a diet containing 1% cholesterol, their hepatic level of dolichols is elevated, resulting in an increased glycosylation of protein. Their observation has been interpreted to be caused by an increased channelization of mevalonate for dolichol synthesis owing to the partial suppression of cholesterol synthesis *de novo*. On the other hand, conditions such as removal of serum or addition of delipidized serum (as in the present study) will deprive the cells of a variety of lipids, thereby enhancing the synthesis of lipids *de novo*. Although it is difficult to compare these two studies, information leading to a better understanding of the co-ordinate changes in individual lipid components and the carbohydrate-peptide linkage at the cell surface is important in the study of the mechanisms of cell proliferation.

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