Effects of dibutyryl cyclic AMP on the syntheses of dolichol-linked saccharides and glycoproteins in cultured hepatoma cells

Correlation with the effect on the adhesiveness of the cells

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(Received 4 January 1983/Accepted 2 March 1983)

When the hepatoma cells (AH 70Btc, Clone 10-5) were cultured in the presence of 1 mM-dibutyryl cyclic AMP for 2 days, the incorporation of [14 C]glucosamine into protein was increased over 2-fold. At the same time, dibutyryl cyclic AMP increased the incorporation of [14 C]glucosamine into dolichol-linked *N*-acetylglucosamine and *NN'*-diacetylchitobiose about 1.5-fold and into dolichol-linked oligosaccharides about 3-fold. Analysis of cellular glycoproteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after reduction showed that dibutyryl cyclic AMP specifically enhanced the glycosylation of a fibronectin-like glycoprotein with an apparent mol.wt. of 220 000 and two other high-molecular-weight glycoproteins (apparent mol.wts. 270 000 and 185 000). Increased glycosylation of the glycoproteins with mol.wts. of 220 000 and 185 000 was shown to be linked to increased synthesis of the polypeptide portion. In addition to the above effects, dibutyryl cyclic AMP enhanced the adhesiveness of AH 70Btc cells to glass surfaces. Both the effects on the glycosylation pathway and on adhesiveness of cells were reversed by further treatment of the cells with 1 μ g of tunicamycin/ml. The results indicated that dibutyryl cyclic AMP increased the synthesis of dolichol-linked oligosaccharides and *N*-glycosylation of proteins in AH 70Btc cells. The enhancement of adhesiveness may be mediated by the increased synthesis of dolichol-linked oligosaccharides and also may be related to the increased synthesis of fibronectin.

Cell-surface glycoproteins are implicated in many cellular functions, including intracellular metabolism and cell–cell interactions (Atkinson & Hakimi, 1980; Gahmberg, 1981). *N*-Glycosidically linked saccharide chains of glycoproteins are synthesized via dolichol-linked oligosaccharides (Hubbard & Ivatt, 1981) and inhibition of the initial reaction in the synthesis of the intermediates has been reported to affect morphology, adhesiveness to substratum and growth in cultured mammalian cells (Duksin & Bornstein, 1977; Duksin *et al.*, 1978; Kohno *et al.*, 1979). Thus the synthesis of dolichol-linked oligosaccharides seems to be essential for the expression of the biological properties of the cells.

Bt₂ cyclic AMP exerts biological effects such as

Abbreviations used: Bt₂ cyclic AMP, *N*⁶*O*^{2'}-dibutyryl adenosine 3':5'-cyclic phosphate; SDS, sodium dodecyl sulphate.

inhibition of growth (Wicks *et al.*, 1973; Baserga, 1976; Hamman *et al.*, 1980) and enhancement of adhesiveness (Pastan & Willingham, 1978) on various malignant cells. Also, the nucleotide is known to modulate cell-surface glycoproteins (Gahmberg & Hakomori, 1973; Truding *et al.*, 1974; Imada *et al.*, 1980; Nielson & Puck, 1980). It seems likely that Bt₂ cyclic AMP changes the metabolic activities that introduce *N*-glycosidically linked saccharide chains into glycoprotein molecules. We investigated how the changes in the syntheses of dolichol-linked oligosaccharides and of glycoproteins are correlated with the biological effects exerted by Bt₂ cyclic AMP in cultured hepatoma cells. The cell line AH 70Btc, Clone 10-5 used in the present study was derived from rat ascites hepatoma AH 70B and characterized morphologically to be epithelial and to possess tumourigenicity (Nishina, 1975).

Materials and methods

Materials

Eagle's minimal essential medium was purchased from Nissui Seiyaku Co., Tokyo, Japan. Calf serum and trypsin were obtained from GIBCO Laboratories, Grand Island, NY, U.S.A., and Difco Laboratories, Detroit, MI, U.S.A., respectively. Bt₂ cyclic AMP and tunicamycin were purchased from Sigma, St. Louis, MO, U.S.A. RNA polymerase B (marker protein) was from Seikagaku Kogyo Co., Tokyo, Japan. D-[1-¹⁴C]Glucosamine hydrochloride (sp. radioactivity 57.9 Ci/mol) and L-[1-¹⁴C]leucine (sp. radioactivity 62 Ci/mol) were the products of The Radiochemical Centre, Amer-sham, Bucks., U.K.

Cell culture

AH 70Btc, Clone 10-5, cells were maintained in glass dishes (diameter, 6 cm) in Eagle's minimal essential medium supplemented with 10% (v/v) heat-treated (at 56°C for 30 min) calf serum, 100 µg of streptomycin sulphate/ml and 100 units of potassium penicillin G/ml. Incubation was performed at 37°C in a humidified atmosphere of air/CO₂ (19:1).

At the time of subculture, the cells were detached from the dish by treatment with 0.2% (w/v) trypsin in Ca²⁺, Mg²⁺-free phosphate-buffered saline [the phosphate-buffered saline used was that originally reported by Dulbecco (Patterson, 1979) and CaCl₂ and MgCl₂ were omitted in this case] at 37°C for 5 min and 5 × 10⁵ cells suspended in 3 ml of the medium were seeded on to a new dish.

Cell number was estimated by counting the number of cell nuclei by the method of Katsuta *et al.* (1957). Thus the term 'cell number' used in the present study indicates, to be precise, the number of cell nuclei.

Treatment of cells with Bt₂ cyclic AMP and tunicamycin

Cells (5 × 10⁵–10⁶) suspended in 3 ml of the medium were seeded on to each dish. On day 1 after seeding, Bt₂ cyclic AMP dissolved in 30 µl of Ca²⁺, Mg²⁺-free phosphate-buffered saline was added to the dish to make the final concentration 1 mM. When the culture was continued over 3 days, the medium and Bt₂ cyclic AMP were changed on day 3 after seeding. And where indicated, tunicamycin dissolved in 30 µl of 20 mM-NaOH was added on day 3 to make a final concentration of 1 µg/ml.

Assay of adhesiveness

Adhesiveness assay was performed essentially by the method of Duksin *et al.* (1978). The cells were cultured in the presence or absence of 1 mM-Bt₂ cyclic AMP for 2 days. They were detached from

the dishes by the treatment with 0.25% (w/v) trypsin/0.1% (w/v) EDTA at 37°C for 5 min. The cells were resuspended in media either in the presence or in the absence of 1 µg of tunicamycin/ml (the media for Bt₂ cyclic AMP-treated groups contained, in addition, Bt₂ cyclic AMP at this step) and pre-incubated for 2 h in glass tubes. Each batch of 10⁶ cells suspended in 3 ml of the same medium was then seeded on to a glass dish. At 6 h after seeding, the medium was removed and the dish was rinsed twice with 2 ml of phosphate-buffered saline. The cells still attached to the dish were harvested with a rubber policeman and the cell number was counted.

Paper chromatography

Descending paper chromatography was carried out on Toyo no. 51 (equivalent to Whatman no. 1) paper with the following solvents: solvent A, butan-1-ol/pyridine/water (6:4:3, by vol.); solvent B, isobutyric acid/conc. NH₃/water (57:4:39, by vol.); solvent C, butan-1-ol/acetic acid/water (12:3:5, by vol.).

Incubation of cells with [¹⁴C]glucosamine and separation of radioactive products

The cells in each culture dish were incubated in the presence of 0.4–1.0 µCi of [¹⁴C]glucosamine for 30–240 min. At the end of the incubation, each dish was cooled on ice and the cells were detached from the dish with a rubber policeman. A small portion of the cell suspension was used for the determination of the cell number. The remainder of the cell suspension was transferred to a glass tube, centrifuged at 100 g for 5 min and the supernatant was removed.

To the cell pellet, 4 ml of chloroform/methanol (2:1, v/v) was added. Then, the chloroform/methanol (2:1, v/v)-soluble fraction, the chloroform/methanol/water (10:10:3, by vol.)-soluble fraction and the insoluble protein were prepared as described previously (Okamoto & Akamatsu, 1981). The chloroform/methanol (2:1, v/v)-soluble fraction was dried under N₂, hydrolysed in 0.01 M-HCl at 100°C for 15 min and separated into aqueous and organic phases (Okamoto & Akamatsu, 1981). The aqueous phase was termed the 'acid-labile CM extract'.

The radioactive substances in the acid-labile CM extract were assumed to be the saccharide moieties of dolichol-linked *N*-acetylglucosamine, *NN'*-diacetylchitobiose and trisaccharide, on the basis of the following criteria. (1) The lability to mild acid is a distinctive characteristic of dolichol derivatives (Behrens & Tábor, 1978). (2) The recovery of radioactivity in the extracts was not affected even when the chloroform/methanol (2:1, v/v)-soluble fraction was treated with alkali (in 0.1 M-NaOH at 37°C for 10 min) and the saponified materials were

removed (Lahav *et al.*, 1969) before acid treatment. Dolichol derivatives are resistant to such alkali treatment (Behrens & Tábor, 1978). (3) Paper chromatography of the extract in solvents A, B and C revealed the presence of radioactive *N*-acetylglucosamine, *NN'*-diacetylchitobiose and one more compound that moved slower. The R_F values of the latter compound in these solvents were similar to those of raffinose and the relative movement in solvent A, compared with that of *N*-acetylglucosamine and *NN'*-diacetylchitobiose, was the same as that of mannosyl-*NN'*-diacetylchitobiose reported by Waechter & Harford (1979).

The radioactive substances in the chloroform/methanol/water (10:10:3, by vol.)-soluble fraction were assumed to be dolichol-linked oligosaccharides. This was based on the acid-lability and the alkali-stability of the substances and the fact that the radioactive materials released by mild acid hydrolysis behaved in the same way as oligosaccharides on paper chromatography (Okamoto & Akamatsu, 1981).

To the supernatant after the centrifugation of the cell suspension (culture medium), 0.5 vol. of ice-cold 30% (w/v) trichloroacetic acid was added. After centrifugation at 1000 *g* for 10 min, protein fraction was prepared by washing the precipitate with 4 ml each of ice-cold 10% (w/v) trichloroacetic acid, methanol, chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, by vol.) successively.

Alkaline-treatment of proteins

The protein sample was incubated with 0.1 M-NaOH at 37°C for 48 h to release *O*-glycosidically linked saccharide chains (Carson & Lennarz, 1981). The incorporation of [14 C]glucosamine into the precipitate insoluble in ice-cold 10% (w/v) trichloroacetic acid was used as an indication of *N*-linked glycoprotein synthesis.

SDS/polyacrylamide-gel electrophoresis

The cells labelled with 1 μ Ci of [14 C]glucosamine or 1 μ Ci of [14 C]leucine for 4 h were harvested using a rubber policeman, washed three times with ice-cold phosphate-buffered saline containing 2 mM-phenylmethanesulphonyl fluoride and solubilized in 2% (w/v) SDS containing 10 mM-sodium phosphate buffer (pH 7.0) and 2 mM-phenylmethanesulphonyl fluoride. Where indicated, the cells were treated with 10 μ g of trypsin/ml in phosphate-buffered saline at 37°C for 10 min before solubilizing in SDS. The solubilized cellular materials were dialysed against distilled water for 72 h, freeze-dried and dissolved in the sample buffer solution for electrophoresis as described by Laemmli (1970). Then the samples were reduced by the addition of dithiothreitol to make a final concentration of 0.1 M and heating at

100°C for 2 min. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) using 5 or 7.5% (w/v) polyacrylamide for the separation gel and 3% (w/v) polyacrylamide for the stacking gel. As molecular-weight standards, RNA polymerase B subunits [mol.wts. of 180 000, 140 000, 100 000, 42 000 and 39 000 (Date *et al.*, 1975)] were used. Proteins were detected by staining with Coomassie Brilliant Blue. When cellular proteins were analysed in the 7.5% gels, a protein band with an apparent mol.wt. of 220 000 was most deeply stained in the molecular-weight range 200 000–250 000. It was easy to detect the corresponding protein band in the 5% gels by comparing the electrophoretic pattern in the 5% gels with that in the 7.5% gels. Therefore, the mobility of the 220 000-mol.wt. protein band as well as that of 180 000- and 140 000-mol.wt. subunits of RNA polymerase B was used as a guide to estimate molecular weights in the 5% gels. The unstained gels were sliced to 1.2 or 2 mm thickness and radioactivity in each slice was determined.

Measurement of radioactivity

Radioactivity in lipid, protein and water-soluble samples was determined as previously described (Okamoto & Akamatsu, 1977, 1981). Each gel slice was solubilized in 0.5 ml of NCS/water (9:1, v/v) by heating at 50°C for 2 h. Then, 10 ml of 0.6% (w/v) 2,5-diphenyloxazole in toluene/Triton X-100 (2:1, v/v) was added, the sample was left in the dark over 2 days and radioactivity was determined.

Results

Essentially the same results were observed in two separate batches of samples.

Changes in the biological properties of AH 70Btc cells after treatment with Bt₂ cyclic AMP

Bt₂ cyclic AMP (1 mM) slowed cell proliferation, enhanced adhesiveness to glass dishes and changed the cell shape from original epithelial type to more elongated form in AH 70Btc cells.

Incorporation of [14 C]glucosamine into dolichol-linked saccharides and protein

Bt₂ cyclic AMP increased incorporation of [14 C]-glucosamine into protein over 2-fold (Fig. 1). The sum of the incorporation into cellular and medium proteins is shown in the Figure; the incorporation into cellular and medium proteins was equally increased by Bt₂ cyclic AMP. The incorporation of [14 C]glucosamine into acid-labile CM extract and dolichol-linked oligosaccharides was also increased in the Bt₂ cyclic AMP-treated cells (Fig. 2). The stimulation of [14 C]glucosamine incorporation was reproducible as shown below in Table 2. Although

Fig. 1. Time course of incorporation of [¹⁴C]glucosamine into proteins

The cells (5×10^5) suspended in 3 ml of culture medium were seeded on to each culture dish. Bt₂ cyclic AMP was added on day 1 after seeding. On day 3, the cells in each dish were incubated with $0.8 \mu\text{Ci}$ of [¹⁴C]glucosamine for 30–240 min. Cell number per dish at this step was about 1.9×10^6 in the non-treated group and about 1.3×10^6 in the Bt₂ cyclic AMP-treated group. The incorporation of radioactivity into protein was measured as described in the Materials and methods section. Each point shows the sum of the incorporation into cellular and medium proteins in a single dish. O, Non-treated cells; ●, Bt₂ cyclic AMP-treated cells.

Fig. 2. Time course of incorporation of [¹⁴C]glucosamine into acid-labile CM extract and dolichol-linked oligosaccharides

The incorporation of radioactivity into acid-labile CM extract and dolichol-linked oligosaccharides in the same samples as those described in the legend to Fig. 1 was measured as described in the Materials and methods section. The incorporation into acid-labile CM extract was determined in the combined samples from two dishes. Each point represents a single determination. Δ, Acid-labile CM extract, non-treated cells; ▲, acid-labile CM extract, Bt₂ cyclic AMP-treated cells; ○, dolichol-linked oligosaccharides, non-treated cells; ●, dolichol-linked oligosaccharides, Bt₂ cyclic AMP-treated cells.

the cells increased in size after Bt₂ cyclic AMP treatment, the increase in protein content of the cells (about 40%) alone could not explain the enhanced incorporation.

N-Acetylglucosamine and *NN'*-diacetylchitobiose were separated from the acid-labile CM extract by paper chromatography. The incorporation of [¹⁴C]glucosamine into these saccharides was lower compared with that into oligosaccharides and the increase of the incorporation after Bt₂ cyclic AMP treatment was about 1.5-fold (Table 1). On the other hand, the incorporation into oligosaccharides increased about 3-fold after the same treatment. These observations were also reflected in a smaller stimulation of [¹⁴C]glucosamine incorporation into acid-labile CM extract compared with that into dolichol-linked oligosaccharides (Fig. 2). The results suggest that Bt₂ cyclic AMP may activate some step(s) in the elongation of sugar chains from dolichol-linked *NN'*-diacetylchitobiose to oligosaccharides. The increased incorporation of [¹⁴C]glucosamine into dolichol-linked oligosaccharides may not be merely due to the increased specific radioactivity of UDP-*N*-acetylglucosamine, the precursor in the biosynthesis of dolichol-linked

saccharides, since such a change in the specific radioactivity must first be reflected in the incorporation into dolichol-linked *N*-acetylglucosamine.

The above data suggested that Bt₂ cyclic AMP increased the synthesis of glycoproteins in AH 70Btc cells and at least a part of this increase was linked to the increased synthesis of dolichol-linked oligosaccharides.

Effect of tunicamycin on the incorporation of [¹⁴C]glucosamine

Tunicamycin is known to inhibit the synthesis of dolichyl diphosphate *N*-acetylglucosamine (Elbein, 1981). The effects of tunicamycin ($1 \mu\text{g/ml}$) on the incorporation of [¹⁴C]glucosamine in control and Bt₂ cyclic AMP-treated cells were examined (Table 2). In both groups of cells, tunicamycin decreased the incorporation of [¹⁴C]glucosamine into the acid-labile CM extract, dolichol-linked oligosaccharides and *N*-linked glycoproteins. As a result, the incorporation into dolichol-linked oligosaccharides in the cells treated with Bt₂ cyclic AMP and tunicamycin

Table 1. *Incorporation of [¹⁴C]glucosamine into dolichol-linked saccharides*

Cells were seeded and treated with Bt₂ cyclic AMP as described in the legend to Fig. 1. On day 3 after seeding, cells in each dish were incubated with 0.8 μ Ci of [¹⁴C]glucosamine for 4 h. Acid-labile CM extract and dolichol-linked oligosaccharides were prepared as described in the Materials and methods section. The samples from two or three dishes were combined. *N*-Acetylglucosamine and *NN'*-diacetylchitobiose were separated from the combined acid-labile CM extract by paper chromatography in solvent A. The results of two separate experiments are shown. Values in parentheses are averages of the two experiments.

Dolichol-linked saccharides	Experiment no.	Incorporation of radioactivity (d.p.m./10 ⁶ cells)		Ratio (b)/(a)
		Non-treated (a)	Bt ₂ cyclic AMP-treated (b)	
<i>N</i> -Acetylglucosamine	1	88	138	1.6
	2	71	96	1.4 (1.5)
<i>NN'</i> -Diacetylchitobiose	1	79	99	1.3
	2	130	194	1.5 (1.4)
Oligosaccharides	1	880	2057	2.3
	2	813	3103	3.8 (3.1)

Table 2. *Effect of Bt₂ cyclic AMP and/or tunicamycin on the incorporation of [¹⁴C]glucosamine*

Cells (5 $\times$ 10⁵) suspended in 3 ml of culture medium were seeded on to each culture dish. Bt₂ cyclic AMP and tunicamycin were added on day 1 and day 3 after seeding respectively. At 2 h after the addition of tunicamycin, 0.4 μ Ci of [¹⁴C]glucosamine was added to each dish and the cells were further incubated for 4 h. Then the incorporation of radioactivity into acid-labile CM extract, dolichol-linked oligosaccharides and *N*-linked glycoproteins was determined as described in the Materials and methods section. The incorporation (d.p.m./10⁶ cells) in each experimental group is also expressed as the ratio to that in non-treated cells and shown in parentheses. Values are means $\pm$ S.E.M. of triplicate determinations. The differences between non-treated and Bt₂ cyclic AMP-treated cells are all statistically significant at $P < 0.05$. * $P < 0.01$, compared with non-treated cells; † $P < 0.05$, compared with non-treated cells; ‡ $P < 0.01$, compared with cells treated only with Bt₂ cyclic AMP; § $P < 0.05$, compared with cells treated only with Bt₂ cyclic AMP.

Treatment	Incorporation of radioactivity (d.p.m./10 ⁶ cells) into:		
	Acid-labile CM extract	Dolichol-linked oligosaccharides	<i>N</i> -linked glycoproteins
None	271 $\pm$ 2 (1.00)	578 $\pm$ 75 (1.00)	6530 $\pm$ 130 (1.00)
Tunicamycin	171 $\pm$ 5* (0.63)	343 $\pm$ 8† (0.59)	5220 $\pm$ 260† (0.80)
Bt ₂ cyclic AMP	525 $\pm$ 74 (1.93)	1628 $\pm$ 144 (2.82)	17900 $\pm$ 2100 (2.74)
Bt ₂ cyclic AMP + tunicamycin	411 $\pm$ 25 (1.52)	553 $\pm$ 137‡ (0.96)	10500 $\pm$ 500§ (1.61)

returned to a similar level to that in non-treated cells. In the above experiment, the incorporation of [¹⁴C]glucosamine was measured during 2–6 h of treatment with tunicamycin. The incorporation of [¹⁴C]glucosamine into lipids other than dolichol-linked saccharides and into *O*-linked glycoproteins changed little during this treatment (results not shown). The inhibition of the incorporation into acid-labile CM extract by the tunicamycin treatment was rather mild (37%), as shown in Table 2,

whereas a prolonged (48 h) treatment with the antibiotic caused a marked (over 80%) inhibition. The same conditions as used in Table 2 or similar conditions were employed in the following experiments (Fig. 3 and Table 4). Such conditions are suitable for investigating how the increase in the synthesis of dolichol-linked saccharides after Bt₂ cyclic AMP treatment is correlated with changes in cellular glycoproteins and in the biological properties of cells.

Analysis of cellular glycoprotein by SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was always performed with reduced samples. Fig. 3 shows the electrophoretic patterns of [^{14}C]glucosamine-labelled cells. In the experimental condition used, mucopolysaccharides are thought to remain close to the origin and glycolipids to move close to the position of Bromophenol Blue (Baig & Roberts, 1973). In the control cells (Fig. 3a), the main radioactive peaks appeared in the molecular-weight range 40 000–60 000 and 100 000–150 000. When the control cells were treated with tunicamycin (Fig. 3b), the radioactivity in the range mol.wt. 100 000–150 000 was significantly decreased and other minor changes were also observed.

In the Bt_2 cyclic AMP-treated cells (Fig. 3c), the radioactivities at position I, II and III (apparent mol.wts. 270 000, 220 000 and 185 000 respectively) were markedly increased. The increases after Bt_2 cyclic AMP treatment were reproducible and were not observed in the cells treated with Bt_2 cyclic AMP and tunicamycin (Fig. 3d). These data indicate that the increased radioactivities at positions I, II and III in Bt_2 cyclic AMP-treated cells are associated with *N*-glycosidically linked saccharide chains of glycoprotein and the increase is mediated by the increased synthesis of dolichol-linked oligosaccharides. The apparent molecular weight of position II (220 000) is coincident with that of a subunit of fibronectin (Yamada & Weston, 1974; Karasaki *et al.*, 1980). When the labelled cells were subjected to a mild-trypsin treatment, as described in the Materials and methods section, the radioactive peak at position II

Fig. 3. SDS/polyacrylamide-gel electrophoresis of [^{14}C]-glucosamine-labelled cells

Cells were seeded, treated with Bt_2 cyclic AMP and/or tunicamycin and labelled with [^{14}C]glucosamine as described in the legend to Table 2, except that the cells in each dish were incubated with $1\ \mu\text{Ci}$ of [^{14}C]glucosamine. Electrophoresis of labelled cellular materials was performed in 7.5% polyacrylamide gel. Radioactivity in each 2 mm gel slice was determined as described in the Materials and methods section. The electrophoretic patterns of (a) non-treated cells, (b) tunicamycin-treated cells, (c) Bt_2 cyclic AMP-treated cells and (d) cells treated with Bt_2 cyclic AMP and tunicamycin are shown. Radioactivities applied to the gels were 6600 d.p.m. (a), 4600 d.p.m. (b), 7800 d.p.m. (c) and 6400 d.p.m. (d). O and B in the Figures indicate the origin and the peak position of Bromophenol Blue respectively. The broken lines in each figure indicate the positions corresponding to mol.wts. (from left to right) 250 000, 200 000, 150 000, 100 000, 60 000 and 40 000. These were estimated from the $R_{\text{Bromophenol Blue}}$ values of standard proteins.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of [^{14}C]-leucine-labelled cells

Cells were seeded and treated with Bt_2 cyclic AMP as described in the legend to Fig. 1. On day 3, cells in each dish were incubated with $1\mu\text{Ci}$ of [^{14}C]-leucine for 4 h. Electrophoresis of labelled cellular materials was performed in 5% polyacrylamide gel and radioactivity in each 1.2 mm gel slice was determined as described in the Materials and methods section. The electrophoretic patterns of (a) non-treated cells and (b) Bt_2 cyclic AMP-treated cells are shown. Radioactivities applied to the gels were 17900 d.p.m. (a) and 24600 d.p.m. (b). The cellular materials labelled with [^{14}C]glucosamine were also analysed in the 5% gels and the positions at which the incorporation of [^{14}C]glucosamine was increased by Bt_2 cyclic AMP are indicated in the Figures for I, II and III. The broken lines in each Figure indicate the positions corresponding to mol.wts. (from left to right) 300000, 250000, 200000 and 150000. Other details are as described in the legend to Fig. 3.

disappeared (results not shown). Since such a sensitivity to trypsin is characteristic of cell-surface fibronectin (Olden *et al.*, 1978; Karasaki *et al.*, 1980), the increased radioactivity at position II is thought to be associated with this glycoprotein.

To examine whether the increased glycosylation

Table 3. Effect of Bt_2 cyclic AMP and/or tunicamycin on the attachment of cells to the dish

Cells (5×10^5) suspended in 3 ml of culture medium were seeded on to each culture dish. Bt_2 cyclic AMP and tunicamycin were added on day 1 and day 3 after seeding respectively. On day 5, the medium was removed and the dish was rinsed twice with 2 ml of phosphate-buffered saline. The cells still attached to the dish were harvested with a rubber policeman. Both detached and attached cells were counted and the number of attached cells is expressed as a percentage of the total number of the cells. Values are means $\pm$ S.E.M. of triplicate determinations. * $P < 0.01$, compared with non-treated cells; † $P < 0.01$, compared with cells treated only with tunicamycin or with Bt_2 cyclic AMP.

Treatment	Attached cells (%)
None	98.7 $\pm$ 0.3
Tunicamycin	8.3 $\pm$ 1.3*
Bt_2 cyclic AMP	93.5 $\pm$ 1.5
Bt_2 cyclic AMP + tunicamycin	65.7 $\pm$ 6.9†

of the three glycoproteins after Bt_2 cyclic AMP treatment was linked to the increased synthesis of the polypeptide portion or not, [^{14}C]leucine-labelled cells were analysed by gel electrophoresis (Fig. 4). The numerals I, II and III in the Figures correspond to the positions at which the incorporation of [^{14}C]glucosamine was increased by Bt_2 cyclic AMP. In the control cells (Fig. 4a), no significant radioactive peak was observed at these positions. On the other hand, distinct peaks appeared at positions II and III in the Bt_2 cyclic AMP-treated cells (Fig. 4b). The radioactive peak at position I was not so significant. Although another peak appeared corresponding to apparent mol.wt. 160000, the corresponding peak of [^{14}C]glucosamine incorporation was not detected (Fig. 3c). The total radioactivity applied to the gel in the Bt_2 cyclic AMP-treated cells was 1.4-fold higher compared with that in the control cells and the radioactivities at position II and III increased over 2.5-fold in the treated cells. From these data, it was inferred that the increased glycosylation of the glycoproteins with apparent mol.wts. of 220000 and 185000 may be linked to the increased synthesis of the polypeptide portion.

Effect of tunicamycin on the adhesiveness of the cells

When the control cells were treated with tunicamycin for 2 days, the cells became rounded and over 90% of the cells were detached from the dish (Table 3). Since most of the detached cells were not stained with Trypan Blue and colonies were formed upon transferring the cells into fresh medium, the detachment may not be due to cytotoxicity of tunicamycin. On the other hand, when the Bt_2 cyclic

Table 4. *Effect of Bt₂ cyclic AMP and/or tunicamycin on the adhesiveness of the cells*

Cells (10⁶) suspended in 3 ml of culture medium were seeded on to each culture dish. Bt₂ cyclic AMP was added on day 1 after seeding. On day 3, the cells were harvested and adhesiveness in the presence or in the absence of tunicamycin was assayed as described in the Materials and methods section. The number of cells attached to the dish during the 6 h plating period is expressed as a percentage of the total number of cells. Values are means $\pm$ S.E.M. of triplicate determinations. * $P < 0.05$, compared with non-treated cells; † $P < 0.01$, compared with cells treated only with Bt₂ cyclic AMP.

Treatment	Attached cells (%)
None	38.7 $\pm$ 4.4
Tunicamycin	29.0 $\pm$ 5.8
Bt ₂ cyclic AMP	55.3 $\pm$ 0.9*
Bt ₂ cyclic AMP + tunicamycin	42.7 $\pm$ 1.8†

AMP-treated cells were cultured with tunicamycin, over 60% of the cells remained attached to the dish (Table 3). Therefore, the adhesiveness of the cells to glass surfaces is altered by treatment with Bt₂ cyclic AMP and/or tunicamycin.

As an index of the adhesiveness, the rate of cell attachment to the dish during a plating period of 6 h was determined (Table 4). Since the experiment was performed on cells treated with trypsin/EDTA, the cell attachment reflected the recovery of adhesive factor(s) on the cell surface. The attachment was significantly increased in the Bt₂ cyclic AMP-treated cells compared with the control cells. Tunicamycin treatment of the control cells somewhat decreased the attachment and that in the cells treated with Bt₂ cyclic AMP and tunicamycin returned to the control level. The differences in the attachment between each group were coincident with those in the incorporation of [¹⁴C]glucosamine into dolichol-linked oligosaccharides (Table 2), indicating that the enhancement of the adhesiveness by Bt₂ cyclic AMP may be mediated by the increased synthesis of the intermediates.

Discussion

In the present paper, it has been shown that Bt₂ cyclic AMP increases the incorporation of [¹⁴C]-glucosamine into protein and that the increase is linked to the enhancement of the synthesis of dolichol-linked oligosaccharides. Since cell-surface glycoproteins are implicated in many cellular functions, as outlined in the introduction, the stimulation of the glycosylation pathway by Bt₂ cyclic AMP would be expected to exert many secondary effects on the cells.

Analysis of [¹⁴C]glucosamine-labelled cells by SDS/polyacrylamide-gel electrophoresis indicated that Bt₂ cyclic AMP specifically enhanced the glycosylation of glycoproteins with apparent mol.wts. 270 000, 220 000 and 185 000. The increased glycosylation was not observed in cells treated with Bt₂ cyclic AMP and tunicamycin. In the experimental conditions used, tunicamycin decreased the incorporation of [¹⁴C]glucosamine into dolichol-linked oligosaccharides only to a level of the control cells. Accordingly, the increased glycosylation was shown to be mediated by increased synthesis of dolichol-linked oligosaccharides. Also it was shown that, by analysis of [¹⁴C]leucine-labelled cells, increased glycosylation of glycoproteins with apparent mol.wts. of 220 000 and 185 000 may be linked to the increased synthesis of the polypeptide portion.

The enhancement of cell adhesiveness to glass surfaces after Bt₂ cyclic AMP treatment was thought to be mediated by the increased synthesis of dolichol-linked oligosaccharides. The reports of Pouyssegur & Pastan (1976, 1977), showing that Bt₂ cyclic AMP increased the adhesiveness in normal 3T3 cells but not in glycosylation-deficient mutant 3T3 cells, support the above conclusion.

Fibronectin, like Bt₂ cyclic AMP, changes the cell shape and enhances adhesiveness in transformed fibroblasts (Yamada *et al.*, 1976). Among the glycoproteins whose synthesis and glycosylation were increased by Bt₂ cyclic AMP, the one with an apparent mol.wt. of 220 000 was thought to be a subunit of fibronectin. Since the saccharide chains of fibronectin are known to stabilize the protein on the cell surface against proteolytic degradation (Olden *et al.*, 1978), Bt₂ cyclic AMP may increase the surface expression of fibronectin via increased synthesis and glycosylation. Similarly Bt₂ cyclic AMP has been reported to restore fibronectin on the Chinese hamster ovary cell surface (Nielson & Puck, 1980). On the other hand, Karasaki *et al.* (1980) reported that when the oncogenic liver cells, which had stopped synthesizing fibronectin, were treated with Bt₂ cyclic AMP, changes in cell shape and inhibition of growth were observed, whereas the glycoprotein was not restored. The biological effects of Bt₂ cyclic AMP on hepatoma cells cannot be unequivocally related to fibronectin. The particular cellular functions in which the increased glycosylation of the other two high-molecular-weight (270 000 and 185 000) glycoproteins are involved are noteworthy. More detailed studies are required to further clarify this link.

A decrease in overall carbohydrate content, as well as structural changes in saccharide chains of membrane glycoproteins, have been extensively observed in various malignant cells (Smith & Walborg, 1977; Atkinson & Hakimi, 1980). The

decreased content of sialic acid and galactose, which are constituents of outer branches of saccharide chains, has been attributed to the low activities of the corresponding glycosyltransferases (Grimes, 1970; Kim & Isaacs, 1975). It seems possible that the decreased activity in the synthesis of core oligosaccharides via dolichol-linked saccharides may be another reason for the decreased carbohydrate content. The present results suggest that the decreased synthesis of dolichol-linked oligosaccharides may be correlated with the weak adhesiveness of malignant cells.

We are very grateful to Dr. T. Tokiwa, Okayama University Medical School, for his advice on the cell-culture techniques.

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