The role of *N*-linked glycosylation in the regulation of activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and proliferation of SV40-transformed 3T3 cells

Olle LARSSON* and Wilhelm ENGSTRÖM†

*Department of Tumour Pathology, Karolinska Hospital, S-104 01 Stockholm, and †Centre for Biotechnology, Karolinska Institutet, Huddinge Hospital F82, S-141 86 Huddinge, Sweden

The effects of glycosylation inhibitors on the proliferation of SV40-transformed 3T3 cells (SV-3T3) were examined *in vitro*. Whereas swainsonine and castanospermine, which inhibit distal steps in the glycosylational processing, exerted marginal or no effects on cell proliferation, a proximal inhibitor, tunicamycin, efficiently decreased the rate of DNA synthesis and also inhibited the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. The inhibitory effects of tunicamycin on cell proliferation could be partially reversed by addition of dolichol, a metabolite in the pathway regulated by HMG-CoA reductase. This finding suggests that tunicamycin exerts at least one of its effects on cell proliferation by modulating the activity of HMG-CoA reductase.

INTRODUCTION

Normal, non-transformed cells grown under suboptimal culture conditions, such as high cell density or low serum concentration, leave the cell cycle in order to enter a reversible state of quiescence [1]. In contrast, virally-transformed cells respond differently to altered culture conditions. Although the rate of proliferation is gradually retarded, tumour cells cannot enter G_0 . Instead, the cells continue to progress slowly through the cell cycle until they eventually die as a consequence of the environmental restraints [2,3]. The capacity to enter a specific state of quiescence (G_0) is believed to reflect some fundamental growth control mechanisms, which are in operation in normal cells but which are aberrantly relaxed in transformed cells. Therefore, the molecular mechanisms that accompany growth regulatory events in the pre-replicative cell cycle phase are of considerable interest in attempts to understand the cellular defects underlying the uncontrolled growth of tumour cells.

Glycosylation of critical molecules located on the cell surface is generally believed to be involved in the control of cellular growth. In this study, specific inhibitors which prevent *N*-linked glycosylation or interfere with the glycosylational processing of oligosaccharides after their attachment to proteins have been used. In particular, it is reported that tunicamycin inhibits the proliferation of a simian-virus-40-transformed cell line. Along with its inhibitory effects on glycosylation, tunicamycin inhibits the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 3.1.2.5). It is further shown that the tunicamycin-induced effects on cell proliferation can be partially reversed by adding dolichol to the cell cultures. It is therefore proposed that this isoprene product may play an important role in the control of cell proliferation.

MATERIALS AND METHODS

Chemicals

Tunicamycin, mevalonolactone and dolichol (dihydrononadecaprenol, > 95 % purity) were purchased from Sigma Chemical Co. Castanospermine and swainsonine were obtained from Boehringer Mannheim. The basal media were purchased in liquid form from Flow Laboratories Inc. Fetal calf serum and trypsin were supplied by GIBCO. All radiochemicals were purchased from Amersham.

Cell culture

Simian virus 40 (SV40)-transformed Swiss mouse 3T3 cells (SV-3T3) were purchased from Flow Laboratories and maintained in monolayer cultures and used for experiments as described elsewhere [3]. DNA synthesis was assayed by autoradiography or liquid scintillation counting after labelling with [3 H]thymidine (5 μ Ci/ml; 5 Ci/mmol) for 1 h prior to fixation as described in detail elsewhere [3].

Assay of HMG-CoA reductase and *N*-linked glycosylation

The activity of HMG-CoA reductase in SV-3T3 cells was determined according to a protocol described elsewhere [3]. The rate of *N*-linked glycosylation was determined by incubating the cells in 50 mm Petri dishes with [3 H]glucosamine (10 μ Ci/ml; 10 mCi/mmol) over a 1 h period. The cells were thereafter rinsed in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS), scraped off the dishes with a rubber policeman, precipitated with trichloroacetic acid and assayed for radioactivity as described in [3].

Time-lapse video analysis (TLV)

Time-lapse cinematography was performed by the use

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; SV40, simian virus 40; SV-3T3, SV40-transformed 3T3 cells; PBS, phosphate-buffered saline; TLV, time-lapse video.

Table 1. Effects of different glycosylation inhibitors on DNA synthesis in SV-3T3 cells

SV-3T3 cells were exposed to media as specified below over 24 h periods. 1 h before the termination of the experiment, the cells were pulse-labelled with [³H]thymidine (5 µCi/ml; 5 Ci/mmol). The cells were then collected for determination of the incorporation of [³H]thymidine (see the Materials and methods section). All figures were related to the control (cells growing in medium without inhibitors) which was given the arbitrary value of 100 % (= 566 c.p.m./A unit of nucleic acid). The figures represent means ± S.D. for three different experiments. Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Ca, castanospermine; Sw, swainsonine; Tu, tunicamycin

Treatment	Incorporation of [³ H]thymidine (% of control)
DMEM + 10 % FCS (control)	100
DMEM + 10 % FCS + Sw (5 µg/ml)	112 ± 20
DMEM + 10 % FCS + Ca (50 µg/ml)	97 ± 11
DMEM + 10 % FCS + Tu (0.4 µg/ml)	22 ± 15

of a video time-lapse recording system as described elsewhere [3].

Determination of cholesterol and dolichol synthesis

Incorporation of [³H]mevalonate into cholesterol and dolichol was determined by scintillation counting following separation of cholesterol by reverse-phase h.p.l.c. as described elsewhere [5].

Statistics

The difference between means was statistically evaluated by using Student's *t* test. The levels of significance are stated in the text or legends to Figures.

RESULTS AND DISCUSSION

Table 1 shows the effects of three different inhibitors of glycoprotein biosynthesis on DNA replication (as measured by scintillation counting of [³H]thymidine incorporation into trichloroacetic acid-precipitable material) in SV-3T3 cells. A 24 h exposure to swainsonine or castanospermine exerted no significant effects on DNA synthesis. The concentrations of swainsonine or castanospermine used in these experiments were chosen on the basis of their growth-inhibitory effects as reported by others [6]. Other concentrations tested did not increase the growth rate of the SV-3T3 cells to any significant extent (results not shown). In contrast, addition of tunicamycin drastically decreased the rate of DNA synthesis (Table 1). It therefore became of interest to examine the effects of this inhibitor in greater detail. This was done by analysing how tunicamycin addition affected the percentage of thymidine-labelled cells. A high dose of tunicamycin (0.8 µg/ml) resulted in a significantly decreased labelling index (from approx. 50 % to 10–15 %, *P* < 0.0005; results not shown). If, however, the cells were supplemented with a lower concentration of tunicamycin (0.4 µg/ml), the inhibitory effect varied greatly between experiments (results not shown). The tunicamycin-induced inhibition of DNA synthesis was

Fig. 1. Effects of tunicamycin on the cell cycle of SV-3T3 cells

(a) SV-3T3 cells grown in medium containing 10 % fetal calf serum for 48 h after plating were rinsed and shifted to serum-free medium. After an additional 24 h, the cells were changed to fresh serum-free medium with the following supplements: no supplements (□), tunicamycin (0.4 µg/ml, ■), or tunicamycin (0.8 µg/ml, ▨). After 24 h incubation, the cells were pulse-labelled with [³H]thymidine, fixed and eventually processed for autoradiography. The percentage of labelled cells was determined by light microscopy (> 400 cells/plate). The values represent the mean values ± S.D. from two separate experiments; * *P* < 0.05. (b) SV-3T3 cells were shifted to serum-free medium 48 h after plating. From this moment the cells were analysed by TLV. After 24 h, the cells were exposed to serum-free medium supplemented with tunicamycin (0.4 µg/ml). After an additional 48 h the experiment was terminated. The cell cycle position (i.e. time elapsed after last mitosis) at the moment of exposure to tunicamycin, as well as generation time of individual cells, was determined from the video recordings.

reversible in the sense that the cells regained their ability to proliferate if they were re-exposed to tunicamycin-free medium (results not shown).

We thereafter examined whether the growth-inhibitory effects of tunicamycin are executed at some defined stage of the cell cycle. This issue was addressed by the use of TLV analysis. This technique enables an accurate determination of the cell cycle position as well as the generation time of individual cells [3]. However, it is significant that it is only with considerable difficulty that SV-3T3 cells can be analysed by TLV if cultured in medium containing serum. This disadvantage is simply due to the striking capacity of serum to promote SV-3T3-cell locomotion on plastic substrates [3]. It is therefore preferable to perform the TLV analysis on cells maintained in serum-free medium. It is confirmed by autoradiography after pulse labelling with [³H]thymidine that the labelling percentage remains high (45 %) in the absence of serum (Fig. 1a). These data support earlier conclusions that SV-3T3 cells have lost their requirement for exogenous serum factors [2]. Tunicamycin addition reduced the proliferative rate drastically and the labelling index dropped from 49 to 4 % in the absence of serum. In serum-free medium, a dose of 0.4 µg of tunicamycin/ml was sufficient to cause a significant decrease in proliferative activity.

Table 2. Effects of tunicamycin on the incorporation of [³H]mevalonate into cholesterol and dolichol

SV-3T3 cells grown in Dulbecco's modified Eagle's medium (DMEM) + 10 % fetal calf serum (FCS) were shifted to new fresh medium (DMEM + 10 % FCS) supplemented with [³H]mevalonate (5 μ Ci/ml; 1.23 Ci/mmol) $\pm$ tunicamycin (0.8 μ g/ml). After an additional 24 h, cells were harvested for lipid isolation. Neutral lipids were separated by normal-phase h.p.l.c. and the radioactivity in peaks corresponding to dolichol and cholesterol was measured by liquid scintillation counting.

Treatment	Incorporation of [³ H]mevalonate (d.p.m./1 $\times$ 10 ⁶ cells) into:	
	Dolichol	Cholesterol
DMEM + 10 % FCS	1104	55200
DMEM + 10 % FCS + tunicamycin	815	62700

Fig. 1(b) shows data from TLV determinations of SV-3T3 cells which had been exposed to tunicamycin over a 48 h period. It was found that only cells younger than approx. 3 h at the moment of tunicamycin addition failed to undergo mitosis. In contrast, cells older than approx. 3 h were fully capable of traversing the cell cycle and completing mitosis. These data imply that tunicamycin treatment induces a cell-cycle-specific block in early G₁. It is further implied that the decrease in the percentage of [³H]thymidine-labelled cells as shown in Fig. 1(a) is the result of this block.

However, it is shown elsewhere that tunicamycin exerts a wide variety of metabolic effects [7], and it is of considerable interest to examine whether it exerts its inhibitory action on DNA synthesis in SV-3T3 cells via some stage-specific regulatory metabolic pathway(s). One such pathway is the biosynthesis of cholesterol and non-sterol isoprenes. The rate-limiting step in this pathway is the conversion of HMG-CoA into mevalonate, which is catalysed by HMG-CoA reductase. Previous findings [7] suggest that tunicamycin inhibits the activity of HMG-CoA reductase, and it remains a distinct possibility that this event accounts for the effect of tunicamycin on cell proliferation. Interestingly, the HMG-CoA reductase activity was drastically depressed in tunicamycin-treated SV-3T3 cells. The enzymic activity dropped from 155 to 7.4 pmol $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ after a 4 h exposure to tunicamycin (0.8 μ g/ml) (mean value from two different experiments). The magnitude of the tunicamycin-induced inhibition of HMG-CoA reductase activity was of the same order as that caused by specific inhibitors of this enzyme [4].

If, however, the inhibitory effects of tunicamycin on DNA synthesis in SV-3T3 cells are in any way mediated by a decrease in HMG-CoA reductase activity, it should be possible to overcome this inhibition by adding metabolites which appear distally to the metabolic step regulated by HMG-CoA reductase. Addition of 0.1 mM-mevalonate to tunicamycin-treated cells exerted only a limited counteractive effect. In fact, there was a great variability between five different experiments, and hence it was impossible to demonstrate that the counteractive

Fig. 2. Effects of dolichol on proliferation of tunicamycin- and 25-hydroxycholesterol-treated cells

SV-3T3 cells grown in Dulbecco's modified Eagle medium (DMEM) + 10 % fetal calf serum (FCS) were shifted to fresh medium (DMEM + 10 % FCS) supplemented with tunicamycin (0.8 μ g/ml) or 25-hydroxycholesterol (5 μ g/ml) $\pm$ dolichol (1 μ g/ml) for indicated times. 1 h before termination of the experiments, the cells were labelled with [³H]thymidine (5 μ Ci/ml; 5 Ci/mmol). After fixation and autoradiography, the percentage of labelled cells was determined by light microscopy (> 400 cells/plate). The mean values $\pm$ s.d. for three to four separate experiments are shown. $\square$, Tunicamycin (24 h); $\blacksquare$, tunicamycin (48 h); $\hatched$, 25-hydroxycholesterol (48 h); $\dots$, addition of dolichol (1.0 μ g/ml). * P < 0.01; ** P < 0.005.

effect on DNA synthesis in tunicamycin-treated cells was statistically significant. Nor was there any further counteractive effect on DNA synthesis in tunicamycin-treated cells if other concentrations of mevalonate were added (results not shown).

The finding that mevalonate only exerted a weak and non-significant counteractive effect on DNA synthesis might be explained by the intrinsic toxic effects of tunicamycin. In fact, the cell numbers were somewhat lower in the presence of tunicamycin, indicating that this compound affects cell survival as well as blocking the progression through the cell cycle. An alternative explanation might be that tunicamycin also inhibits metabolic steps that appear distally of mevalonate in the isoprene biosynthetic pathway. However, as shown in Table 2, the incorporation of tritiated mevalonate into cholesterol and dolichol, which constitute the two major products in this pathway, was not affected by the addition of tunicamycin.

It also remains a possibility that tunicamycin exerts its inhibitory effects on cell proliferation by some other mechanisms than through inhibiting the activity of HMG-CoA reductase.

Recently it was reported that a cell-cycle-specific

Table 3. Effects of addition of dolichol on *N*-linked glycosylation in tunicamycin-treated cells

SV-3T3 cells grown for 48 h after plating were rinsed and exposed to fresh medium [containing 10 % fetal calf serum (FCS)] with or without tunicamycin (0.8 µg/ml) and/or dolichol (1 µg/ml) over a 24 h period. 1 h before termination of the experiment, cells were labelled with [³H]glucosamine (10 µCi/ml; 10 mCi/mmol). The values are expressed as percentages of the control [i.e. cells growing in serum-enriched (10 %) medium without supplements] and represent the mean values for duplicate determinations in two different experiments.

Treatment	Incorporation of [³ H]glucosamine (% of control)
Control	100
Tunicamycin	18
Dolichol	105
Tunicamycin + dolichol	11

inhibition of SV-3T3 cells follows a 25-hydroxycholesterol-induced depression of HMG-CoA reductase activity [3]. When cholesterol and other isoprene derivatives were investigated for their capacity to overcome the 25-hydroxycholesterol-induced block, it was found that dolichol exerted a significant counteractive effect. We therefore hypothesized that dolichol might also have the capacity to overcome a tunicamycin-induced block. Fig. 2 shows that 1 µg of dolichol/ml partially counteracted the inhibitory effects of both 25-hydroxycholesterol and tunicamycin on DNA synthesis. In addition, Table 3 shows that dolichol does not reverse the inhibitory effects of tunicamycin on the incorporation of tritiated glucosamine into trichloroacetic-acid precipitable material. These findings are generally in line with the notion that tunicamycin's growth-inhibitory effects involve the inhibition of dolichol and isoprene biosynthesis. However, it is significant that dolichol cannot reverse the effects of tunicamycin on glycosylation. Furthermore, tunicamycin does not block the flow of mevalonate into dolichol or cholesterol. One possibility is therefore that dolichol, in a more specific

way, reverses the effect of tunicamycin on HMG-CoA reductase, and that it is the synthesis of isopentenyl adenine or similar compounds which is the crucial step [4].

The finding that tunicamycin efficiently inhibits the activity of HMG-CoA reductase is in good agreement with observations on other cell lines [7]. The mechanisms for this inhibition remain to be elucidated. However, HMG-CoA reductase is in fact a transmembrane glycoprotein located at the endoplasmic reticulum with an active domain facing the cytosol and a high-mannose asparagine-linked oligosaccharide-chain-bearing domain orientated towards the lumen of the endoplasmic reticulum [8,9]. If we then consider that this oligosaccharide is of importance for the function of the enzyme, it is conceivable that tunicamycin-induced inhibition of asparagine-linked glycosylation would depress the activity of HMG-CoA reductase.

This work was supported by grants from the Swedish Cancer Society, the Stockholm Cancer Society, Svenska Läkarsällskapet and Karolinska Institutet.

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Received 29 April 1988/13 February 1989; accepted 13 March 1989