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Selective killing induced by an inhibitor of N-linked glycosylation

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SUMMARY

Treatment with a low dose (0.5 μg/ml) of tunicamycin (an inhibitor of N-linked glycosylation) blocked the cell cycle progression of both normal Balb/c 3T3 cells (A31) and their SV40-transformed derivatives (SVA31) specifically in early G_1 (0-3 h after mitosis). Upon release after an 8-h treatment the A31 cells returned to the cell cycle via a 9-h recovery phase, indicating that they were arrested in G_0. The A31 cells were fully viable after this treatment. In contrast, the postmitotic SVA31 cells, which were unable to arrest in G_0, did not divide after the removal of tunicamycin. Instead, these cells died but this did not occur until 22-34 h after release from the treatment. SVA31 cells that had passed the postmitotic phase of G_1 survived during the parental generation and divided normally. However, a large portion of these cells died during the next cycle, and in total during a 48-h period approximately 50% of the cells were killed as a consequence of an 8-h exposure to tunicamycin. In contrast, treatment with inhibitors of protein synthesis and HMG CoA reductase activity as well as inhibitors of modification of N-linked oligosaccharide chains did not result in cell death.

Key words: cell cycle, killing, glycosylation

INTRODUCTION

Untransformed cells are generally dependent on serum growth factors in the tissue culture medium for continuous proliferation (Holley, 1975; Baserga, 1976). Cells exposed to suboptimal conditions, e.g. serum depletion or a high cell density, leave the cell cycle after mitosis and enter a state of quiescence (G_0) (Nilhausen and Green, 1965; Temin, 1971; Pardee, 1974). When optimal conditions are restored, e.g. serum or growth factors are added back, the cells re-enter the cell cycle via G_1 after a certain lag period has been completed (Pardee, 1974; Brooks, 1976; Pardee et al., 1981; Zetterberg and Larsson, 1985). In fibroblasts this lag period measures approximately 8 h (Zetterberg and Larsson, 1985, 1991).

In contrast, cells of tumor origin or cells transformed in vitro can be grown under suboptimal conditions (e.g. serum depletion) without leaving the cell cycle (Bartholomew et al., 1976; Martin and Stein, 1970). Although cell proliferation is gradually slowed down, the cells do not readily escape into a state of quiescence (G_0), but instead remain in the cell cycle, which they traverse slowly, until most cells eventually die as a result of the environmental conditions (Zetterberg and Sköld, 1969; Pardee and James, 1975; Paul, 1973). Even if the transformed cells can be blocked in G_1 by the use of metabolic inhibitors, e.g. 25-hydroxycholesterol (Larsson and Zetterberg, 1986; Zetterberg and Larsson, 1991), they do not behave as if they were arrested in G_0. In particular, the transformed cells, as distinguished from normal cells, re-enter the cell cycle more or less immediately, without traversing any lag phase, upon the removal of the inhibitor (Larsson and Zetterberg, 1986; Zetterberg and Larsson, 1991).

The ability of non-transformed cells as opposed to the inability (or reduced ability) of transformed cells to enter a specific G_0 state in response to changed environmental conditions most likely reflects one of the fundamental growth control mechanisms that operates stringently in normal cells, but is improperly relaxed in tumor cells. The knowledge of this difference between normal and transformed cells might be useful in the search for anti-tumor agents. In this study, which is concerned with this matter, we have investigated the effects of a specific inhibitor of N-linked glycosylation (tunicamycin) on growth and survival of normal and SV40-transformed Balb/c 3T3 cells, i.e. A31 and SVA31. Since a great portion of cellular proteins, with widely different functions, is glycosylated in this way (Struck and Lennarz, 1980), one would expect that an inhibition of this processing may affect both cell cycling and viability. We found that a transient exposure to tunicamycin results in a temporary G_0 arrest in A31, whereas it kills a substantial portion of the transformed cells.

MATERIALS AND METHODS

Materials

Radiochemicals were from Amersham. Castanospermine and swainsonine were purchased from Boehringer. Tunicamycin mixture and tunicamycin homologues were from Sigma. Mevinolin was obtained from Merck, Sharp and Dohme, and was converted to its sodium salt before experimental use.

Cell culture

Murine Balb/c 3T3 (A31) and SV40-transformed Balb/c 3T3 (SVA31) were purchased through Flow Inc. The cells were grown in monolayers in tissue culture flasks maintained in a 90% air/10% CO_2 atmosphere at 37°C in a humidified incubator. The medium used was Dülbecco's modified Eagle's Medium (DMEM) supplemented with glutamine and 10% (v/v) fetal calf serum (FCS).

Time-lapse video recording

Determinations of cell cycle position (time elapsed after completion of mitosis) and generation time for individual cells, were performed by the use of time-lapse video recording (TLV). This was done by placing a 25 cm^2 flask containing exponentially growing cells in an upright microscope with an attached video camera system for time-lapse analysis. The temperature was maintained at 37°C by a stage heater. A more detailed description of the technique is presented elsewhere (Zetterberg and Larsson, 1991).

DNA synthesis

The level of DNA synthesis of cultured cells in 35 mm dishes (2 ml medium/dish), following the indicated experimental conditions, was assayed by autoradiography or scintillation counting after a 0.5- or a 1-h pulse labelling with [^{3}H]thymidine (0.5 or 2 μCi/ml, 5 Ci/mmol). After termination of the experiments the cells were rinsed with phosphate-buffered saline (PBS) and treated with 10% (w/v) trichloroacetic acid (TCA), whereupon DNA synthesis was assayed.

Protein synthesis and N-linked glycosylation

Protein synthesis and glycoprotein synthesis was measured as the total incorporation of [^{3}H]leucine and [^{14}C]- or [^{3}H]glucosamine, respectively, into acid-insoluble cell material, essentially according to the protocol of Carson and Lennarz (1981). The cells in 35 mm dishes were, after the indicated experimental conditions, pulse-labelled (0.5 h or 2 h) with DL-[4,5-^{3}H]leucine (1 μCi/ml, 53.4 Ci/mmol) or D-[6-^{3}H]glucosamine (1 μCi/ml, 25.2 Ci/mmol) or D-[1-^{14}C]glucosamine (2 μCi/ml, 57.9 mCi/mmol). The radioactivity of the TCA-precipitable material was determined by scintillation counting.

Sodium dodecyl sulphate/polyacrylamide gelelectrophoresis (SDS-PAGE) and protein gel slicing

Cells grown in 14 cm dishes were after labelling with [^{3}H]glucosamine (10 μCi/ml, 25.2 Ci/mmol) rinsed twice with ice-cold PBS and thereafter collected by a cell scraper. The obtained cell pellet was then subjected to a buffered solution containing 0.0625 M Tris-HCl (pH 6.8), 20% glycerol, 2% sodium dodecyl sulphate, 2-mercaptoethanol and bromophenol blue (according to the description given by the supplier). Identical amounts of proteins from each sample was analyzed by SDS-PAGE with a 4% polyacrylamide stacking gel and a 7.5% polyacrylamide separation gel (Ornstein, 1964; Laemmli, 1970; Maniatis et al., 1989). Molecular mass markers ranging from 14.4 to 97.4 kDa were run simultaneously. The gel was stained and fixed in 45% methanol, 10% glacial acetic acid and 0.25% (w/v) Coomassie brilliant blue, and destained in the same mixture excluding the dye (Maniatis et al., 1989). Eventually the gel was put in 20% glycerol and cut in 1 mm slices with a slicing device. The slices were then dissolved in 30% H_2O_2 (Findlay and Geisow, 1989), whereupon 0.2 M NaOH was added to eliminate the excess of H_2O_2. Material from each slice was put in a 20 ml scintillation vial containing 5 ml scintillation cocktail (Instagel, Canberra-Packard). The radioactivity was determined using a liquid scintillation counter.

Determination of HMG CoA reductase activity

Cells cultured in 50 mm dishes were, after the indicated experimental conditions, rinsed twice in prewarmed (37°C) PBS (Ca^{2+}- and Mg^{2+}-free) and harvested for determination of cellular activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, essentially as described elsewhere (Larsson and Zetterberg, 1986).

Determination of cellular protein content

The protein concentration of cell lysates was determined by a dye binding assay (Bradford, 1976), with a reagent purchased from Bio-Rad. Bovine serum albumin was used as the standard.

Additions of inhibitors and chemicals

25-Hydroxycholesterol and tunicamycin were dissolved in 99.5% ethanol prior to use. Equivalent volumes of ethanol were added to the controls. The volume of ethanol added to a 2 ml dish was 0.5-5 μl. MVA was dissolved in medium or PBS.

Cell viability

Cell death was recognized microscopically as detachment or lysis of cells in the monolayer cultures and was confirmed by uptake of trypan blue.

RESULTS

The aim of the present study was to investigate the effects of inhibition of N-linked glycosylation and HMG CoA reductase activity on cell cycle progression and cell viability of normal and tumor-transformed fibroblasts. These studies were performed using Balb/c 3T3 cells (A31) and their SV40-transformed derivatives (SVA31). First, we measured the inhibitory effects of 25-hydroxycholesterol (a HMG CoA reductase inhibitor) and tunicamycin (an inhibitor of N-linked glycosylation) on DNA synthesis, HMG CoA reductase activity, N-linked glycosylation and protein synthesis in cultures of A31 and SVA31 (Table 1). The effects of both 2-h and 24-h incubations were tested. 25-hydroxycholesterol (2 μg/ml), added for 24 h, blocked DNA synthesis in both cell types. This effect was related to a substantial depression (80-85%) of HMG CoA reductase activity and a 70-80% and 30-40% decrease in glycosylation and protein synthesis, respectively. After only 2 h of treatment, when DNA synthesis had not yet been affected, 25-hydroxycholesterol had induced a 65-75% decrease in HMG CoA reductase activity and a 10-30% decrease in glucosamine incorporation and protein synthesis.

Treatment with tunicamycin also blocked DNA synthesis in both cell types. The SVA31 line was most sensitive in this respect, and a dose of 0.2 μg/ml was as effective as 0.5 μg/ml tunicamycin was in A31 cells. When 1 μg/ml of tunicamycin was administered to SVA31 for 24 h, it induced cytotoxic effects. Such effects were not seen in A31. As expected, the glucosamine incorporation was drastically reduced in both cell lines following treatment with tunicamycin, and there were essentially no differences in the dose-response pattern between the cell lines.

Interestingly, tunicamycin also induced a considerable depression of HMG CoA reductase activity. A treatment (0.5 μg/ml) of as short a duration as 2 h reduced the level

Table 1. The effects of 25-hydroxycholesterol and tunicamycin on HMG CoA reductase activity and macromolecular synthesis

Cell type and treatment	2 h				24 h			
	TdR[1]	HMGR[2]	GlcN[3]	Leu[4]	TdR[5]	HMGR[6]	GlcN[7]	Leu[8]
A31								
25-OH (2 μg/ml)	105.7	36.0	69.4	89	15.2	16.6	22.4	58
Tu (0.2 μg/ml)	105.7	42.5	68.6	97	36.4	32.8	44.1	79
Tu (0.5 μg/ml)	100.0	26.3	52.1	87	9.1	17.5	24.5	64
Tu (1.0 μg/ml)	85.7	14.2	47.1	n.d.	3.0	13.5	18.9	n.d.
Tu (0.5 μg/ml)+ MVA (0.77 μM)	97.1	23.9	52.9	94	9.1	14.8	22.4	68
SVA31								
25-OH (2.0 μg/ml)	93.3	27.0	75.6	91	7.0	22.3	28.7	61
Tu (0.2 μg/ml)	88.9	54.8	60.7	94	4.7	19.4	30.2	78
Tu (0.5 μg/ml)	93.3	10.3	41.9	80	2.3	14.4	22.0	68
Tu (1.0 μg/ml)	33.3	n.d.	n.d.	n.d.	–[9]	–[9]	–[9]	–[9]
Tu (0.5 μg/ml) + MVA (0.77 μM)	97.8	14.1	44.6	86	7.0	30.2	21.6	63

The effects of treatment with 25-hydroxycholesterol or tunicamycin on DNA synthesis (TdR), HMG CoA reductase activity (HMGR), N-linked glycosylation (GlcN) and protein synthesis (Leu) were investigated in A31 and SVA31 cells. Exponentially growing cells were exposed to 25-hydroxycholesterol (25-OH) and tunicamycin (Tu) at indicated doses for 2 or 24 h, whereupon cells were harvested for assay (for details see materials and methods). All values represent % of the control (i.e. untreated cells). The values are means of duplicate determinations. In all cases the standard deviations (s.d.) were less than 15% of the means.

Control values of A31/SVA31,respectively: (1) 35%/45%; (2) 24.7/12.6 pmol/min per mg protein; (3) 12,100/29,500 dpm/mg protein; (4) 110 /170 dpm/μg protein; (5) 33%/43%; (6) 22.9/13.9 pmol/min per mg protein; (7) 14,300/26,800 dpm/mg protein; (8) 136/183 dpm/μg protein. (9) Cell death. n.d., not determined.

of HMG CoA reductase activity by 75-90%. This finding raises the question as to whether the growth-inhibitory effects of tunicamycin may be mediated through the depression of HMG CoA reductase activity rather than by the inhibition of N-linked glycosylation in itself. However, as is also shown in Table 1, addition of mevalonate (MVA) to tunicamycin-treated cells did not overcome the inhibitory effects on DNA synthesis at 24 h, which it would have done if the growth inhibition was mediated by the decrease in HMG CoA reductase activity. Therefore, it appears that the growth-inhibitory effects of tunicamycin instead are the result of its inhibitory effects on N-linked glycosylation. On the other hand, the 25-hydroxycholesterol-induced growth-inhibition might be the result of decreased N-linked glycosylation. Tunicamycin was also found to induce slight inhibitory effects on protein synthesis (approximately 20%) in both cell types (Table 1).

Table 2 demonstrates the effects on cell viability of cultures of A31 and SVA31 following treatment with 25-hydroxycholesterol and tunicamycin. The inhibitors were added either for a continuous 48-h period, or for transient periods (2 or 8 or 24 h), whereupon the cells were shifted back to inhibitor-free medium. After 48 h cell viability was determined. 25-hydroxycholesterol caused a slight (5%) or no decrease at all in cell viability. Whereas continuous treatment with tunicamycin induced a substantial cell killing (40% at most) in SVA31, this treatment only led to a 5% decrease in cell viability in A31. Interestingly, a transient 8-h exposure was sufficient to produce a 35-50% decrease in cell viability in SVA31 but had no effects at all on A31. The decrease in SVA31 viability was not reduced by incubating the treated cells at low temperature (4°C). If the duration of the tunicamycin exposure was reduced to 2 h there was no extensive cell killing in SVA31. We also tested the effects of an inhibitor of protein synthesis, i.e. cycloheximide, on cell viability. An 8-h exposure to cycloheximide (1 μg/ml) was found to decrease the level of protein synthesis by 82.8%. As is shown in Table 2, cycloheximide did not kill either cell type, irrespective of whether it was present during the whole period or only for 8 h.

Table 2. Cell viability following treatment with metabolic inhibitors

Treatment and duration (h)	Duration of inhibitor-free period (h)	Cell viability (%)	
		A31	SVA31
25-OH, 48	0	95-100	90-95
25-OH, 24	24	100	100
25-OH, 8	40	100	100
Tu, 48	0	90-95	60-75
Tu, 24	24	95-100	50-60
Tu, 8	40	100	50-65
Tu, 2	46	100	90-95
Tu, 8	40 (at 4°C)	–	55-65
CHX, 48	0	100	100
CHX, 8	40	100	100

Exponentially growing cells in 35 mm dishes were shifted to fresh medium containing metabolic inhibitors, i.e. 25-hydroxycholesterol (25-OH) (2 μg/ml), tunicamycin (Tu) (0.5 μg/ml) and cycloheximide (CHX) (1 μg/ml), for various periods (2-48 h). After the 2-, 8- or 24-h exposures the cells were rinsed three times with prewarmed PBS and then shifted to inhibitor-free medium. The cell number in marked areas in the dishes was counted in a light microscope at different time points. All experiments were terminated after 48 h and the percentages of cell viability as compared to untreated control cells were determined. The indicated values are from two independent experiments.

On the basis of the data above we conducted a deeper investigation into how an 8-h inhibition of N-linked glycosylation interferes with cell growth and viability. Hence, we were especially interested in analyzing whether the cyto-

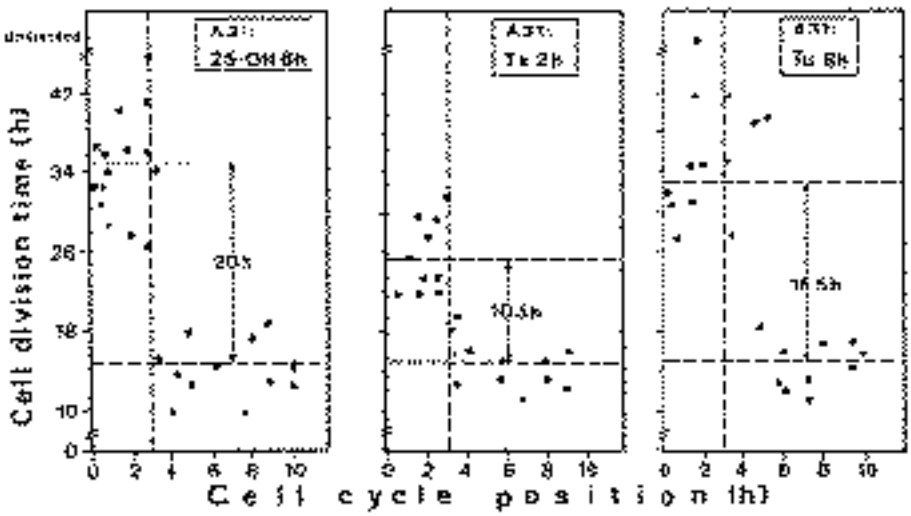

Fig. 1. Effects of inhibition of HMG CoA reductase activity or inhibition of N-linked glycosylation on the cell cycle progression of A31 cells. Exponentially growing A31 cells were treated with 25-hydroxycholesterol (25-OH) (2 μg/ml) for 8 h, or with tunicamycin (Tu) (0.5 μg/ml) for 2 or 8 h. After these treatments the cells were rinsed and shifted back to inhibitor-free medium for an additional 40 or 46 h. Cell cycle position, at onset of treatment, and cell division time of individual cells was determined by TLV. Each dot represents one cell division.

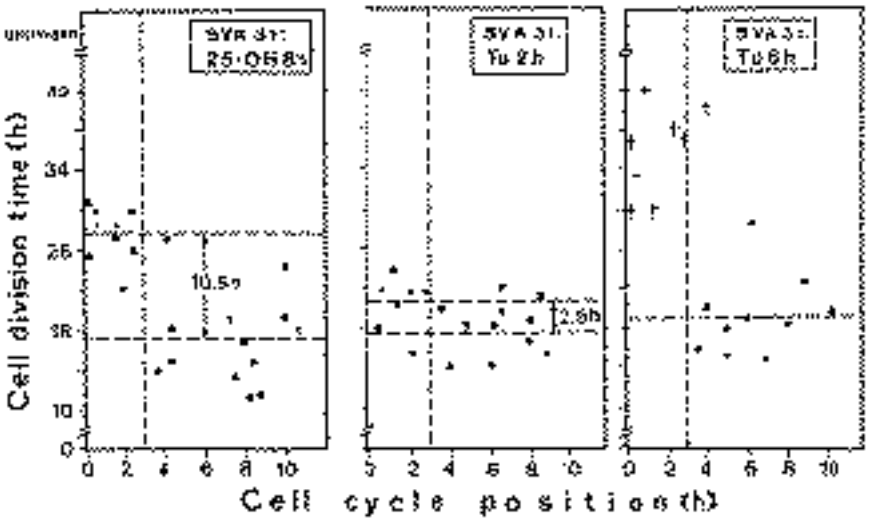

Fig. 2. Effects of inhibition of HMG CoA reductase activity or inhibition of N-linked glycosylation on the cell cycle progression of SVA31. Exponentially growing SVA31 were exposed to 25-hydroxycholesterol (2 μg/ml) for 8 h or tunicamycin (0.5 μg/ml) for 2 and 8 h. Dots indicate cell division, and † indicates cell death.

toxic effects induced by a transient 8-h exposure of SVA31 cells to tunicamycin were confined to a specific cell cycle stage. These studies were performed by the use of time-lapse video recording. This method makes it possible to map the cell cycle with regard to response to environmental manipulations of the cell cycle progression of individual cells in a unperturbed, asynchronously growing cell population (Zetterberg and Larsson, 1991). The mean generation times of exponentially growing untreated A31 and SVA31 cells were found to be 14.5 h and 18 h, respectively. In Fig. 1 it is shown that an 8-h exposure of A31 cells to 25-hydroxycholesterol resulted in a substantial delay (20 h) in cell division time. However, this occured only in cells that were located in cell cycle positions of 0-3 h, measured as the time spent after the last mitosis, whereas older cells were unaffected. In Fig. 1 it is also shown that brief exposures (2 h or 8 h) to tunicamycin promoted a similar cell cycle delay in postmitotic A31-cells. Consistent with the data in Table 2, no cytotoxic effects appeared in A31 following treatment with any of these inhibitors.

Fig. 2 illustrates the effects of the inhibitors on the cell cycle progression of SVA31 (compare with Fig. 1). As can be seen, postmitotic SVA31-cells responded by a cell cycle delay following an 8-h exposure to 25-hydroxycholesterol as well as a 2-h exposure to tunicamycin. However, as compared to the case of A31-cells, the cell cycle delay was considerably shorter in length. Interestingly, the postmitotic SVA31 cells were unable to undergo a new cell division upon a transient 8-h treatment with tunicamycin. Instead all these postmitotic cells were killed. However, cell death of these cells did not occur during the time when tunicamycin was present. Instead this occurred as late as 22-34 h after the shift to tunicamycin-free medium (Fig. 2). In contrast, all cells older than 3 h, with the exception of a single cell, survived and underwent mitotis on schedule.

In order to survey the differences in the cell cycle kinetics of A31 and SVA31 cells with regard to the responsiveness to 25-hydroxycholesterol and tunicamycin, the division-time distributions of these cells were compared. Fig. 3A shows the division-time distributions following an 8-h exposure to 25-hydroxycholesterol. As is clearly shown, the division times of postmitotic 25-hydroxycholesterol-treated A31 were additionally delayed by 11 h (i.e. in

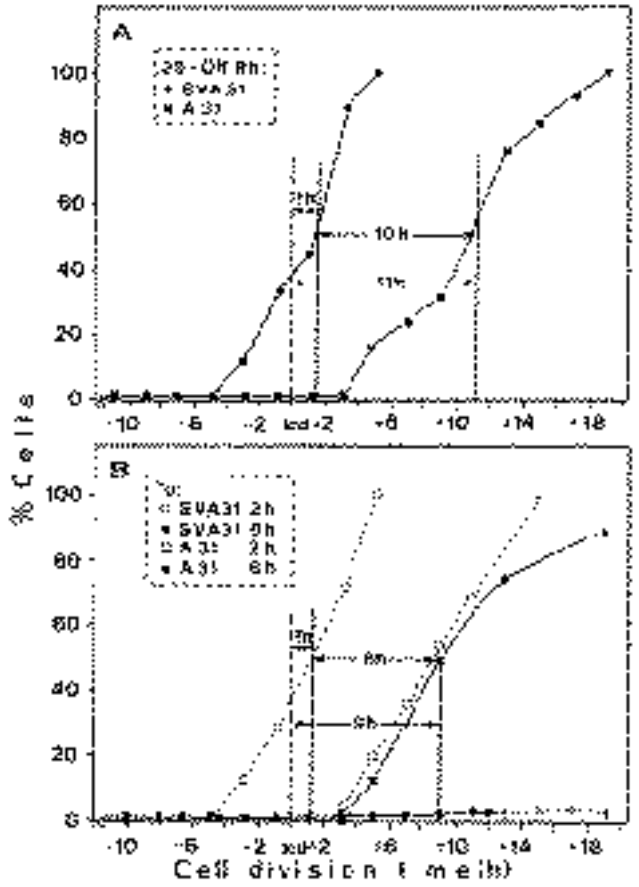

Fig. 3. Kinetics of cell cycle progression of postmitotic A31 (squares) and SVA31 (circles) exposed to an 8-h (filled symbols) treatment with 25-hydroxycholesterol (A), or 2-h (open symbols) or 8-h (filled symbols) treatments with tunicamycin (B). The data are derived from the TLV recordings, and show the distributions of cell division times of postmitotic cells (i.e. cells younger than 3 h). The actual lengths of treatments were subtracted from the observed division times. The division-time distribution curves are related to the mean cell division time (indicated as *tcd* and broken vertical lines) of untreated cells. The vertical continuous lines show the mean cell division time of treated cells. The horizontal arrowed continuous lines indicate the difference in mean cell division time between the two cell lines, as well as the difference between mean cell division time of treated and untreated cells.

Table 3. The level of glycosylation and DNA synthesis after release of tunicamycin

Treatment and duration (h)	Inhibitor-free period (h)	Incorporation (dpm/mg protein)	
		[^{14}C]glucosamine	[^{3}H]thymidine
Tu, 8	0	3,277±633	1,050±57
Tu, 8	2	8,563±865	3,786±456
Tu, 8	4	11,172±987	7,930±658
Tu, 8	24	757±120	1,571±60
Control	–	19,568±1,763	14,078±1,099

Exponentially growing A31 were shifted to medium containing 0.5 μg tunicamycin (Tu)/ml for a 8-h period. Thereafter the cells were rinsed three times with PBS and changed to tunicamycin-free medium for 0, 2, 4 or 24 h periods. During the last 30 min of the periods [^{14}C]glucosamine (1.0 μCi/ml) and [^{3}H]thymidine were present in the medium. The incorporation into acid-insoluble material was determined by scintillation counting. The mean values ± s.d. of the incorporation of [^{14}C]glucosamine of duplicate determinations are indicated.

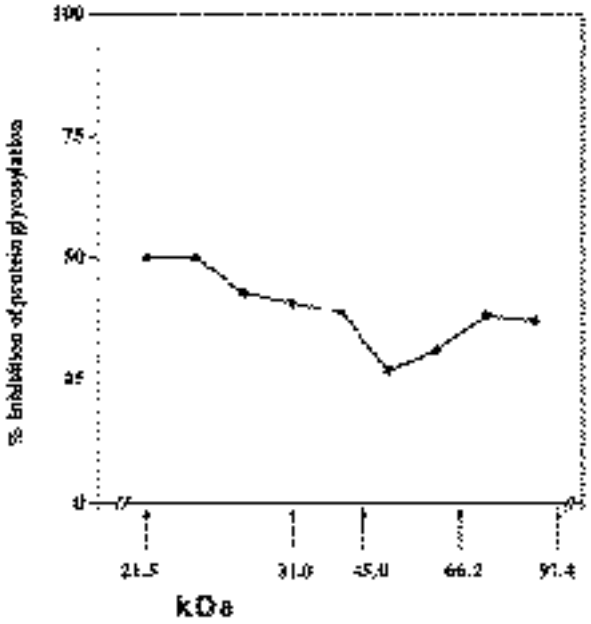

Fig. 4. The effects of tunicamycin on incorporation of [^{3}H]glucosamine into proteins separated by SDS-PAGE. Cells were subjected to inhibitor-free medium or medium containing tunicamycin, and labelled with [^{3}H]glucosamine for a 4-h period, after which the cell material was harvested for SDS-PAGE. The gel was then sliced and the radioactivity of each slice (i.e. slice numbers 6-50, corresponding to 21.5-97.4-kDa proteins) was determined. By comparing the incorporation data between tunicamycin-treated and untreated cells, the inhibitory effect of tunicamycin could be calculated. Each dot represents a mean value obtained from 5 consecutive slices (i.e. slices 6-10; 11-15 etc.).

addition to the treatment time). These data imply that these cells are arrested and therefore require an 11-h phase in order to resume the cell cycle upon removal of 25-hydroxycholesterol. In contrast the SVA31 cells were only delayed 1 h implying that these cells re-enter the cell cycle almost immediately after termination of the treatment. Fig. 3B shows how 2-h and 8-h exposures to tunicamycin result in a similar delay in A31 to that following 25-hydroxycholesterol treatment. Conversely, SVA31 either enter the cell cycle directly, as after a 2-h treatment, or are killed, as after an 8-h treatment.

On the basis of the cytotoxic effects on SVA31, the remaining experiments will be focused on this cell line. In Table 3 it is shown that the cells were viable at the time of termination of the 8-h tunicamycin treatment, since they exhibited macromolecular synthesis. In fact the cells increased their incorporation of [^{14}C]glucosamine and [^{3}H]thymidine multi-fold during the first 4 h after the removal of tunicamycin. These data confirm that the removal of the drug was successful, as well as excluding the possibility that the inhibitory effects of tunicamycin on N-linked glycosylation were irreversible. However, 24 h after release from the treatment the incorporation of both

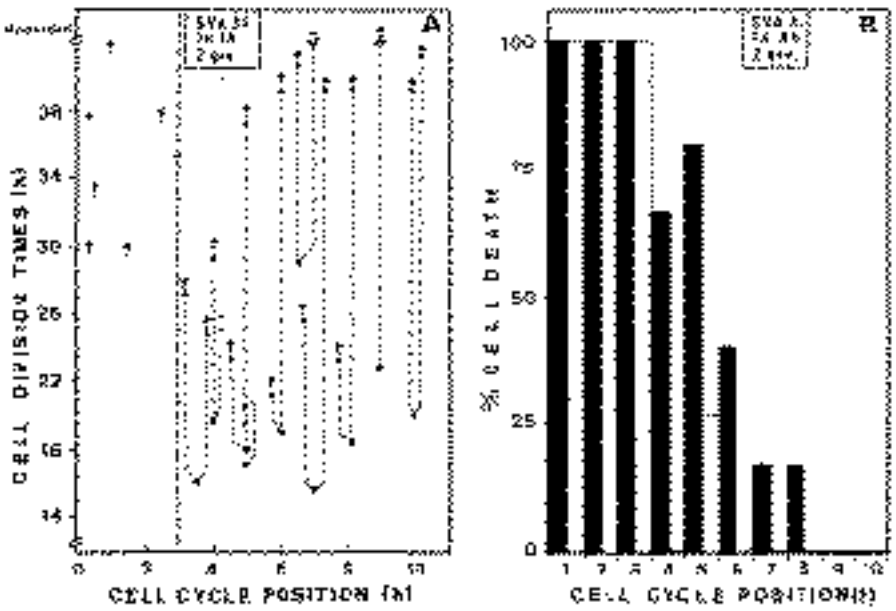

Fig. 5. (A) The effects of an 8-h transient treatment with tunicamycin (0.5 μg/ml) on progeny cell division and cell viability in SVA31, as analyzed by TLV. Progeny cell cycles are indicated as vertical arrowed broken lines. Dots indicate cell divisions, and † cell death. Cells that had not undergone division during the observation period are seen in the "undivided" region of the figure. In two cases the fate of only one daughter cell could be determined on the TLV recordings. (B) Percentages of cell death in the parental generation (open bars) or the accumulated cell death during two generations (filled bars), as related to cell cycle position of the parental cell at exposure to tunicamycin.

substances was found to be drastically depressed. This suggests that a large portion of the cells had undergone severe damage during the middle or late part of the tunicamicin-free period. In Fig. 4 it is confirmed that a short exposure (here 4 h) to 0.5 μg tunicamycin/ml was enough to induce a substantial decrease in *protein* glycosylation. As can be seen, the incorporation of [^{3}H]glucosamine into 21.5-97.4 kDa proteins, which were separated by SDS-PAGE, was lowered by approximately 40% (Fig. 4).

Since essentially all SVA31 cells older than 3 h at onset of the 8-h tunicamycin treatment survived and underwent mitosis, it was interesting to investigate the fate of these cells during the next generation. This is demonstrated in Fig. 5A. As can be seen, a substantial portion of the progeny cells died, and this occurred 3-40 h after mitosis. Some of the surviving cells subsequently underwent a new mitosis while the remainder did not divide during the observation period. Interestingly, the survivers in the second generation were all originated from cells that were located in cell cycle positions exceeding 6.5 h at the time of treatment. Thus, whereas all cells younger than 3 h were killed during the first generation, all cells with cell ages of 3-6.5 h were killed in the next generation. This is summarized in Fig. 5B, which shows the percentages of cell-cycle-specific cell death in the first generation as well as the accumulated cell death during two generations.

The batch of tunicamycin used in the experiments presented so far is composed of a mixture of tunicamycin homologue A (3%), homologue B (36%), homologue C (38%) and homologue D (20%). In Table 4 the effects of each of these tunicamycin homologues (i.e. tunicamycins A_1, B, C_1 and D_1) on protein synthesis, N-linked glycosylation and cell viability of SVA31 are demonstrated. As is shown, 8-h exposures to tunicamycins B, C_1 and D_1 reduced the levels of glycosylation and cell viability to equal extents, 80-81% and 52-59%, respectively. These effects are comparable with those induced by the tunicamycin mixure. Tunicamycin A1 decreased N-linked glycosylation only moderately (50%), and its cytotoxic effects were marginal (20%). Thus, there is a relationship between the level of the inhibitory effect on glycosylation and the obtained cytotoxic effects. None of the homologues caused any large decrease in the level of protein synthesis. The strongest effect on protein synthesis was obtained by tunicamycin C_1 (26%) and the tunicamycin mixure (21%), whereas tunicamycin B reduced the level of protein synthesis by as little as 5%.

Table 4. The effects of different glycosylation inhibitors on protein synthesis, glycosylation and cell viability

Compound	Protein synthesis (%)	Glycosylation (%)	Cell viability (%)
None (control)	100*	100*	100
Tunicamycin (mixed) (0.5 μg/ml)	79	14	40
Tunicamycin A1 (0.5 μg/ml)	97	50	80
Tunicamycin B (0.5 μg/ml)	95	20	41
Tunicamycin C1 (0.5 μg/ml)	74	20	48
Tunicamycin D1 (0.5 μg/ml)	83	19	42
Castanospermine (100 μg/ml)	−	−	100
Swainsonine (10 ng/ml)	−	−	100
Mevinolin (5 μM)	−	77	95

In parallel experiments SVA31 cells were exposed to the indicated compounds for 8 h, after which the cells were either pulse-labelled with [^{3}H]leucine or [^{3}H]glucosamine in order to assay the level of protein synthesis and N-linked glycosylation, or shifted to inhibitor-free medium for an additional 40 h in order to determine the cell viability. The obtained values are expressed as percentages of the control.

*Absolute control values: Incorporation of [^{3}H]leucine (protein synthesis) was 58,000 dpm/mg protein; incorporation of [^{14}C]glucosamine (N-linked glycosylation) was 70,000 dpm/mg protein.

We also tested the effects of mevinolin, which is a competitive inhibitor of HMG CoA reductase. As can be seen, this inhibitor reduced the uptake of glucosamine by only 23% (Table 4), although it depressed the HMG CoA reductase activity by as high as 90-95% (data not shown). Seemingly, this effect was not enough to induce cytotoxic effects (Table 4). The inability of mevinolin to induce a substantial decrease in glycosylation might be explained by the

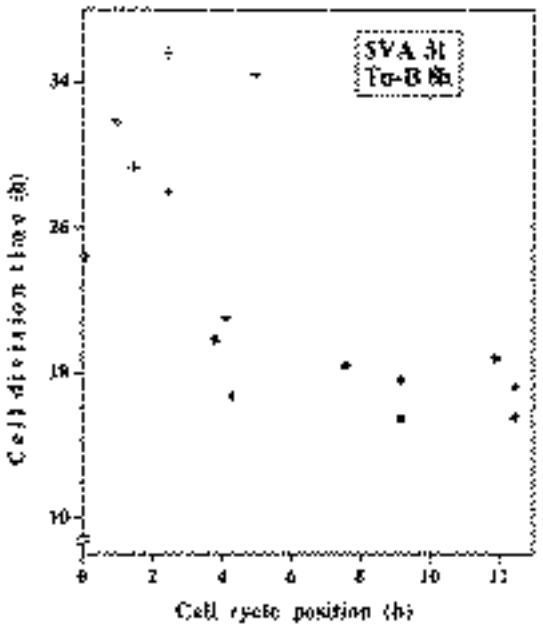

Fig. 6. Effects of an 8-h transient exposure to tunicamycin B on the cell cycle progression and cell viability of SVA31. Exponentially growing SVA31 were exposed to tunicamycin B (0.5 μg/ml) for 8 h, whereupon the cells were shifted back to inhibitor-free medium (compare with Fig. 2 and Fig. 3). Dots indicate cell division, and + indicates cell death.

high substrate affinity in the dolichyl phosphate biosynthetic pathway (James and Kandutsch, 1979).

Table 4 also shows the effects of 8-h exposures to castanospermine and swainsonine on cell viability of SVA31. Castanospermine is an inhibitor of glucosidase I and swainsonine of Golgi mannosidase II (Elbein, 1987). Glucosidase I and mannosidase II are important enzymes in the processing of N-linked oligosaccharides. However, none of these compounds decreased the viability of SVA31. The castanospermine and swainsonine concentrations were chosen on the basis of other studies (Hadwiger et al., 1986; deSantis et al., 1987). Higher concentrations of castanospermine and swainsonine than those shown in Table 4 were also tested, but did not result in any measurable cell killing either.

In Fig. 6 it is demonstrated that tunicamycin B, which exerted less inhibitory effects on protein synthesis (see Table 4), induces cell cycle-specific cell death, comparable to that induced by the mixed type of tunicamycin (see Fig. 2), in SVA31 cells.

DISCUSSION

In general all eukaryotic cells are capable of the synthesis of glycoproteins that are destined to become components of the plasma membrane (Struck and Lennarz, 1980; Lennarz, 1987). However, N-linked glycosylation is not restricted to a specific functional type of glycoprotein. Instead it occurs in several membrane proteins (Bharathan et al., 1990; Segarini et al., 1992), growth factors (Tominaga et al., 1990; Arakawa et al., 1991; Kris et al., 1991), enzymes (Turnay et al., 1989), secretory proteins without enzymatic functions (Nilsen-Hamilton et al., 1980), membrane channels (Zona et al., 1990) and immunoglobulins (Lund et al., 1990). Thus, N-linked glycosylation affects several important functions in the cell. N-linked glycosylation can be specifically inhibited by tunicamycin. Tunicamycin, which is a naturally occuring antibiotic, inhibits UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-1-phosphate transferase and thereby blocks the production of *N*-acetylglucosamine-pyrophosporyldolichol (Heifetz et al., 1979). This leads to underglycosylated glycoproteins by preventing the formation of intermediates necessary for the synthesis of *N*-glycosidic linkages (Heifetz et al., 1979). The use of tunicamycin has enabled a lot of manipulative studies concerning the role of N-linked glycosylation in biological systems to be performed. In sea urchin tunicamycin has been shown to specifically block gastrulation, which requires the biosynthesis of dolichyl-dependent glycoproteins, without impairing earlier developmental steps (Struck and Lennarz, 1980). However, tunicamycin is not without side-effects. For instance, tunicamycin has been shown to decrease the rate of de novo protein synthesis to some extent (Struck and Lennarz, 1980).

After attachment of the oligosaccharide chain to the asparagine residue of the protein, the N-linked oligosaccharide is usually subjected to modifications (Schachter and Roseman, 1980; Hubbard and Ivatt, 1981). These processes occur in the endoplasmic reticulum and in the Golgi apparatus (Schachter and Roseman, 1980; Hubbard and Ivatt, 1981), and several enzymes, e.g. glucosidase I and Golgi mannosidase II, are involved (Schachter and Roseman, 1980; Hubbard and Ivatt, 1981). The extent of processing depends on the protein and on the location of the asparagine residue within the protein to which the oligosaccharide is attached (Schachter and Roseman, 1980; Hubbard and Ivatt, 1981). Therefore the final state of the N-linked oligosaccharide may vary between different types of glycoproteins. By the use of inhibitors of certain modification steps, e.g. castanospermine (inhibitor of glucosidase I) and swainsonine (inhibitor of Golgi mannosidase II), it has been confirmed that the processing of N-linked oligosaccharides may influence the function of glycoproteins (Hadwiger et al., 1986; deSantis et al., 1987).

The major finding of the present study is that treatment with tunicamycin selectively kills SV40-transformed Balb/c 3T3 cells (SVA31) whereas the normal cells (A31) are temporarily arrested in G_0. We compared the effects of tunicamycin with an inhibitor of HMG CoA reductase, i.e. 25-hydroxycholesterol, which has previously been shown to arrest Swiss 3T3 fibroblasts in G_0 (Larsson and Zetterberg, 1986). We found that A31 responded similarly to these drugs in the sense that early G_1 cells were substantially delayed in their cell cycle progression, and hence required a 8-11 h lag period in order to return the cell cycle after the removal of the inhibitors. These cells did *not* exhibit any cytotoxic effects either during or after exposures to tunicamycin. In contrast, SVA31 did not need any recovery phase in order to re-enter the cell cycle. Even if the postmitotic (0-3 h after mitosis) SVA31 cells were blocked by an 8-h exposure to 25-hydroxycholesterol or a 2-h expo-

sure to tunicamycin, they returned to the cell cycle immediately upon the removal of the inhibitors. As a consequence of an 8-h tunicamycin exposure, the postmitotic SVA31 cells failed to divide. Instead all these cells died. This did not occur during the time of treatment but as late as 20-34 h *after* its release. If it is assumed that postmitotic cells have to build up certain glycoproteins in order to survive the traverse through the cell cycle, one mechanism contributing to the tunicamycin-induced selective killing of SVA31 cells may be ascribable to the inability of these cells to become arrested. Since the postmitotic A31 cells are arrested as a response to tunicamycin they re-enter the cell cycle through an 8-10 h recovery period. Thereby these cells may have sufficient time in which to restore the loss of glycoproteins. In contrast, the tumor-transformed cells, which enter the cell cycle directly, would be vulnerable, since they have had no time in which to compensate for the glycoprotein deficit.

Our data strongly suggest that the cytotoxic effect of tunicamycin depends on its inhibitory effects on N-linked glycosylation and not on its reduction of protein synthesis. In fact we provide direct evidence that decreased protein synthesis is not responsible for the killing of the SV40 transformed cells: firstly, the low doses of the tunicamycin mixture used in our experiments only reduced the rate of protein synthesis marginally (approximately 20%); secondly, the tunicamycin homologue B was as effective in killing SVA31 as the the other tunicamycin types were, although it reduced the level of protein synthesis hardly at all (only 5%); thirdly, treatment with an inhibitor of protein synthesis (cycloheximide) failed to induce cytotoxicity.

In contrast to the case of a direct inhibition of the N-linked glycosylation such as that following treatment with tunicamycin, two inhibitors of N-linked oligosaccharide modification (i.e. castanospermine and swainsonine) did not induce cell death in the virus transformed cells.

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