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Growth inhibition of human pancreatic cancer cells by sphingosylphosphorylcholine and influence of culture conditions

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Abstract. Sphingosylphosphorylcholine (SPC) has been shown to be a potent mitogen for Swiss 3T3 fibroblasts and also to be an inhibitor of cell growth of some cancer cells, suggesting cell-selective action of the lipid. We examined the effects of SPC, and its structurally-related sphingosine (SP), sphingosine 1-phosphate (S1-P) and membrane-permeable derivatives of ceramides on cell growth of four strains of human pancreatic cancer cells, MIA PaCa-2, PANC-1, PK-1 and PK-9. Under the reported conditions for SPC-induced stimulation of 3T3 fibroblasts, where cells were grown to confluency in the presence of 10% fetal bovine serum (FBS) in culture prior to experiments and insulin was supplemented in experimental culture, none of the agents tested stimulated DNA synthesis in MIA PaCa-2 cells and ceramide at high concentration even inhibited it. On the other hand, in reduced FBS concentration in preculture and in the absence of insulin in experimental culture, SP, S1-P and ceramides suppressed cell growth of all the cells tested including Swiss 3T3 fibroblasts. However, under these conditions, SPC inhibited three out of four species of pancreatic cancer cells but stimulated Swiss 3T3 fibroblasts in terms of both DNA synthesis and cell proliferation. Cell cycle analysis showed that SPC stimulated cell cycle progress from the G_1 to the S phase in Swiss 3T3 fibroblasts but inhibited it in PANC-1 cells in reduced FBS concentrations. We suggest that extracellular SPC can inhibit cell growth of human pancreatic cancer cells through regulation of the cell cycle process depending upon both the cell species and environmental conditions.
Key words. Sphingolipids; pancreatic cancer; growth modulation.

Abbreviations. SPC = sphingosylphosphorylcholine, S1-P = sphingosine 1-phosphate, C2-ceramide = N-acetyl-sphingosine, C6-ceramide = N-hexanoyl-sphingosine.

Pancreatic cancer is the fourth or fifth leading cause of cancer death in the United States [1–3], probably because pancreatic cancer is clinically characterized by poor prognosis and unresponsiveness to intensive therapeutic efforts [4–6]. Under the circumstances, characterization of the growth regulation of pancreatic cancer cells is required to provide a basis for improving prognosis and for developing effective therapies.

Some investigators have suggested roles for sphingolipids in cell growth regulation [7–9]. The bioactivity of sphingolipids and their metabolites has been widely noticed, especially in terms of their effects on cell proliferation and death. Among these sphingolipids, sphingosylphosphorylcholine (SPC), accumulated in tissues of patients with Niemann-Pick disease [10], was originally demonstrated to be a potent mitogen for a variety of cells [11, 12], and its stimulatory action on cell proliferation in vivo as a wound healing agent was recently reported [13]. On the other hand, another research group reported the inhibitory actions of SPC on the proliferation of ovarian and breast cancer cells [14], despite the fact that lysophosphatidic acid, another lysophospholipid, markedly stimulates the proliferation of these cells under the same experimental conditions. Sphingosine and its phosphorylated derivative, sphingosine 1-phosphate (S1-P), have also been shown to stimulate [15, 16] and inhibit [17] cell growth of a variety of cells. The effect of these lipids thus seems to be bidirectional, possibly depending on target cell type, although the studies previously reported did not indicate the cause of the bidirectional effects. These findings led us to investigate the effects of these lysosphingophospholipids on the proliferation of pancreatic cancer cells, to characterize the cancer cells regarding their proliferative nature and to obtain a basic idea of how they might be treated.

In the present study, we examined the effects of SPC and its structurally related compounds, sphingosine (SP) and sphingosine 1-phosphate (S1-P), and two membrane-permeable derivatives, on cell growth of established strains of pancreatic cancer cells, MIA PaCa-2, PANC-1, PK-1 and PK-9. We found that under serum-deprived conditions, SPC selectively inhibits cell growth of some pancreatic cancer cells but stimulates that of Swiss 3T3 fibroblasts, despite the fact that other sphingolipids tested non-selectively suppress either pan-

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creatic cancer cells or Swiss 3T3 fibroblasts. SPC stimulates progress of the cell cycle from the G_1 to the S phase in Swiss 3T3 fibroblasts but inhibits it in pancreatic cancer cells, suggesting that the bidirectional action of the sphingolipid is mediated through this cell cycle modulation.

Materials and methods

Chemicals. Sphingosine, SPC, N-acetyl-sphingosine (C2 ceramide) and N-hexanoyl-sphingosine (C6 ceramide) were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Sphingosine 1-phosphate (S1-P) was prepared by treatment of SPC with phospholipase D as previously described [15, 18]. Propidium iodide, ribonuclease and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO). [^{3}H]thymidine was purchased from DuPont New England Nuclear (Boston, MA).

Cells and cell culture. Swiss 3T3 cells and two human pancreatic cancer cell lines, PANC-1 and MIA PaCa-2 cells, were purchased from ATCC (Rockville, MD). Other human pancreatic cancer cell lines, PK-1 and PK-9, were kindly provided by the Cancer Cell Repository, Tohoku University (Sendai, Japan). For the purpose of cell propagation, cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and without antibiotics at 37° C in a humidified atmosphere of 5% CO_2 in air. For experimental purposes, cells were inoculated to give a cell density of 5% confluency, and cultured in 10% FBS-supplemented DMEM for the first 24 h. For the depletion of serum factors, the medium was then changed to 0.5% FBS-supplemented DMEM and the culture was continued for 72 h. Usually the cells were then 80% confluent. After the medium was discarded, the cells were treated for 24 h with the agent to be tested in 0.5% FBS-supplemented DMEM. In some experiments, cells were cultured according to the protocol of Spiegel et al. [15, 19–21]. Briefly, cells were cultured in 10% FBS-supplemented DMEM for 72–120 h until they became confluent. During the cultivation, the medium was changed once after 48 h cultivation. Quiescent cells were treated for 24 h with DMEM supplemented with 30 μg/ml BSA, 4 μg/ml insulin and the agent to be tested.

Analysis of DNA synthesis. After the first 12 h of cell treatment with or without a test agent in 0.5% FBS-supplemented DMEM, 1 μCi of [^{3}H]thymidine was added, and the cultivation was continued for the next 12 h. The cells were washed once with 0.5 ml of PBS, then twice with 0.5 ml each of 5% trichloroacetic acid (TCA) and solubilized with 0.75 ml of lysis buffer composed of 0.1% sodium dodecyl sulfate, 0.1N NaOH and 2% Na_2CO_3. TCA insoluble radioactivity was counted using a liquid scintillation counter, LSC-3000 (Aloka, Tokyo).

Analysis of changes in viable cell number. Number of viable cells cultured under the serum-deprived conditions was estimated by the MTT(3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide) cell growth assay kit (Chemicon International Inc., Temecula CA), as previously described [22]. Briefly, cells were plated at about 1×10^5 cell/ml in a 48-well microplate (Costar, Cambridge, MA) and cultured with or without a test agent under the serum-deprived conditions as described in the previous section. After the first 21 h or 69 h, 30 μl of 5 mg/ml MTT was added to each well and the incubation was continued for 3 h at 37° C. MTT formazan produced by living cells was solubilized with isopropanol containing 0.04N HCl. We determined the concentration of formazan solubilized by measuring its optical density at 550 nm using a MTP-32 CORONA microplate reader (Corona Electric Co., Ltd., Ibaragi, Japan). Numbers of cells in culture were expressed by relative optical densities of the specimens, taking the value for each type of cell in a well at the beginning of experimental culture as 1.0.

Cell cycle analysis. Cells were harvested by a brief trypsinization and centrifugation at $400 \times g$ for 5 min and fixed with 70% ethanol at 4° C for 15 min. The fixed cells were washed twice with PBS and treated at 37° C for 15 min with PBS which contains 40 μg/ml propidium iodide and 1 mg/ml of ribonuclease for DNA staining and for RNA digestion, respectively. After being filtered through a nylon mesh filter ($\varnothing = 40$ μm), the cells collected from the filter were suspended in PBS and subjected to cell cycle analysis in a flow cytometer, EPICS Elite (Coulter, Miami, FL) following the manufacturer's recommended protocol. Distribution of the cells in the different cell cycle phases was calculated from the histogram of cell distribution against fluorescence strength of each cell particle which corresponds to its DNA content, using the computer software, MULTICYCLE (purchased from Phoenix Flow Systems, San Diego, CA). Cells in the G_0 phase cannot be distinguished from those in the G_1 phase by this propidium iodide staining method.

Statistical analysis. Values are expressed as the mean $\pm$SE. The Student's t-test was used for the statistical analysis. A p-value of less than 0.05 was considered to be significant.

Results

Effects of SPC and its structurally-related lipids on DNA synthesis in Swiss 3T3 and MIA PaCa-2 cells under the culture conditions reported

The effect of SPC on Swiss 3T3 cells has been reported by Desai et al. [12]. We confirmed the stimulatory effects of SPC (fig. 1a), S1-P (fig. 1b) and sphingosine (fig. 1c) on 24 h DNA synthesis in Swiss 3T3 cells under the culture conditions reported by Desai.

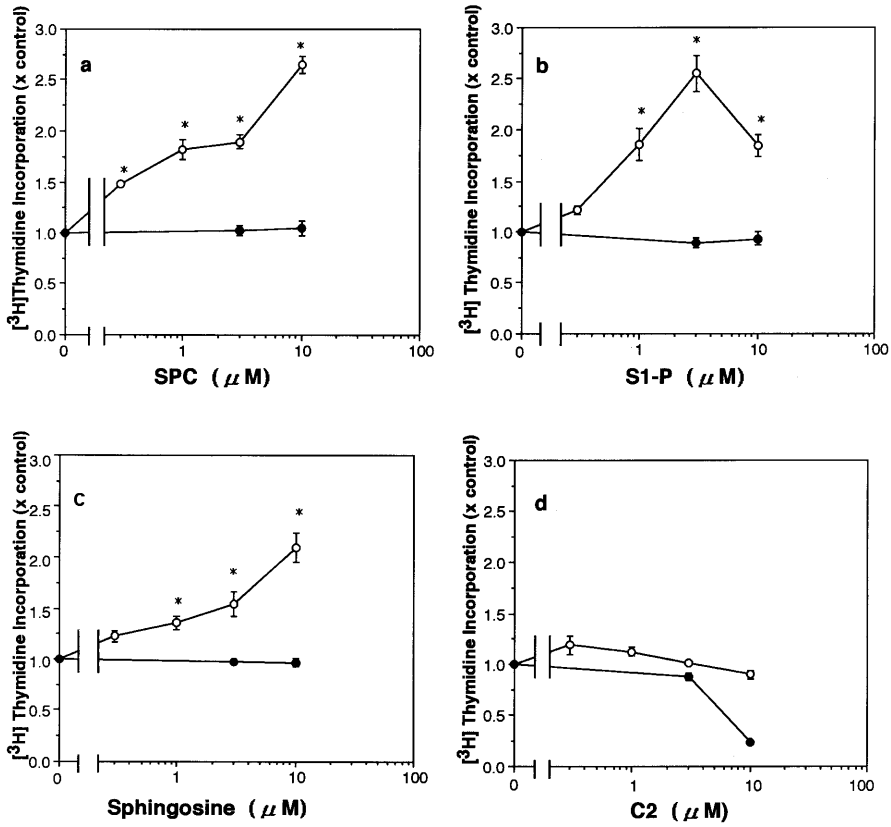

Figure 1. Stimulation of DNA synthesis in MIA PaCa-2 (●) and Swiss 3T3 cells (○) by (*a*) SPC, (*b*) S1-P, (*c*) sphingosine and (*d*) C2-ceramide under the conditions reported by Dasai et al. as described in Materials and methods. DNA synthesis was estimated by [^{3}H]thymidine incorporation into the TCA insoluble fraction of the cells. Each assay was carried out in triplicate. Stimulation was evaluated by folds of the control value for each experiment. Data were expressed as the mean ± SE. * Effect of test agent is significant ($p < 0.05$).

These lipids, however, did not stimulate the DNA synthesis in a pancreatic cancer cell line, MIA PaCa-2 cells, under the same conditions (figs 1a–1c). On the other hand, C2 ceramide, a membrane-permeable derivative of ceramide, the mother molecule of sphingosine, did not appreciably affect either Swiss 3T3 and MIA PaCa-2 cells, but, at a high concentration, actually inhibited the growth of cancer cells (fig. 1d).

Effects of SPC and its structurally-related lipids on DNA synthesis in various pancreatic cancer cells and Swiss 3T3 cells under serum-deprived conditions

In the experiments mentioned above, cells were cultured in the presence of 10% FBS until just before the experimental incubation and, in addition, insulin was supplemented in the experimental culture medium. Therefore, the observed effect of the sphingolipid might have been influenced by the remaining serum factors and/or synergistic effects of exogenous insulin. We investigated the effects of the lipids under serum-deprived conditions on four types of pancreatic cancer cells including PANC-1, PK-1 and PK-9 in addition to MIA PaCa-2.

Figure 2 summarizes the results. DNA synthesis in all four pancreatic cancer cells as well as Swiss 3T3 cells were more or less inhibited by S1-P (fig. 2b), sphingosine (fig. 2c), and C2 (fig. 2d) and C6 (fig. 2e) ceramides at doses greater than 1, 3, 1 and 1 μM, respectively. In the effective dose ranges, inhibition rates were dose-dependent with S1-P and ceramides. The 10 μM sphingosine effects on PK-1 and PK-9 were moderate and those on MIA PaCa-2 and PANC-1 cells were not very significant; instead, this agent seriously impaired DNA synthesis in Swiss 3T3 cells at the same concentration. On the other hand, the pattern of the SPC effect varied dramatically with cell type. As shown in figure 2a, MIA PaCa-2 cells responded to this lipid at concentrations as low as 1 μM, but PK-9 was only slightly influenced at concentrations as high as 10 μM. Furthermore, up to 3 μM SPC did not inhibit but actually stimulated DNA synthesis in Swiss 3T3 cells.

Such differential effects of SPC were confirmed by measuring the change in MTT values that is a measure of cell number. As shown in figure 3, 24 h incubation with SPC increased the MTT value of Swiss 3T3 cells up to 150% of the initial value at 3 μM SPC, whereas the MTT value of PANC-1 cells was not increased but slightly reduced. This slight reduction of the MTT value does not seem to indicate the cytotoxity of SPC to this type of cancer cell, because longer incubation of PANC-1 cells with SPC showed a dose-dependent reduction of

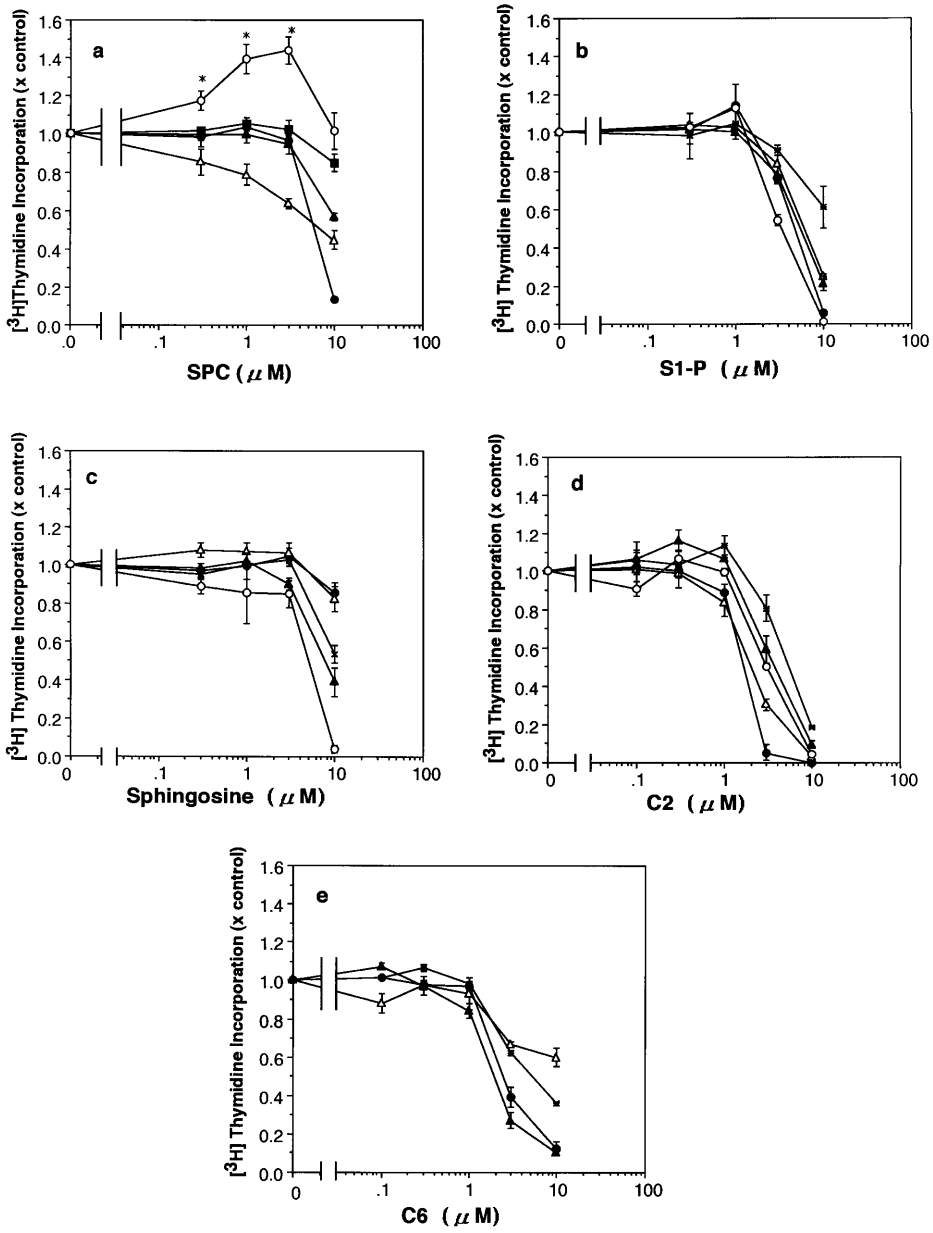

Figure 2. Stimulation of DNA synthesis in MIA PaCa-2 (●) PANC-1 (△), PK-1 (▲), PK-9 (×) and Swiss 3T3 cells (○) by (*a*) SPC, (*b*) S1-P, (*c*) sphingosine, (*d*) C2-ceramide and (*e*) C6-ceramide under the serum-deprived conditions as described in Materials and methods. Assay procedure and data expression were the same as those described in the legend to figure 1.

cell proliferation rate but no decrease in number of cells from the beginning of cell culture (fig. 3). Thus it seems to be clear that SPC induces bidirectional changes in the cell cycle mechanism.

Effects of the sphingolipids on cell cycle progress

To characterize the modulation of cell proliferation by SPC, we examined the effects of the lipid on cell cycle progress under the serum-deprived conditions. As shown in figure 4a, SPC at the maximal effective dose (10 μM) significantly increased the proportion of the PANC-1 cells in the G_1 phase. This increase was compensated by the decrease in the proportion of cells in the S phase, suggesting the induction of G_1 arrest by the lipid. Conversely, the treatment of Swiss 3T3 cells by SPC at its maximal stimulatory dose (3 μM) resulted in a decrease in percentage of the cells in the G_1 phase and an increase in those in S phase (fig. 4b). These results suggest that SPC can modulate the rate of cell cycle progress from the G_1 to the S phase positively or negatively depending upon the response mechanisms of target cells.

The effects of C2 and C6 ceramides on the cell cycle progress in PANC-1 cells were similar to that of SPC, that is, the cell population in the G_1 phase was in-

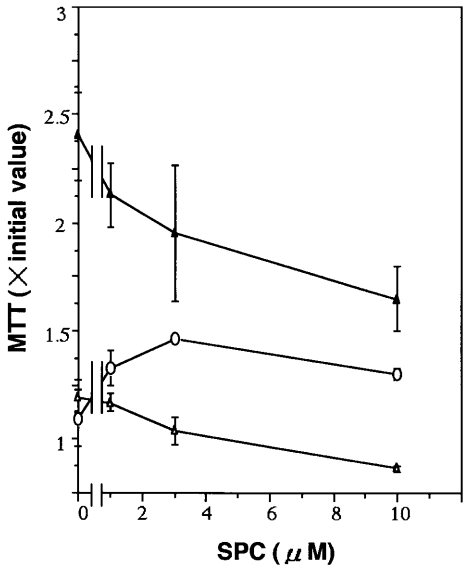

Figure 3. Modulation of cell proliferation by SPC of Swiss 3T3 cells (○) or PANC-1 cells (△, ▲) in the culture for 24 h (○, △) or 72 h (▲). The cells were plated and cultured in 48-well culture plates. The culture conditions were the same as described in the legend to figure 2. The change in cell number was estimated by the MTT cell growth assay as described in Materials and methods. Values on the vertical axis are relative MTT values corresponding to viable cell numbers. The MTT value of each type of cell at the beginning of the experiment was taken as 1.0.

creased while that in the S phase was decreased (fig. 4c). This suggests that under these conditions, these ceramides also induce G_1 arrest but not the DNA fragmentation which occurs in other cells [23].

Discussion

The present study is the first report of growth inhibition of a variety of human pancreatic cancer cells by sphingolipids. In addition, we demonstrated a unique aspect of the action of SPC. After 72 h cultivation with a reduced concentration of FBS prior to experimental culture, SPC selectively suppressed DNA synthesis in three out of four strains of human pancreatic cancer cells tested, but stimulated Swiss 3T3 fibroblasts; whereas sphingosine, S1-P and membrane-permeable ceramides non-selectively inhibited cell growth in all the cancer cells examined and in Swiss 3T3 cells. This may indicate the usefulness of SPC as a selective inhibitor of the propagation of certain pancreatic cancer cells under certain conditions.

In most of the experiments, we minimized the influence of serum or other growth factors by reducing FBS concentration from 10 to 0.5% in the culture prior to the experiments. Therefore, SPC effects under the serum-deprived conditions seem to be mainly due to the intrinsic activity of SPC. As mentioned at the start, SPC was originally shown by Desai et al. to be a potent mitogen for Swiss 3T3 cells [12]. In their report, there

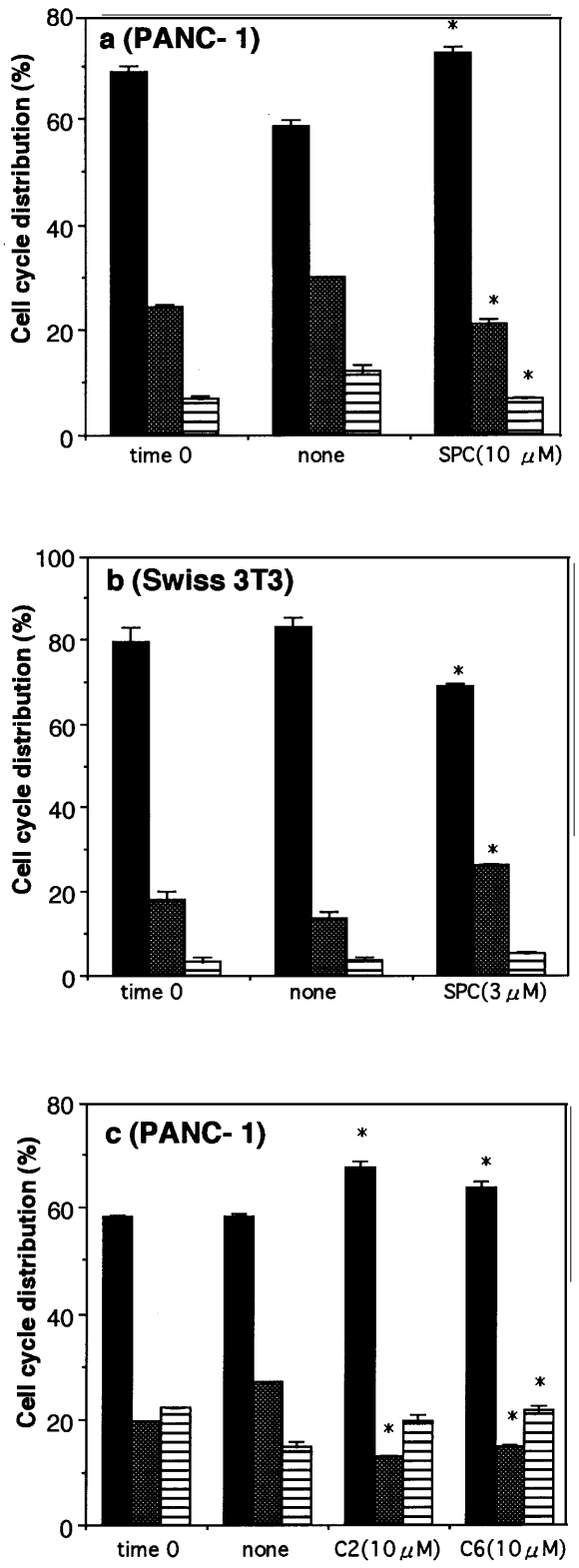

Figure 4. Effect of SPC, C2-ceramide and C6-ceramide on cell cycle progress under the serum-deprived conditions. (*a*) Effect of SPC on PANC-1 cell lines, (*b*) effect of SPC on Swiss 3T3 cell lines and (*c*) effect of C2-ceramide and C6-ceramide on PANC-1. Distribution of cells in the (■) G_0/G_1, (▩) S and (▤) G_2/M phases were analysed by a flow cytometer and estimated by the computer program, MULTICYCLE. * Effect of test agent is significant from 'none' ($p < 0.05$).

was no sign of the inhibitory nature of SPC; not only were Swiss 3T3 and various non-transformed cells stimulated, but two other types of transformed cells were weakly but appreciably stimulated in their cell growth. Later, Xu et al. reported an inhibitory effect of SPC on cell growth [14], without discussion of a discrepancy between their work and Desai et al.'s [12]. Xu et al. [14], like us, used cells precultured longer than 20 h without FBS to avoid the influence of serum constituents and/or growth factors and observed the inhibitory actions of SPC on the growth of cancer cells. Desai et al. used cells grown in the presence of 10% FBS for experiments in a medium containing insulin, and observed stimulatory effects on cancer cells. In fact, in the present study, when we used cells treated according to the protocol of Desai et al., the inhibitory action of SPC on cell growth in the pancreatic cancer cells disappeared, although no stimulation was observed in contrast to the transformed cells reported by Desai et al. [12]. On the other hand, under the serum-deprived condition, SPC still stimulated the growth of Swiss 3T3 cells but the rate of stimulation was lower than that in the cells cultured under the conditions described by Desai et al. These observations suggest that the level and/or direction of growth modulation may depend on both cell type and culture conditions, probably because of the difference in cellular mechanisms responding to the agent and/or in synergistic interaction between SPC and serum factors and/or paracrine factors.

On the basis of reports of the growth inhibitory actions of SPC on transformed cells [14], including the present paper, it is suggested that cancer cells or transformed cells (if not all cells) are targeted by the inhibitory rather than the stimulatory action of SPC. On the other hand, SPC-induced stimulation of cell growth would be a general feature of normal tissue cells or non-transformed cells [13]. In relation to this, it is noted that cell proliferation of keratinocytes and other injury-related tissue was stimulated by this lipid both in vitro and in vivo, and SPC was proposed as a new wound healing agent which accelerates the growth of cells surrounding an injured region [13]. If this is the case, it would also be reasonable to imagine a selective inhibition of malignant cells, including certain pancreatic cancer cells by SPC under controlled conditions.

The present study demonstrates that the growth stimulation of Swiss 3T3 cells and the inhibition of pancreatic cancer cells by SPC as well as ceramide are mediated through the up- and down-regulation of the process from the G_0/G_1 phase to the S phase. In our preliminary experiments (data not shown), however, sphingosine and S1-P showed no indication of cell cycle modulation under the present serum-deprived conditions. This result may suggest that different mechanisms, including cytotoxic actions [24], are exerted by sphingosine and S1-P under the present serum-deprived conditions.

Ceramides have been assumed to act as second messengers for several membrane receptor ligands [25] and mediate ligand-dependent apoptosis [23]. However, cell cycle analysis of the ceramide-treated pancreatic cancer cells showed no sign of apoptosis or appreciable decrease in number of cells, but a change in cell cycle pattern, suggesting the occurrence of G_0/G_1 arrest consistent with the inhibition of DNA synthesis under the serum-deprived conditions.

Pancreatic cancer is one of the most refractory human tumors despite intensive efforts [26]. The present results may provide a basis for understanding the growth control of pancreatic cancer cells.

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