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Sphingosine inhibits attachment of murine Lewis lung carcinoma cells to laminin and type IV collagen

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The effect of sphingosine (SPH) on the adhesive properties of Lewis lung carcinoma (3LL) cells was investigated using plastic precoated with the extracellular matrix proteins, laminin, fibronectin, or type IV collagen. Treatment of 3LL cells with SPH (0.5–10 μM) resulted in a dose-dependent decrease in the ability to bind to laminin and type IV collagen but had little or no effect on atachment to fibronectin. Phorbol 12-myristate 13-acetate (PMA) selectively enhanced attachment of 3LL cells to laminin and collagen. The inhibitory effect of SPH on attachment to both proteins was competitively antagonized by PMA. These results suggest that SPH acts as a negative effector for cell attachment to laminin and collagen, and that the cell attachment process to both proteins might be regulated in part by protein kinase C.

Sphingosine; Cell adhesion; Protein kinase C; Laminin; Collagen type IV; Lewis lung carcinoma

1. INTRODUCTION

The interaction between basement membrane components, such as laminin, fibronectin, and collagen, and their cell surface receptors may play a key role in the mechanism of tumor invasion and metastasis [1]. In animal models, tumor cells selected for their ability to attach via laminin produced 10-fold more metastases following i.v. injection [2]. Moreover, increased expression of laminin and collagen receptors in various cancer cells have been associated with increased invasive and metastatic potentials [3–6]. These results suggest the roles of these cell surface receptors in malignancy. In our recent work, an inhibitor of UDP-glucose:*N*-acylsphingosine glucosyltransferase (EC 2.4.1.80), D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), was found to inhibit the attachment of B16 melanoma cells to laminin and type IV collagen, but not to fibronectin [7]. Moreover, treatment of Lewis lung carcinoma (3LL) cells with D-PDMP resulted in significant inhibition of the attachment ability to laminin and experimental metastatic potential [8].

Since D-PDMP treatment not only blocks all glycosphingolipid synthesis but also induces the accumulation of ceramide and sphingoid bases [9], the observed effects of D-PDMP might be due to the accumulation of these precursors of glucosylceramide. Sphingoid bases including sphingosine (SPH) have recently attracted considerable attention, primarily because – added exogenously to cells – they inhibit the activity of the Ca^{2+}/phospholipid-dependent enzyme protein kinase C (PKC) and regulate various cellular events which depend on its activation [10,11]. In the present study, we demonstrate that SPH acts as a negative effector for cell attachment to laminin and collagen, and that the cell attachment process to both proteins might be regulated in part by protein kinase C.

Abbreviations: SPH, sphingosine; 3LL, Lewis lung carcinoma; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; RPMI, Roswell Park Memorial Institute; BSA, bovine serum albumin; HPF, high power field; D-PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

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2. MATERIALS AND METHODS

2.1. *Cells and culture conditions*

3LL cells were from Dr. T. Taniguchi (Kyushu University, Institute of Bioregulation, Fukuoka, Japan). The cells were maintained in Roswell Park Memorial Institute (RPMI) Medium 1640 in 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (all from Grand Island Biological Co., Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Staining with Hoechst 33258 showed all cells to be free of mycoplasma. To ensure reproducibility, we performed all experiments with cultures grown <6 weeks after recovery from frozen stocks.

2.2. *Stock solutions of SPH and PMA*

PMA and *trans*-D-erythro-SPH prepared from bovine brain sphingomyelin were from Sigma Chemical Co. (St. Louis, MO). The purity of SPH was assessed by TLC (Silica Gel plates) with chloroform/methanol/7M NH_4OH (80:20:2) [9], and the spot was visualized by impregnating the plates with a modified charring reagent [12], 100 g of $CuSO_4 \cdot 5H_2O$ in conc. H_3PO_4/water/MeOH (100/750/400 ml). The sample (100 μg) yielded a single spot coinci-

dent with SPH, showing that there was no visible contamination by dimethyl-SPH. The stock solution of SPH was prepared as equimolar concentrations of SPH and fatty acid free BSA by the method of Merrill et al. [13] for the purpose of reducing cytotoxicity of SPH [14]. The PMA was added as a 1% solution in dimethylsulfoxide.

2.3. *Cell attachment assay*

Mouse laminin, human fibronectin, and type IV collagen were from Iwaki Glass Co. (Osaka, Japan). The attachment assay was performed as described [8] with slight modification. The proteins were diluted with PBS and were allowed to bind to 96-well polystyrene microtiter plates (Flow Laboratories, McLean, VA) overnight at 4°C. The wells were then coated further with 1% BSA in PBS for 2 h. Cells from the culture flasks were detached by the addition of EDTA to the culture medium (2 mg/ml), pelleted, washed, and resuspended at 10^5 cells/ml in the assay medium (RPMI 1640 containing 0.1% BSA). Fifty microliters of the cell suspensions were placed in each well containing 50 μl of assay medium in the presence of various concentrations of SPH–BSA complex and/or PMA. Control and experimental solutions both contained equivalent final concentrations of BSA and dimethylsulfoxide. In some case, the cell suspensions were incubated with SPH–BSA complex for 1 h at 37°C and then the treated cells were resuspended at 10^5 cells/ml in the assay medium. Subsequently, the cell attachment assay was performed in the absence of SPH. The cells were allowed to adhere at 37°C in a humidified incubator containing 5% CO_2. After 60 min, the plates were inverted to facilitate the removal of unbound cells, and all wells were washed with 100 μl PBS. Adhering cells were fixed with 2.5% glutaraldehyde for 10 min and stained with Giemsa. Each condition was evaluated in quadruplicate and the fixed cells were counted by two different investigators in at least five microscopic fields (100× HPF) per well.

2.4. *Statistical calculations*

Each assay described above was repeated three or four times. Means ± SD were calculated and analyzed using Student's *t*-test for unpaired populations.

3. RESULTS

3.1. *Effect of SPH on cell attachment to extracellular matrix proteins*

We first examined the attachment of 3LL cells to plastics coated with various concentrations of laminin, fibronectin, or type IV collagen. The coating concentration required to induce maximal cell binding was 25 μg/ml for laminin and 10 μg/ml for fibronectin. 3LL cells failed to bind to type IV collagen at a concentration of 10 μg/ml, as reported previously [8]. When the coating concentration of type IV collagen was raised to 100 μg/ml, the cells attached and spread on this protein. These concentrations were therefore used in this study.

We determined the attachment properties of 3LL cells to laminin, fibronectin, and type IV collagen in the presence of SPH at the concentrations ranging from 0.5 to 10 μM (Fig. 1A). SPH decreased the ability of 3LL cells to bind to laminin and type IV collagen in a dose-dependent manner. The concentrations of SPH required to induce a 50% decrease in the attachment to laminin and collagen were about 5 and 1.2 μM, respectively. On the other hand, attachment of the cells to fibronectin was not inhibited by SPH at concentrations as high as 10 μM. To exclude the possibility that SPH may interact directly with these extracellular matrix proteins rather than the cells during the attachment assay, 3LL cells were preincubated with various concentrations of SPH for 1 h at 37°C, and then the attachment assay was performed in the absence of SPH (Fig. 1B). As expected, the SPH-pretreated cells lost their attachment abilities to laminin and collagen in very much the same manner as observed in Fig. 1A. This indicates that the cells used in Fig. 1A had absorbed the SPH very rapidly as observed with other cell lines [13,15].

When 3LL cells were incubated with SPH for 60 min at 37°C, SPH concentrations below 10 μM had no effect on cell viability, as assessed by the exclusion of Trypan blue dye (data not shown). When we incubated cells, previously exposed to 2.5 μM SPH, in SPH-free

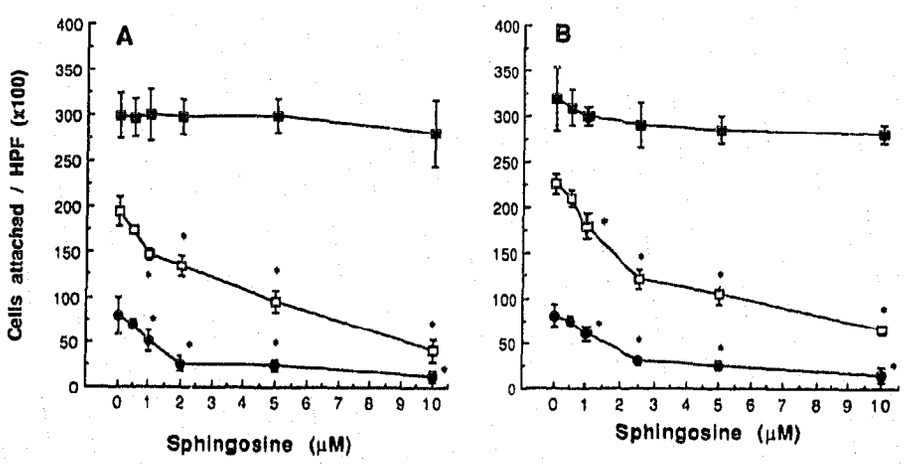

Fig. 1. Effect of SPH on 3LL cell attachment to laminin (□), type IV collagen (●), and fibronectin (■). (A) The cell attachment assay was performed in the presence of the indicated concentrations of SPH. (B) In order to allow more time for uptake of SPH into 3LL cells, the cells were incubated with SPH at the concentrations ranging from 0.5 to 10 μM for 1 h at 37°C. After this treatment, the cell suspensions were centrifuged to remove SPH in the medium and the cell pellets were resuspended with RPMI 1640 containing 0.1% BSA for the attachment assay. * $P < 0.0005$ as compared to the corresponding value in the absence of SPH.

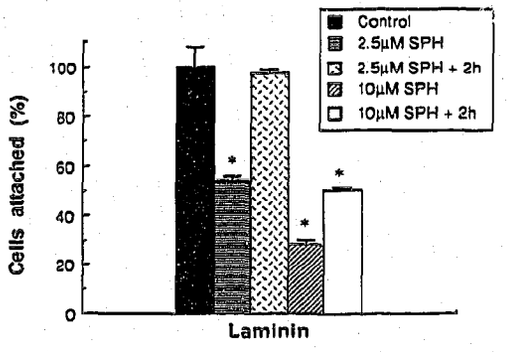

Fig. 2. The reversibility of SPH-mediated inhibition on 3LL cell attachment to laminin substrate. 3LL cells were preincubated with 2.5 or 10 μM SPH for 1 h at 37°C. Subsequently, the cell attachment assay was performed in the absence of SPH. In order to see the recovery of attachment abilities after the SPH treatment, the treated cells were further incubated for 2 h at 37°C in growth medium (RPMI 1640 containing 10% fetal calf serum) before the assay. Control cells were preincubated the same way in the absence of SPH. * $P < 0.0005$ as compared to the control value.

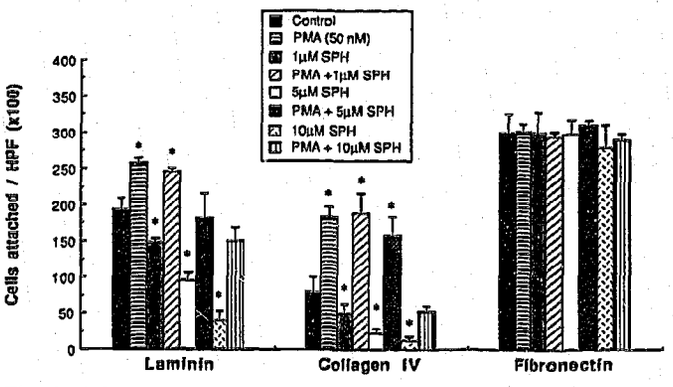

Fig. 3. Effect of PMA and/or SPH on 3LL cell attachment to laminin, type IV collagen and fibronectin substrates. The cell attachment assay was performed in the presence of PMA (50 nM) and/or the indicated concentration of SPH. * $P < 0.0005$ as compared to the value in the absence of PMA and SPH.

medium for 2 h, they completely recovered their ability to bind to laminin (Fig. 2). Cells that had been exposed to 10 μM SPH recovered only partially, from 28% binding to 51% binding. Similar results were obtained with collagen instead of laminin (data not shown). These data eliminate cytotoxicity as a factor in the inhibitory effect.

3.2. *Effect of PMA on 3LL cell attachment to laminin and collagen, and antagonism of the inhibition of SPH by PMA*

If SPH was indeed acting through the inhibition of PKC, PMA would presumably be able to antagonize the inhibitory effect of SPH on cell attachment to laminin and collagen. This prediction was tested in the following experiments. In the presence of 50 nM PMA, the attachment abilities of 3LL cells to laminin and collagen were significantly increased, and these increases were, in turn, antagonized by SPH in a dose-dependent manner (Fig. 3). On the other hand, attachment to fibronectin was not affected appreciably by either PMA or SPH.

The nature of this antagonism was further investigated by examining the SPH inhibition of cell attachment to laminin and collagen over a range of PMA concentrations (Fig. 4). PMA increased the ability of 3LL cells to bind to laminin by 40% at 170 nM. Further stimulation was not seen with higher PMA concentrations (data not shown). The half-maximally effective dose of PMA for laminin was ~33 nM (curves with solid circles). For collagen, the half-maximal dose was ~2.5 nM (curves with solid circles). Maximal stimulation by PMA, an increase of ~200%, was obtained at ~5 nM. In all cases, increasing concentrations of SPH caused a progressive shift in the PMA dose/response curves to the right, suggesting that there is a competitive mode of antagonism between PMA and SPH.

4. DISCUSSION

Our data presented here show for the first time that SPH inhibits the attachment of 3LL cells to laminin and type IV collagen in a reversible and dose-dependent

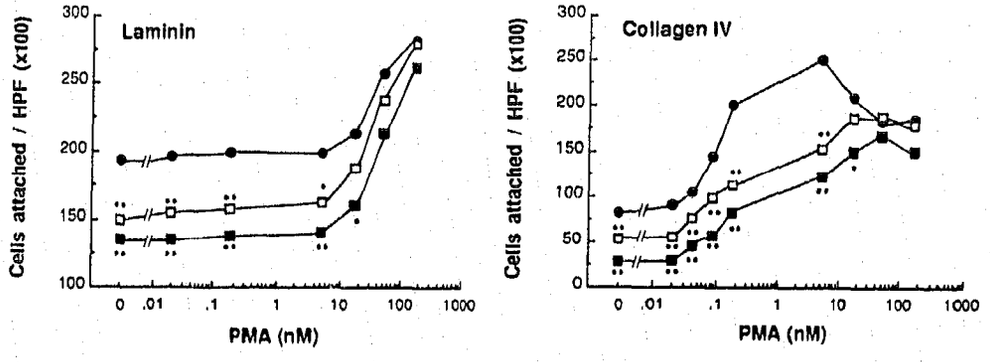

Fig. 4. 3LL cell attachment to laminin or type IV collagen as a function of PMA and SPH concentrations. (●) In the absence of SPH; (□) 1 μM SPH; (■) 2 μM SPH. * $P < 0.0025$; ** $P < 0.0005$ as compared to the corresponding value obtained at each PMA concentration in the absence of SPH.

manner. The inhibition exhibits selectivity, because SPH had little or no effect on attachment to fibronectin. In addition, PMA selectively enhanced attachment of 3LL cells to laminin and collagen, and SPH-mediated inhibition of attachment to both proteins was competitively antagonized by PMA, suggesting the direct interaction of SPH with PKC.

A possible involvement of PKC in mediation of cell attachment to extracellular matrix proteins has been suggested. For example, it has been reported that PMA quickly enhanced attachment of macrophages to laminin but not to fibronectin [16]. PMA also enhanced the attachment of NIH/3T3 cells to laminin and type IV collagen, but not to fibronectin [17]. Moreover, PMA stimulation of human neutrophils resulted in a massive translocation of laminin receptors from an intracellular compartment to the cell surface [18]. Recently, Shaw et al. demonstrated that the $\alpha 6\beta 1$ integrin complex functions as a macrophage laminin receptor and that PMA-dependent adhesion to laminin was mediated by phosphorylation of the $\alpha 6$ subunit [19]. These observations suggest that the functions of laminin and collagen receptors on the cell surface are regulated by PKC, and that protein phosphorylation including these receptor proteins is probably an initial event in the process of cell adhesion. However, the possibility cannot be ruled out that phosphorylation of cytoskeletal proteins such as vinculin and talin by PKC may affect their interaction with receptors for extracellular matrix proteins so as to change the affinity and/or specificity of the receptors [20–22].

Our findings suggest that SPH may be one of the endogenous factors that regulate cell attachment to laminin and collagen mediated through PKC. However, the actual biochemical mechanism(s) remains to be elucidated. Study of the effect of SPH on phosphorylation of cell surface receptors for these extracellular matrix proteins and its role in the receptor functions is of immediate interest. A complicating factor in interpreting PMA experiments arises from the many reports of additional effects beyond the PKC stimulation and down-regulation. One effect of PMA is to stimulate the synthesis of glucosylceramide and higher glycosphingolipids [23] and that of sphingomyelin [24]. PMA has also been found to speed the conversion of sphinganine to ceramide [25], so it could neutralize the effect of SPH by lowering the intracellular concentration. If one speculates further, since SPH is rapidly metabolized to dimethyl-SPH [26] or SPH-1-phosphate [27], it might also be possible that the observed effects are due not to free SPH, but to one of these metabolic products.

Recent work has shown that metastatic tumor cells have increased affinity to laminin and collagen, suggesting a major role of these proteins and their receptors in tumor metastasis [1–6,28]. In fact, the highly metastatic clone of 3LL, the H59 cell line, was highly adherent to laminin compared to the parental one [28]. Thus, the affinity toward laminin of this cell line appears to be correlated directly with its metastatic potential as well as in the other cell lines [2]. Moreover, it has been reported that the treatment of a weakly metastatic clone of 3LL, the P29 cell line, with PMA resulted in a marked increase in the ability to form lung tumor nodules after i.v. inoculation of the treated cells [29]. Together with the results of our present and previous reports [7–9], the accumulation of sphingoid bases, which was caused by the inhibition of glucosylceramide synthase with D-PDMP, may result in the inhibition of cell attachment to laminin and/or collagen and that of experimental metastasis.

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