Inhibition of Experimental Metastasis of Murine Lewis Lung Carcinoma by an Inhibitor of Glucosylceramide Synthase and Its Possible Mechanism of Action

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ABSTRACT

In view of the increasing evidence that glucosphinolipids (GSLs) on tumor cell surfaces play an important role in tumor metastasis, an inhibitor of glucosylceramide synthase, d-threo-l-phenyl-2-decanoylamino-3-morpholino-l-propanol (D-PDMP) was used to evaluate the role of GSLs in this respect. Treatment of Lewis lung carcinoma cells with 5 μM D-PDMP resulted in a time-dependent marked decrease in levels of all cellular GSLs (glucosylceramide, lactosylceramide, ceramide trihexoside, globo side, and ganglioside GM3). By 6 days, the total GSL content was reduced to ~20% of the level in the untreated control cells and at the same time the lung-colonizing capacity of the PDMP-treated cells in inoculated mice was greatly reduced. Closely associated with the degree of GSL depletion, the ability of the cells to invade reconstituted basement membranes in vitro was also reduced, suggesting that GSLs in tumor cell membranes modulate the cell surface interaction with basement membrane components. In order to assess a possible contribution of the defective capacities to drug-induced suppression of experimental metastasis and invasion, we tested the effect of D-PDMP on attachment and migration to laminin and fibronectin and found that the inhibitor specifically reduced the laminin-mediated attachment and migration, whereas it had no effect on fibronectin-mediated attachment and migration. These effects of the inhibitor on lung colonizing capacity in vivo and the invasion, adhesion, and migration properties of the cells in vitro were reversible within 24 h after removal of the drug. By contrast, L-PDMP (the enantiomeric form of D-PDMP), which has no inhibitory activity on glucosylceramide synthase, did not cause any of the changes produced by D-PDMP. Together, these results suggest that GSLs in tumor cell membranes are essential for the metastatic spread of tumor cells through basement membranes, modulating the interaction of laminin and its receptors.

INTRODUCTION

A better understanding of the factors influencing the development of metastasis is one of the major challenges in cancer research. An important part of the metastatic process is the invasion by tumor cells through extracellular matrix, a complex process that involves cellular adhesions, chemotactic responses, and hydrolysis of specific basement membrane components (1-5). Growing evidence suggests that carbohydrate residues on cell surface GSLs play an important role in the metastatic spread of tumor cells since GSLs on the cell membranes have been implicated as functionally important molecules in tumor cell attachment (6, 7). Furthermore, comparison of two cell strains, metastatic and nonmetastatic, showed distinct differences in GSL composition, and the metastatic cell line generally had greater amounts of GSLs (8-10). These findings suggest that it may be possible to modify the metastatic potential of tumor cells by altering GSL metabolism.

The glucose-containing glucosphinolipids (called GSLs or glucolipids here) are derived from the primary GSL, glucosylceramide, which is formed enzymatically from ceramide and UDPglucose. The attachment of galactose and other sugars to GlcCer results in a complex family of structures, such as the gangliosides (ganglio, globo, neolacto, and lacto series). We developed an analogue of ceramide, D-PDMP, which inhibits UDPglucose:N-acetylglucosamine glucosyltransferase (EC 2.4.1.80), the synthase that makes GlcCer (11). The inhibitor and PDMP are similar in structure. They have the general structure R—CHO—CH(NH—CO—R')—CH2—R". R is a long aliphatic chain in the natural lipid and a benzene ring in PDMP. R' is a fatty acid in amide linkage, C16-20 in the lipid and C10 in PDMP. R" is glucose in the lipid and morpholine in PDMP. The hydroxyl group in PDMP has a configuration opposite to the hydroxyl group in PDMP, which has no inhibitory effect on GlcCer synthesis, which does not cause any of the changes produced by D-PDMP. Together, these results suggest that GSLs in tumor cell membranes are essential for the metastatic spread of tumor cells through basement membranes, modulating the interaction of laminin and its receptors.

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2 The abbreviations used are: GSLs, glucosphinolipids; 3LL, Lewis lung carcinoma; BSA, bovine serum albumin; GalCer, galactosylceramide; GlcCer, glucosylceramide; HFP, high power field; LacCer, lactosylceramide; PBS, divalent cation-free Dulbecco’s phosphate-buffered saline (137 mM NaCl-2.7 mM KCl-8.1 mM Na2PO4-1.5 mM KH2PO4, pH 7.4); PDMP, threo-l-phenyl-2-decanoylamino-3-morpholino-1-propanol; TLC, thin layer chromatography.

MATERIALS AND METHODS

Cells and Culture Conditions. 3LL cells were from Dr. T. Taniguchi, Kyushu University, Institute of Bioregulation (Fukuoka, Japan). The cells were maintained in RPMI 1640 supplemented with 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% CO₂, 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.

Treatment of 3LL Cells with PDMP. D-PDMP hydrochloride and its enantiomer, L-PDMP, were synthesized as previously described (11). Ten mm solutions were prepared in ethanol and stored at −20°C until use. Portions of the solution were evaporated to dryness in sterile screw cap glass tubes with a nitrogen stream, the culture medium was added to the tubes, and the tubes were sonicated for 10 min in an ultrasonic water bath. The cells were cultured for 3 or 6 days in the culture medium with or without PDMP. The subconfluent cultures were detached with 0.05% EDTA, then washed with PBS and pelleted. The cells were resuspended in PBS, and 5 × 10⁶ viable cells/ml for the pulmonary colonization assay or in RPMI 1640 containing 0.1% BSA at 3 × 10⁶ cells/ml for the in vitro assays of invasion, attachment, and migration.

Glycolipid Analysis. Cells (>5 × 10⁶) were collected, washed twice with PBS, and lyophilized. The neutral and acidic GSLs of each sample were purified chromatographically and developed and visualized on high-performance TLC as previously described (7). The quantity of lipid in each band was measured at 500 nm with a dual-wavelength flying spot scanner (CS-9000, Shimadzu, Kyoto, Japan) in the reflectance mode with integrated areas. We confirmed the presence of carbohydrate in the GSL spots by also spraying the plates with sugars-detecting reagents.

Pulmonary Colonization Assay. Six-week-old pathogen-free female C57BL/6 mice (Shizuoka, Experimental Animal Farm, Shizuoka, Japan) were quarantined for 1 week and used over a range of 7 to 8 weeks. Five mice/experimental group were inoculated with 2 × 10⁶ cells (kept at 25°C) via the tail vein. The injections were alternated between control and experimental animals in order to minimize time variances during the injection. Twenty days later, the animals were sacrificed in ether, and the number of pulmonary metastases was determined by infiltration with India ink according to the method of Wexler (18). Extrapulmonary metastases were also checked.

In Vitro Chemoinvasion Assay. The assay of in vitro invasiveness was done essentially as described by Albini et al. (19). The basement membrane, Matrigel, prepared from the Engelbrecht-Holm-Swarm tumor, was from Collaborative Research, Inc. (Bedford, MA). Polycarbonate filters (8 μm pores; Nucleopore, Pleasanton, CA) were coated with 50 μg of Matrigel and placed in a modified Boyden blind well chamber (NeuroProbe, Inc., Cabin John, MD). Conditioned medium (0.2 ml), obtained by incubating NIH 3T3 cells for 24 h in serum-free medium containing 50 μg ascorbate/ml, was placed in the lower compartment of the Boyden chamber. The 3LL cells (3 × 10⁶/0.8 ml) were added to the upper chamber and incubated for 6 h at 37°C in a humid 5% CO₂ atmosphere. After the incubation the filters were removed from the chambers, and fixed and stained with Diff-i-Quik as described above. Each sample was assayed in quadruplicate and the fixed cells in at least five microscopic fields (×100 HPF) per well were counted.

Migration Assay. The assay was carried out by using polycarbonate filters and Boyden chamber as previously described (20). In brief, laminin or fibronectin (25 μg/ml) was dissolved in RPMI 1640 containing 0.1% BSA and added to the lower well as chemoattractants. Cells (3 × 10⁶/0.8 ml in RPMI 1640 containing 0.1% BSA) were placed in the upper compartment and incubated for 4 h at 37°C in a humid 5% CO₂ atmosphere. After the incubation the filters were removed from the chambers, and fixed and stained with Diff-i-Quik as described above. Each sample was assayed in quadruplicate and the chemotactic activity was measured by counting the number of cells that had migrated to the lower side of the filter. At least five HPFs were counted per filter.

Statistical Calculations. Each assay described above was repeated 2 or 3 times with reproducible results. Means ± SEM were calculated and analyzed using Student's t test for unpaired populations.

RESULTS

Assessment of Drug Cytotoxicity. In view of the many reported involvements of GSLs in physiological phenomena (15, 21-26), one would expect that prolonged depletion of GSLs would eventually interfere with vital cell functions. Thus it is difficult to decide whether a deleterious effect by a GSL inhibitor is due to the specific, known mechanism of action of the inhibitor, or whether some unknown function of the drug is the cause. In several studies, the changes produced by D-PDMP were paralleled by a disappearance of GSLs and were prevented by including GlcCer or another GSL in the cell medium (7, 12-14), so it appears likely that PDMP acts primarily to block GlcCer synthesis. The corrective effects of GlcCer could not be mimicked by GalCer, a very close analogue of GlcCer, which can be presumed to undergo hydrolysis (like exogenous GlcCer) to ceramide, fatty acid, and sphingosine.

In this study, we checked the viability and growth of 3LL cells after 6 days of exposure of the cells to 5–20 μM D-PDMP. In addition, the effects of the enantiomer of the inhibitor, L-PDMP, were also determined. The growth of 3LL cells was inhibited by D-PDMP in a concentration-dependent fashion to a maximum degree of 64% (Table 1). Treatment of the cells with 5 μM L-PDMP did not inhibit cell growth but cells exposed to higher concentrations exhibited a dose-dependent decrease. However, the effect was not as great as that seen with the D

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cell no. (× 10⁶)</th>
<th>Cell viability (% unstained)</th>
<th>Tumorigenicity (tumor wt, g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>38.0 ± 1.5⁴</td>
<td>97</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>5 μM D-PDMP</td>
<td>33.1 ± 2.3 (13)⁴</td>
<td>97</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>5 μM L-PDMP</td>
<td>38.6 ± 1.8 (0)⁴</td>
<td>95</td>
<td>3.7 ± 1.1</td>
</tr>
<tr>
<td>7.5 μM D-PDMP</td>
<td>29.5 ± 0.8 (22)⁴</td>
<td>96</td>
<td>ND (n.d.)</td>
</tr>
<tr>
<td>7.5 μM L-PDMP</td>
<td>32.0 ± 0.9 (16)⁴</td>
<td>95</td>
<td>ND (n.d.)</td>
</tr>
<tr>
<td>10 μM D-PDMP</td>
<td>25.9 ± 1.3 (32)⁴</td>
<td>95</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>10 μM L-PDMP</td>
<td>29.5 ± 0.5 (22)⁴</td>
<td>96</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>20 μM D-PDMP</td>
<td>13.8 ± 0.6 (64)⁴</td>
<td>86</td>
<td>ND</td>
</tr>
<tr>
<td>20 μM L-PDMP</td>
<td>15.3 ± 0.9 (60)⁴</td>
<td>90</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Suspensions of 3LL cells (5.0 × 10⁶ cells) were seeded into 175-cm² culture flasks and left to adhere overnight. The following day, the medium was substituted with standard medium or medium containing PDMP. The cells were cultured for 6 days, then detached with EDTA, and counted.

** Numbers in parentheses, percentage of inhibition of cell growth.

P < 0.025 as compared to control.

ND, not determined.

P < 0.005 as compared to controls.

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isomer at the lower concentrations (Table 1). A similar difference in sensitivity of GSL synthesis to the two enantiomers has been observed for other cells and it is likely that the L isomer inhibits some other important step in GSL metabolism.

Although the growth rates of 3LL cells were decreased by the drugs, there were no effects on the viability of cells grown in <20 μM PDMP (more than 95% nonstaining) or on their ability to grow after s.c. injection (Table 1). All the inoculated mice ultimately developed a tumor at the site of injection. Cell viability at 20 μM was decreased to about 86%. However, the growth potential of the drug-treated cells in inhibitor-free medium was not impaired by the drugs even after depletion with 20 mM PDMP for 6 days (data not shown). In order to maximize secondary effects, we decided to use PDMP at 5 μM in the rest of this study.

Effects of PDMP on Glucolipid Content in 3LL Cells. Drug-mediated inhibition of GSL biosynthesis in 3LL cells was assessed by high performance TLC and the quantity of GSL in each band was measured densitometrically (Table 2). The control cells contained GlcCer, LacCer, and Gm3 as their major GSLs, with only minor amounts of ceramide trihexoside and globoside (or paragloboside). TLC with borate-impregnated plates showed there was no detectable GalCer present. Treatment of 3LL cells for 3 or 6 days with 5 mM D-PDMP resulted in a time-dependent marked decrease in the levels of all their neutral GSLs (GlcCer, LacCer, ceramide trihexoside, and globoside) and ganglioside Gm3. The total amount per cell of GSL was decreased to 21% of the amount in the control cells when they were treated for 6 days with 5 μM D-PDMP; the GlcCer, particularly, was decreased to only 4% of normal. After this treatment, removal of the inhibitor and incubation for 24 h in inhibitor-free medium resulted in rapid partial restoration of the major and total GSLs (Table 2, last line). This indication of rapid GSL turnover had been seen also with B16 melanoma cells after the PDMP had been removed (7).

Treatment of the cells for 3 days with the enantiomer, L-PDMP, did not affect the levels of the major GSLs. Interestingly, further incubation for another 3 days resulted in an increase in the levels of all GSLs (about 30% increase in the total amount). Most of this increase was due to a 67% rise in the level of LacCer. We reported previously that the activity of GlcCer synthase in brain microsomes was inhibited 50% by 5 μM PDMP, whereas L-PDMP produced only a very slight or insignificant stimulation at the same concentration (11). We did see a marked accumulation of LacCer with L-PDMP incubated with B16 melanoma cells (7). This effect of L-PDMP could not be attributed to an inhibitory action on LacCer hydrolysis since the two β-galactosidases acting on LacCer were not inhibited by L-PDMP (7). Moreover, there is little possibility that the accumulated LacCer is actually Gal(α1→4)GalCer since neither D- nor L-PDMP could inhibit the α-galactosidase purified from liver membranes.

Effects of PDMP on Experimental Metastasis of 3LL Cells. Treatment of 3LL cells for 6 days with D-PDMP markedly inhibited their pulmonary colonization capacity, evaluated by i.v. injection of the cells into C57BL/6 mice (Fig. 1; Table 3). This inhibition was highly significant ($P < 0.005$), judging from both the total number of pulmonary colonies and the number of large colonies having a diameter $> 4$ mm (Table 3). In addition, complete elimination of the incidence of 3LL extra-pulmonary colony formation was observed with cells pretreated for 6 days, indicating that tumor colonization had not been shifted to other organ sites. When the cells were treated for 6 days with L-PDMP, only the total number of metastatic colonies decreased, but not significantly. The number of large colonies and the incidence of extrapulmonary metastases were not affected.

After 6 days of exposure to 5 μM D-PDMP, removal of the inhibitor and incubation for another 24 h in inhibitor-free medium, the cells regained their capability of forming pulmonary colonies (Table 3), and the total GSL content of the cells was largely recovered to the level of cells treated with PDMP for 3 days (Table 2).

The inhibition of pulmonary colonization by D-PDMP was found only with prolonged exposure of the cells to the inhibitor (6 days); incubation of the inhibitor for 3 days had almost no effect (Table 3). This suggests that extensive depletion of GSLs in 3LL cells is needed in order to block pulmonary colony formation. Treatment of the cells with L-PDMP, which has no inhibitory activity on GlcCer synthesis, did not significantly affect the capacity for pulmonary colonization (Table 3). From these results we conclude that GSLs in tumor cells are essential for successful completion of at least one obligatory step in the metastatic cascade.

Effect of PDMP on Invasiveness of 3LL Cells in Vitro. We used an in vitro invasion assay (19) which has been found to strongly correlate with metastatic ability in vivo. The method uses a reconstituted basement membrane (Matrigel) for a barrier and fibroblast-conditioned medium for a chemoattractant. We found that treating 3LL cells with D-PDMP under similar conditions as in Table 2 resulted in a marked decrease in the invasive ability, whereas the treatment of the cells with L-PDMP had no such effect (Fig. 2). As there was a correlation

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**Table 2** Effects of PDMP on glucolipid content of 3LL cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GlcCer</th>
<th>LacCer</th>
<th>CTH</th>
<th>Globoside</th>
<th>Gm3</th>
<th>Total GSLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.21</td>
<td>0.81</td>
<td>0.17</td>
<td>0.0</td>
<td>0.05</td>
<td>5.4</td>
</tr>
<tr>
<td>D-PDMP for 3 days</td>
<td>0.81</td>
<td>2.14</td>
<td>0.16</td>
<td>0.19</td>
<td>1.55</td>
<td>6.0</td>
</tr>
<tr>
<td>D-PDMP for 6 days</td>
<td>0.68</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>L-PDMP for 3 days</td>
<td>0.97</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>L-PDMP for 6 days</td>
<td>0.65</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td>D-PDMP for 6 days + 24 h without PDMP</td>
<td>1.76</td>
<td>1.55</td>
<td>0.31</td>
<td>0.19</td>
<td>18.60</td>
<td>23.2 (57)</td>
</tr>
</tbody>
</table>

* Value of each glucolipid is expressed as the mean of two separate determinations.
* CTH, ceramide trihexoside.
* Numbers in parentheses, percentage of control value.

A. Abe and N. S. Radin, unpublished work.
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Fig. 2. Inhibition of 3LL cell invasion in vitro by D-PDMP. The in vitro chemoinvasion assay was performed as in "Materials and Methods." Before the cells were added to the upper well of the Boyden chamber, they were pretreated as described in Table 2. The result is from an experiment performed in parallel with Table 3. * P < 0.01; ** P < 0.005 compared to the untreated control.

Fig. 3. Effects of D- and L-PDMP on binding of 3LL cells to laminin and fibronectin. The cells were pretreated as in Table 2 and allowed to attach to microtiter wells which had been coated with 10 µg/ml of laminin or fibronectin described in "Materials and Methods." *, P < 0.025; **, P < 0.005 compared to the untreated control.

Table 3 Effects of PDMP on metastasis following i.v. injection of 3LL cells

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>No. of total lung tumor colonies (mean ± SE)</th>
<th>No. of large lung tumor colonies* (mean ± SEM)</th>
<th>No. of mice with extrapulmonary tumors/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28.0 ± 3.9</td>
<td>4.0 ± 1.5</td>
<td>5/5</td>
</tr>
<tr>
<td>D-PDMP for 3 days</td>
<td>24.0 ± 4.2</td>
<td>3.5 ± 1.8</td>
<td>4/5</td>
</tr>
<tr>
<td>L-PDMP for 3 days</td>
<td>30.3 ± 5.5</td>
<td>4.2 ± 1.7</td>
<td>5/5</td>
</tr>
<tr>
<td>D-PDMP for 6 days</td>
<td>8.5 ± 2.2*</td>
<td>0.5 ± 0.6*</td>
<td>0/5</td>
</tr>
<tr>
<td>L-PDMP for 6 days</td>
<td>15.0 ± 8.7</td>
<td>3.3 ± 1.9</td>
<td>5/5</td>
</tr>
<tr>
<td>D-PDMP for 6 days + 24 h without drug</td>
<td>26.6 ± 4.5</td>
<td>3.7 ± 0.9</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Tumor colonies >4 mm in diameter were counted.

levels of two individual GSLs, GlcCer and ceramide trihexoside, were distinctly higher than in the cells exposed to D-PDMP for 3 days. This suggests that one or both of these lipids is closely involved in invasive process.

Effects of PDMP on Attachment and Migration of 3LL Cells.

We first tested the adhesive properties of 3LL cells to laminin, type IV collagen, fibronectin, and heparan sulfate. 3LL cells bound to laminin and fibronectin whereas the cells failed to bind to type IV collagen and heparan sulfate (data not shown), confirming the previously reported observation for this cell line (27). Therefore, we tested the effects of GSL depletion on adhesion only to laminin and fibronectin. As shown in Fig. 3, the capacity of the cells to adhere to laminin was specifically and significantly reduced by the treatment with D-PDMP for either 3 or 6 days but not with L-PDMP. The inhibitory effect of D-PDMP on the attachment to laminin was reversed after drug removal (Fig. 3). When cells treated with D-PDMP were tested for their adhesion to wells coated with various concentrations of laminin or fibronectin, the adhesion to the laminin was found to be altered by GSL depletion in a dose-dependent fashion at all levels of laminin. However, fibronectin binding was unaffected by the level of the matrix protein or of PDMP, confirming the specific action of PDMP on laminin attachment (Fig. 4).

Chemokinetic assay measuring the migration of the drug-...
in 3LL cells to laminin and fibronectin. 3LL cells were pretreated with either D-PDMP or L-PDMP as mediated inhibition of ceramide glucosylation in 3LL cells metastatic properties. It is clear from our data that D-PDMP-treatment caused extensive depletion of the cellular GSLs and resulted in a marked reduction in the potential to form pulmonary colonies after i.v. injection of the drug-pretreated cells. In contrast, L-PDMP, which has no inhibitory activity on GlcCer or GSL synthesis at the level used, did not significantly affect pulmonary colonization, further indicating the importance of GSLs in this process. The inhibition of lung tumor colonization by D-PDMP was not due to cytotoxicity since incubation of cells with 5 µM D-PDMP did not affect their viability, tumorigenicity, or subsequent proliferation rate in fresh medium. Moreover, incubation in inhibitor-free medium restored the capability of 3LL cells to form pulmonary colonies.

Since GSLs function in a wide variety of biological activities (6, 21–26), blockage of GSL biosynthesis by D-PDMP could result in a complex pattern of interference with cellular events, thereby making it difficult to identify the lipid species involved. The recent development in many laboratories of specific monoclonal antibodies directed against the carbohydrate moieties of GSLs (22, 28–32) has provided important information about the biological functions of those molecules that are exposed on the membrane surface. One possible mechanism of action of tumor cell GSLs in metastasis could be in mediation of the interactions between tumor cell and extracellular matrix, including basement membranes. For example, it was demonstrated that monoclonal antibodies to gangliosides GD2 and GD3 of human melanoma inhibited melanoma cell attachment to various extracellular matrix proteins, including laminin, fibronectin, collagen, and vitronectin (31).

Tumor invasion of basement membranes is a crucial step in the complex multistage process which leads to metastasis. Malignant cells must penetrate the vascular basement membrane several times when entering and exiting the circulation. In the “three-step” invasion hypothesis (1–3), the first step involves attachment of tumor cells to the basement membrane through laminin; during the second step, proteolytic enzymes from the tumor cells digest the matrix; and the third step involves cell migration through the basement membrane. Our present data on 3LL cells suggest that tumor GSLs play a key role in the first and third of these steps. Our previous work (7) demonstrated that depletion of GSLs, particularly GlcCer, in B16 melanoma cells by D-PDMP results in a significant loss of the attachment abilities to the basement membrane components, laminin and type IV collagen, but not to fibronectin. The results presented in this study regarding the effects of D-PDMP on 3LL cell adhesion are generally consistent with our previous report. The attachment ability of 3LL cells to laminin was decreased with decreasing GSL content of the cells by treatment with D-PDMP, suggesting that the inhibitor inhibits the first step of the invasion into basement membrane.

Our finding that PDMP blocked binding to laminin seems inconsistent with the reports that this protein does not bind to GSLs but rather to a lipid having the properties of a galactolipid sulfate ester, sulfatide (33). However, it should be noted that the identification of sulfatide as the active lipid in laminin binding did not establish the identity of the sugar moiety. The occurrence of sulfated GlcCer in the brain of an infant with Gaucher disease has been reported (34), so it is possible that the active lipid (at least in tumors) is actually a glucolipid sulfate.

Recent work has shown that malignant tumor cells have increased affinity to laminin and supported a major role for laminin and its receptors in tumor metastasis (27, 35–42). In fact, the highly metastatic clone of 3LL, the H59 cell line, was
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highly adherent to laminin compared to the parental one (27). Thus, the affinity toward laminin of this cell line appears to be correlated directly with its metastatic potential as well as the other cell lines (35). Moreover, it was reported that tumor cell binding to laminin through its receptors enhances the production of type IV collagenase in tumor cells and thereby induces the collagenolytic dissolution of basement membrane; however, augmentation of the enzyme production was not induced by fibronectin (43). Consequently, it is possible that D-PDMP could also inhibit the subsequent second step of invasion by interfering with production of type IV collagenase in tumor cells.

Concerning the third step, it has been reported that the rates of migration of 3LL cells out of glass capillary tubes suggested that higher cell motility is related to higher metastatic potential (44) and the addition of gangliosides to 3LL cells resulted in enhanced metastatic potential and migration of the cells (17). Therefore, we tested the effects of the drugs on 3LL cell migration to laminin and fibronectin and found a specific inhibitory effect of D-PDMP on the migration toward laminin.

At present the mechanism of impairment of the affinity of 3LL cells to laminin by the inhibitor is currently unknown but one possible explanation is that GlcCer or a closely related GSL acts to control the distribution of the laminin receptors in the cell surface, and thereby control its reactivity.

Since treatment of the cells with the inhibitor did not affect their subsequent proliferation rate in fresh medium (data not shown) and the growth rates in a s.c. region of mice (Table 1), one might conclude that depleted cells can rapidly synthesize new GSL molecules. If this is true, an explanation must be sought for the slow metastasis of the depleted cells in our tumor colonization test in vivo. It is possible that many of the depleted cells were attacked and destroyed by the immunosurveillance system of the mouse before they could regenerate a sufficient amount of GSL. Several reports indicate that tumor GSLs prevent the host from using its full immunodefense mechanisms (15).

Interference in GSL metabolism and functions with PDMP should throw further light on the mechanisms of metastasis of tumor cells.

REFERENCES


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