Inhibition of Experimental Metastasis and Cell Adhesion of B16F1 Melanoma Cells by Inhibitors of Protein Kinase C

Jennifer A. Dumont, Winton D. Jones, Jr., and Alan J. Bitonti
Marion Merrell Dow Research Institute, Cincinnati, Ohio 45215

ABSTRACT

Phorbol esters which activate protein kinase C (PKC) have been shown to enhance experimental lung metastasis. Therefore, it was reasoned that inhibitors of PKC might also modulate metastasis. We have investigated this possibility using a PKC inhibitor, MDL 27,032 [4-propyl-5(4-pyridinyl)-2(3H)-oxazolone], as well as staurosporine and H-7. Treatment of B16F1 murine melanoma cells with MDL 27,032 for 24 h in culture and subsequent i.v. injection of the cells into mice resulted in >90% inhibition of lung metastasis. Inhibition of metastasis was time dependent, with 90% of maximum inhibition occurring by 8 h of incubation. The 50% inhibitory concentration (IC50) for inhibition of metastasis with MDL 27,032 was 7 μM, a value similar to that for the inhibition of B16F1 membrane-associated PKC (IC50 = 13 μM) but not cytosolic PKC (IC50 = 54 μM). B16F1 cells treated with MDL 27,032 for 24 h were less adherent than untreated cells to extracellular matrix/basement membrane proteins. Adhesion to fibronogen and collagen IV was inhibited (IC50 = 6 μM and 48 μM, respectively) by MDL 27,032, whereas adherence to laminin and fibronectin was not affected, indicating that the drug affects specific adhesion molecules. MDL 27,032-treated cells were also found to be less adherent than untreated cells to human umbilical vein endothelial cells. The phosphorylation of an 80-kDa B16F1 cell plasma membrane protein was stimulated under conditions known to stimulate PKC activity, and MDL 27,032 inhibited this phosphorylation in a dose-dependent manner. MDL 27,032 was more potent than H-7 for the inhibition of metastasis but was significantly less potent than staurosporine. These results support the hypothesis that there is a critical role for PKC-mediated phosphorylation of cell surface adhesion receptors in metastasis.

INTRODUCTION

Most deaths due to cancer are not a result of primary tumor growth but, rather, are caused by the dissemination of tumor cells to secondary sites as a result of a series of events known collectively as the metastatic cascade (1, 2). During this process, cells escape from the primary tumor into the circulation through loss of adhesive properties, while the establishment of new colonies at distant sites depends upon the increased ability of these cells to adhere to vascular endothelium and, subsequently, to extracellular matrix proteins (3). Although the mechanisms by which tumor cells break and establish adhesive contacts are not completely understood, some key aspects of these processes have been identified and are the subject of vigorous current investigation.

Cell adhesion receptors, called integrins, have been associated with tumorigenicity and metastasis (4, 5). These receptors are cell surface glycoproteins that bind to extracellular adhesion proteins such as fibronin, fibronectin, laminin, and collagen with relatively low affinity. The importance of integrins to metastasis has been inferred in experiments in which relatively large amounts of synthetic peptides containing the sequence arginine-glycine-aspartic acid (RGD) were injected simultane-

ous with tumor cells into mice, resulting in a decreased number of metastatic foci (6). More recently, the gene for the integrin very late antigen (VLA)-2 was transfected into rhabdomysosarcoma cells, which are not normally metastatic, and metastasis was induced (7). Thus, while the role of integrins in metastasis seems clear, it is not known how the function of integrins, and ultimately cell adhesion, is regulated during tumor progression to the metastatic state.

Phosphorylation of protein receptors often induces conformational changes in receptors, which can affect binding characteristics for the ligands (8). Integrins have been shown to be substrates for both protein tyrosine kinase (9) and protein kinase C (10, 11). The latter enzyme is a calcium-activated and phospholipid-dependent kinase, which also requires diacylglycerol for full catalytic activity (12). PKC is the major intracellular receptor for the phorbol ester tumor promoters, which bind to and activate the enzyme in a manner analogous to diacylglycerol (13, 14). Tumor-promoting phorbol esters and compounds that activate calcium mobilization (which also results in activation of PKC) have shown to increase metastasis in experimental animal models and also to enhance the adherence potential of tumor cells. Lewis lung carcinoma cells treated with PMA display an enhancement of adhesion to endothelial cells, which is correlated with increased phosphorylation of a specific intracellular protein (15). Similarly, treatment of B16 murine melanoma cells in vitro with PMA and subsequent i.v. injections into mice result in increased numbers of metastatic foci in the lungs (16). Spontaneous metastasis of SP1 mouse mammary adenocarcinoma cells, which do not normally metastasize, is induced by treatment with either PMA or the calcium ionophore A23187 (17). These data suggested that activation of PKC activity plays a role in determining the metastatic phenotype. It was logical, therefore, that inhibition of PKC might reduce the metastatic potential of tumor cells. In the present study, we have investigated the effect of MDL 27,032 [4-propyl-5(4-pyridinyl)-2(3H)-oxazolone] (18) and other PKC inhibitors on experimental metastasis of B16F1 melanoma cells and adhesion of these cells to vascular endothelial cells and extracellular matrix/basement membrane proteins. The data suggest that MDL 27,032, through inhibition of PKC, affects cell adhesion properties that may be related to integrin phosphorylation.

MATERIALS AND METHODS

Chemicals. Phenylmethylsulfonyl fluoride, leupeptin, histone H1 (type III-S), phosphatidyserine, 1,2-diolein (1,2-dioleoyl-sn-glycerol), collagen IV (human placenta), and phorbol-12-myristate-13-acetate were purchased from Sigma; staurosporine from Kamiya Biomedical; H-7 from Molecular Probes; laminin (human placenta) from Calbiochem; fibronectin (human serum) from Boehringer Mannheim; fibrinogen (human serum) from Behring Diagnostic; integrin monoclonal antibodies from Coulter and the American Type Culture Collection; and anti-integrin antibodies from Boehringer Mannheim. Anti-bactericidal permeability increasing protein (BPI) and tumor necrosis factor (TNF) were purchased from Collaborative. Phorbol-12-myristate-13-acetate (PMA), staurosporine, and the calcium ionophore A23187 were provided by Marion Merrell Dow. The PKC inhibitors were supplied by Dr. S. Schreiber, DuPont. All other chemicals were obtained from Sigma.

Received 7/24/91; accepted 12/16/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at Marion Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, OH 45215.

2 The abbreviations used are: PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; MEM, minimum essential medium; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; [3H]HLDU, 5-[3H]Hljodo-2'-deoxyuridine; BSA, bovine serum albumin; TCA, trichloroacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazin METH2.0sulfonic acid; IC50, 50% inhibitory concentration.
ogen (human plasma) from Kabi; and [γ-32P]ATP (10 Ci/mmol) and 
[3H]thymidine (78.5 Ci/mmol) from New England Nuclear. MDL 27,032 and MDL 27,044 [4-methyl-5-(4-pyridinyl)-2(3/-)-oxazolone] were synthesized at the Marion Merrell Dow Research Institute (Fig. 1).

Cell Culture. B16F1 cells (ATCC 6233) were cultured in MEM (GIBCO) supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 5 mg/liter gentamicin. New cultures were started every 3 months, to maintain stable expression of the metastatic phenotype.

HUVEC were obtained from Clonetics Corporation and cultured in monolayers, using endothelial growth medium-umbilical vein supplied with the cells. After four or five passages, new cultures were initiated, because growth of these primary cultures is much slower following several passages.

Experimental Metastasis Assay. Experimental metastasis was performed as described (19). Subconfluent B16F1 cells were harvested by brief trypsinization, followed by washing with HBSS. Viable cells were counted after dilution with trypan blue. Female C57Bl/6 mice, 6 to 8 weeks old, were given iv. injections, via the tail vein, of 1 x 105 viable cells in 0.2 ml. Three weeks later, the lungs were excised, and the number of metastatic nodules was counted.

Quantitation of [3H]Thymidine-labeled B16F1 Cells in Lungs. The procedure was performed as described previously (19). The drinking water of C57Bl/6 mice was supplemented with 0.1% KI for 3-4 days prior to inoculation of tumor cells. Subconfluent B16F1 cells were labeled with 0.3 μCi/ml medium [3H]thymidine (specific activity, 2200 Ci/mmol; New England Nuclear) for 24 h at 37°C. The labeled cells were washed and prepared for i.v. injection as described above for experimental metastasis. Following injection, the lungs were excised and placed in 70% ethanol. Ethanol was changed twice, and then the radioactivity associated with the lungs was quantitated in a gamma counter.

Colony-forming Assay. The viability of drug-treated tumor cells was assessed by the ability of the cells to form colonies following 24 h drug treatment. B16F1 murine melanoma cells (1 x 104) were plated 24 h prior to drug treatment. Drug solutions were then added and incubated for 24 h at 37°C. Following the drug treatment, the cells were briefly trypsinized, washed twice with HBSS, and resuspended in MEM. One hundred viable cells, as determined by trypan blue exclusion, were plated in each 35-mm-dish and incubated at 37°C. Dishes were prepared in triplicate for each drug concentration. Ten days later, colonies with a diameter of ≥2 mm were counted.

Preparation of Membrane and Cytosolic Fractions for PKC Assay. Cytosolic and membrane-associated PKC activity was prepared as described (20). Subconfluent cultures of B16F1 cells were washed twice with PBS (0.125 M NaCl, 10 M NaHPO4, 3 M KH2PO4, pH 7.2) and then twice with buffer A (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 M ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid, 0.33 M sucrose, 2 M phenylmethylsulfonfonyl fluoride, 2 μg/ml leupeptin). Approximately 4 x 105 cells were scrapped into 3 ml of buffer A and homogenized with 40 strokes (Wheaton Dounce homogenizer, type A pestle). Centrifugation of the homogenate was at 100,000 x g for 1 h at 4°C. The supernatant was stored on ice while the pellet was washed twice with buffer A and homogenized with 10 strokes, as described above. The insoluble membrane and cytosolic PKC fractions were applied to separate columns, followed by washing of the columns twice with 3 ml of buffer B. Protein Kinase C Assay. Protein kinase C activity was assayed by the transfer of γ-32P-labeled phosphate from ATP to histone, as described (20). The assay contained 20 mM Tris, pH 7.5, 0.75 mM calcium chloride, 10 mM magnesium acetate, 0.1 μg/ml histone H1 (type III-S), 250 μg/ml leupeptin, 100 μg [γ-32P]ATP (120 pmol), 96 μg/ml phosphatidylinerine, and 6.4 μg/ml 1,2-diolein, in a total assay volume of 250 μl. A new lipid mixture was prepared daily for each experiment. In 20 mM Tris, pH 7.5, HVE was added to the assay just prior to the addition of enzyme, which initiated the reaction. Blank samples were incubated in the absence of calcium and phospholipids. Incubation was for 5 min at 30°C. The reaction was terminated by the addition of 1 ml of cold 25% TCA, followed by filtration of the sample on Whatman GF/B filters. The tubes were washed twice with 2 ml of cold 5% TCA and applied to the filters. The filters were then washed 5 times with 2 ml of cold 5% TCA and counted in 10 ml of Beckman Ready Protein.

Adhesion of B16F1 Cells to Immobilized Proteins. Adhesion proteins (2 μg/well) were coated onto Immulon 1 Removawells (Dynatech, catalog no. 011-010-6301) and incubated for 1 h at 37°C. Laminin, fibronectin, and fibrinogen were dissolved in water or buffer, while collagen IV was dissolved in 0.05% acetic acid. After 1 h, the solution was aspirated and the wells were blocked with 400 μl of PBS, pH 7.2, containing 0.1% BSA, for an additional 1 h at 37°C. Before addition of the cell suspension, the PBS solution was aspirated.

Subconfluent cells, incubated for 24 h at 37°C with 1 μCi/ml [3H]thymidine, were washed twice with HBSS and then removed from the flasks by incubation for 10 min at 37°C with PBS, pH 7.2, 2 mM EDTA, 0.05% BSA. The cells were washed twice with MEM containing 20 mM HEPES and 0.1% BSA and were resuspended in the same medium, and cell density was adjusted to 1 x 105 cells/ml. Cells were added to each well in a volume of 50 μl (5 x 104 cells) and incubated for 1 h at 37°C. The liquid in each well was then carefully aspirated, and the wells were washed 3 times with 400 μl of PBS, pH 7.2, containing 0.1% BSA. Individual wells were detached and counted in 5 ml of Beckman Ready Protein. Blanks were measured as non-protein-coated wells blocked as described above.

Adhesion of Tumor Cells to Endothelial Cells. Thermannox tissue culture coverslips from Lux (15 mm round) were placed in Corning tissue culture 12-well clusters (22-mm-diameter wells; catalog no. 25815). HUVEC were obtained from Clonetics Corporation were added to each well and grown to confluency. In preparation for the adhesion assay, the wells containing HUVEC and blanks (coverslips, no cells) were blocked with MEM containing 20 mM HEPES and 1% BSA, for 1 h at 37°C. The medium was aspirated just prior to addition of the melanoma cells.

Subconfluent B16F1 cells were labeled with 1 μCi/ml [3H]thymidine for 24 h. Cell suspensions were prepared by removal from flasks with 2 mM EDTA and 0.05% BSA in PBS, pH 7.2 (incubation for 10 min at 37°C). The cells were washed twice with MEM containing 20 mM HEPES and 1% BSA and were then resuspended to a cell density of 2 x 105 viable cells/ml (viability determined with trypsin blue).

One ml of cells was added per well and incubated for the time indicated, at 37°C. Nonadherent cells were washed from each coverslip in the following manner (21). The coverslip was retrieved from the well with forceps and dipped 10 times into a beaker containing 100 ml of the MEM described above. This was followed by 10 additional dips into a second beaker of MEM. Coverslips were placed in 10 ml of Beckman Ready Protein, and radioactivity was determined by β-scintillation counting.

Phosphorylation of B16F1 Plasma Membranes. Plasma membranes were prepared as described previously (22). B16F1 cells were scraped from 150-mm-diameter Petri dishes, with a rubber policeman, into a solution of 50 mM boric acid, pH 7.2, containing 150 mM NaCl, 1 mM MgCl2, and 1 mM CaCl2, sedimented at 450 x g for 5 min in a

Fig. 1. Structures of protein kinase C inhibitors.
Table 1. Inhibition of B16F1 melanoma cell metastasis by MDL 27,032

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Individual values</th>
<th>Mean ± SE</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>27, 13, 13, 19, 10, 31</td>
<td>18 ± 3.3</td>
<td>100</td>
</tr>
<tr>
<td>1 µM MDL 27,032</td>
<td>7, 18, 2, 21, 9, 18</td>
<td>12 ± 2.8</td>
<td>67</td>
</tr>
<tr>
<td>5 µM MDL 27,032</td>
<td>8, 20, 8, 7, 8, 25, 22</td>
<td>14 ± 3.3</td>
<td>78</td>
</tr>
<tr>
<td>10 µM MDL 27,032</td>
<td>3, 7, 8, 15, 14, 1</td>
<td>7.8 ± 2.1</td>
<td>43</td>
</tr>
<tr>
<td>20 µM MDL 27,032</td>
<td>8, 2, 5, 7, 12, 8</td>
<td>5.4 ± 1.1</td>
<td>30</td>
</tr>
<tr>
<td>50 µM MDL 27,032</td>
<td>0, 4, 2, 2, 4, 1</td>
<td>2 ± 0.6</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2. Effects of MDL 27,032 on B16F1 colony formation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Individual values</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17, 18, 16</td>
<td>17 ± 0.6</td>
</tr>
<tr>
<td>5 µM MDL 27,032</td>
<td>17, 19, 17</td>
<td>18 ± 0.7</td>
</tr>
<tr>
<td>10 µM MDL 27,032</td>
<td>16, 16, 15</td>
<td>16 ± 0.6</td>
</tr>
<tr>
<td>20 µM MDL 27,032</td>
<td>17, 19, 19</td>
<td>18 ± 0.7</td>
</tr>
<tr>
<td>50 µM MDL 27,032</td>
<td>15, 18</td>
<td>16</td>
</tr>
</tbody>
</table>

RESULTS

Effects of MDL 27,032 on Experimental Metastasis and PKC Activity. When B16F1 murine melanoma cells were treated with MDL 27,032, a PKC inhibitor, for 24 h in vitro and subsequently injected i.v. into mice, a significant decrease in metastatic nodules in the lung was observed (Table 1). The concentration at which the number of metastatic foci was reduced to 50% of control (IC50) was approximately 7 µM. B16F1 cells treated with MDL 27,032 were assayed for their ability to form colonies, as a measure of viability. The cells were incubated for 24 h with 0, 5, 10, 20, or 50 µM MDL 27,032. The cells were removed from the flasks, washed, and plated at a density of 10^5 cells/35-mm dish. Ten days later colonies (>2 mm in diameter) were counted. The overall plating efficiency was approximately 17%, and the number of colonies formed by drug-treated cells was the same as control (Table 2).

Other PKC inhibitors were tested in this assay and were also found to inhibit experimental metastasis (Table 3). Staurosporine had an IC50 of 0.03 µM for inhibition of metastasis, while H-7 had an IC50 of 12 µM. The effects of MDL 27,032 and other PKC inhibitors on B16F1 cell membrane and cytosolic PKC activity were determined (Table 3). Incubation of MDL 27,032 with the cytosolic enzyme resulted in an IC50 of 54 µM, while treatment of the membrane enzyme yielded an IC50 of 13 µM. Staurosporine and H-7 were also examined, because they are known inhibitors of PKC. Staurosporine had IC50 values of 0.019 µM and 0.025 µM for the cytosolic and membrane enzymes, respectively, while an IC50 of 180 µM for the cytosolic fraction and an IC50 of 71 µM for the membrane fraction were determined for H-7. A close structural analog of MDL 27,032, MDL 27,044, behaved in a manner similar to H-7. This drug showed a 2-fold difference in its effect on PKC activity in the cytosol (IC50 = 500 µM) versus membrane (IC50 = 250 µM) and was more effective in the experimental metastasis assay, giving an IC50 of 36 µM.

Table 3. Effects of PKC inhibitors on B16F1 PKC activity and experimental metastasis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosol IC50 (µM)</th>
<th>Membrane IC50 (µM)</th>
<th>B16 metastasis IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDL 27,032</td>
<td>54</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>MDL 27,044</td>
<td>500</td>
<td>250</td>
<td>36</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>0.019</td>
<td>0.025</td>
<td>0.03</td>
</tr>
<tr>
<td>H-7</td>
<td>180</td>
<td>71</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Subconfluent B16F1 cells were harvested and membrane fractions were prepared as described in "Materials and Methods." PKC activity was then measured in the presence of the drugs listed.

Subconfluent B16F1 cells were exposed to drugs for 24 h at 37°C. Cells were then harvested, and 1 x 10^5 cells were injected i.v. into mice. The number of metastatic nodules was quantitated 3 weeks later.

Fig. 2. Time dependency of inhibition of experimental metastasis by MDL 27,032. B16F1 cells were treated with MDL 27,032 for the times indicated, and then the cells were harvested by trypsinization and injected i.v. into mice, as described in "Materials and Methods." Metastatic nodules in the lungs were quantitated 3 weeks after inoculation.

Downloaded from cancerres.aacrjournals.org on July 20, 2017. © 1992 American Association for Cancer Research.
Time Course of Inhibition of Metastasis. The time course of the inhibition of metastasis by MDL 27,032 was investigated to determine the onset of the inhibitory effect (Fig. 2). Cells were treated with 10 or 50 µM MDL 27,032 for 0, 1, 2, 4, 8, and 24 h and then harvested and injected into mice. The cells treated with 10 µM MDL 27,032 first showed decreased metastasis at 2 h, while 50 µM MDL 27,032 showed a sharp decrease by 1 h. For both of the drug concentrations, maximum inhibition of the pulmonary nodule formation was achieved by 8 h, and inhibition was sustained for 24 h. For convenience, in experiments in which the maximum effect of the drug was desired, treatment of the tumor cells was for 24 h.

Arrest of B16F1 Cells in Lungs. To ensure that MDL 27,032-treated cells distributed to the lungs as well as untreated cells, the arrest of tumor cells inoculated i.v. in the lungs of C57BL/6 mice was examined using B16F1 cells which were labeled with [3H]thymidine during a 24-h period of treatment with MDL 27,032. The lungs from animals (n = 5) inoculated with untreated B16F1 cells retained 81,427 ± 2,360 cells, and animals (n = 5) injected with cells pretreated for 24 h with MDL 27,032 had 81,453 ± 4,890 cells retained in the lungs. Therefore, cells exposed to MDL 27,032 reached the lungs as well as untreated cells.

Table 4  Inhibition of B16F1 melanoma cell adhesion to HUVEC

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>50 µM MDL 27,032-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20,014 ± 1,328*</td>
<td>7,828 ± 1,422 (39)*</td>
</tr>
<tr>
<td>20</td>
<td>79,400 ± 10,901</td>
<td>48,750 ± 7,024 (60)</td>
</tr>
<tr>
<td>30</td>
<td>130,021 ± 6,541</td>
<td>92,084 ± 5,386 (71)</td>
</tr>
<tr>
<td>60</td>
<td>124,979 ± 10,669</td>
<td>136,950 ± 6,341 (110)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Values in parentheses represent B16F1 cell adhesion expressed as percentage of control.

Adhesion of Tumor Cells to Endothelial Cells. At early time points during the incubation of tumor cells with endothelial cells, adhesion of drug-treated B16F1 cells to HUVEC was markedly diminished (Table 4). With a 10-min incubation adhesion was only 40% of control levels, but as incubation time increased the inhibitory effect disappeared. Maximum adhesion was observed by 60 min, at which time adhesion of MDL 27,032-treated cells was no different than control. It was critical, therefore, to examine more than one time point for this adhesion process.

Adhesion of Tumor Cells to Immobilized Proteins. The adhesion of B16F1 cells to fibrinogen, fibronectin, collagen IV, and laminin immobilized on plastic was examined. Cells treated for 24 h with MDL 27,032 showed decreased adhesion to both fibrinogen and collagen IV, while adhesion to fibronectin or laminin was not affected (Fig. 3). The greatest effect was seen with B16F1 cells exposed to 50 µM drug, which resulted in 83% inhibition of adhesion to fibrinogen. Exposure of B16F1 cells to MDL 27,032 for 5 h resulted in no inhibition of adhesion to the immobilized proteins.

Phosphorylation of B16F1 Membrane Proteins. Phosphorylation of a number of proteins was stimulated by the addition of diacylglycerol, phosphatidylserine, and calcium to incubations containing B16F1 plasma membranes and [γ-32P]ATP. The most prominent stimulation was of an 80-kDa protein (Fig. 4). The phosphorylation of this protein was inhibited in a concentration-dependent manner by MDL 27,032 (Fig. 4, lanes 4 and 5).
DISCUSSION

Previous studies have shown that there is a correlation between membrane-bound PKC activity and metastatic capacity in a number of different tumor cell lines (15-17). Short term stimulation of membrane-bound PKC with phorbol ester resulted in enhanced metastasis, whereas a 24 h exposure to phorbol ester decreased membrane-bound PKC below basal level and decreased metastasis (16). The latter study suggested that basal membrane-bound PKC activity is involved in the induction of the metastatic phenotype. The finding that more highly metastatic tumor cell lines (B16F10, B16BL6) have a higher level of membrane-bound PKC than does the less metastatic B16F1 cell line, while having virtually identical cytosolic PKC activity (16), also suggests that the membrane-bound PKC plays an important role in determining metastatic potential. Accordingly, we postulated that inhibition of basal membrane-associated PKC activity would lead to a decrease in metastatic potential and, in fact, we found this to be the case. MDL 27,032, H-7, and staurosporine inhibited experimental lung metastasis of B16F1 cells exposed to the drugs before being inoculated i.v. into mice.

Exposure of the more highly metastatic B16BL6 cells to MDL 27,032 (5-50 µM) and subsequent inoculation of the treated cells into mice also resulted in inhibition of metastasis of approximately 60-70% (data not shown). The inhibition did not appear to be as great with this cell line as with the less metastatic B16F1, but accurate quantitation of metastasis in untreated mice was difficult, due to the extremely large number of metastatic nodules in the lungs (>200). However, this experiment demonstrates that the effect of MDL 27,032 on metastasis is not restricted to the B16F1 cell line. The reduced effect seen in B16BL6 may reflect the higher level of PKC in these cells (16) that may not be completely inhibited by the drug.

Inhibition of metastasis with MDL 27,032 was related to inhibition of membrane-bound PKC rather than inhibition of cytosolic enzyme. The IC50 for inhibition of metastasis by MDL 27,032 was 7 μM, which is closer to the drug's IC50 for membrane-bound PKC (13 μM) than its IC50 for cytosolic PKC (54 μM). In contrast, staurosporine was uniformly effective at inhibiting membrane-bound and cytosolic PKC and B16F1 metastasis, with IC50 values of 19-30 nM. However, staurosporine may have a different (additional?) mechanism of action than inhibition of either membrane-bound PKC rather than inhibition of cytosolic PKC.

Phosphorylation experiments using plasma membranes from B16F1 cells revealed that a prominent 80-kDa protein was phosphorylated under the same conditions as those used to assay PKC activity. MDL 27,032 inhibited phosphorylation of this protein in a dose-dependent manner, with almost complete inhibition at 50 µM, a concentration of drug which inhibited metastasis by >90%. The molecular weight of the phosphorylated protein is consistent with several common PKC substrates (27). The molecular weight is also consistent with that published for PKC in its phosphorylated active form (28). We suggest that depletion of measurable PKC activity from the membrane fraction with MDL 27,032 might result from inhibition of phosphorylation of PKC, thus resulting in less of the enzyme being in an active state. Because MDL 27,032 is competitive with ATP and is not entirely specific for PKC (18), the inhibition of phosphorylation of the 80-kDa species might be due to inhibition of either autophosphorylation or inhibition of a PKC kinase (28). The phosphorylation of other proteins was also inhibited by MDL 27,032. The regulation of cellular adhesion has been shown to be mediated by integrins, which function as a link between the extracellular matrix and the cytoskeleton.
PKC INHIBITORS BLOCK METASTASIS AND CELL ADHESION

The ability of these cell surface receptors to adhere to specific cell or protein targets has been shown to be related to the phosphorylation state of the subunits (29–31). One or more of these proteins affected by MDL 27,032 may represent subunits of integrin receptors involved in the adhesion of B16F1 cells either to endothelial cells or to the extracellular matrix protein. Further investigation into this possibility is needed to clarify which of the phosphorylated proteins, if any, are involved in the inhibition of adhesion and of metastasis.

Invasion of an artificial basement membrane matrix (Matrigel) by human bladder carcinoma (EJ) cells has been shown recently to be sensitive to inhibition of PKC by staurosporine (32). The invasive cells had more total PKC activity than a related, but noninvasive, cell line. Staurosporine inhibited the motility of the EJ cells but had no effect on cell adhesion to tissue culture slides. We found that neither staurosporine nor H-7 had an effect on the adhesion of B16F1 cells either to endothelial cell monolayers or to the extracellular adhesion proteins collagen IV, fibrinogen, fibronectin, and laminin. The reason for the disparate results with the different PKC inhibitors on cell adhesion is unclear but may reflect the nonspecificity of all of the inhibitors studied. Inhibitors more specific for PKC may assist in further understanding of these phenomena.

In summary, we have shown that inhibitors of PKC inhibit cell adhesion and experimental metastasis of B16 melanoma cells. Although the inhibitors tested are not entirely specific and, therefore, the mechanism of action of MDL 27,032 and the other PKC inhibitors is not well defined, these data, along with those from other laboratories, suggest that it may be possible ultimately to limit the dissemination of tumor cells through interference with specific steps in the metastatic cascade.

REFERENCES


3 J. A. Dumont and A. J. Bitonti, unpublished observations.
Inhibition of Experimental Metastasis and Cell Adhesion of B16F1 Melanoma Cells by Inhibitors of Protein Kinase C

Jennifer A. Dumont, Winton D. Jones, Jr. and Alan J. Bitonti


**Updated version**

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/52/5/1195

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.