Platelet-activating factor and metastasis: calcium-independent phospholipase A\(_{2}\beta\) deficiency protects against breast cancer metastasis to the lung

Jane McHowat,1 Gail Gullickson,1 Richard G. Hoover,1 Janhavi Sharma,1 John Turk,2 and Jacki Kornbluth J

1Department of Pathology, Saint Louis University School of Medicine, St. Louis, Missouri; 2Division of Endocrinology, Metabolism and Lipid Research, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri; and 3Department of Veterans Affairs Medical Center, St. Louis, Missouri

Submitted 8 December 2010; accepted in final form 6 January 2011

McHowat J, Gullickson G, Hoover RG, Sharma J, Turk J, Kornbluth J. Platelet-activating factor and metastasis: calcium-independent phospholipase A\(_{2}\beta\) deficiency protects against breast cancer metastasis to the lung. Am J Physiol Cell Physiol 300: C825–C832, 2011. First published January 12, 2011; doi:10.1152/ajpcell.00502.2010.—We determined the contribution of calcium-independent phospholipase A\(_{2}\beta\) (iPLA2\(_{\beta}\)) to lung metastasis development following breast cancer injection into wild-type (WT) and iPLA2\(_{\beta}\)-knockout (iPLA2\(_{\beta}\)-KO) mice. WT and iPLA2\(_{\beta}\)-KO mice were injected in the mammary pad with 200,000 E0771 breast cancer cells. There was no difference in primary tumor size between WT and iPLA2\(_{\beta}\)-KO mice at 27 days postinjection. However, we observed a 11-fold greater number of breast cancer cells in the lungs of WT mice compared with iPLA2\(_{\beta}\)-KO animals (\(P < 0.05\)). Isolated WT lung endothelial cells demonstrated a significant increase in platelet-activating factor (PAF) production when stimulated with thrombin [1 IU/ml, 10 min, 4,330 ± 555 vs. 15,227 ± 1,043 disintegrations per minute (dpm), \(P < 0.01\)] or TNF-\(\alpha\) (10 ng/ml, 2 h, 16,532 ± 538 dpm, \(P < 0.01\)). Adherence of E0771 cells to WT endothelial cells was increased by thrombin (4.8 ± 0.3% vs. 70.9 ± 6.3, \(P < 0.01\)) or TNF-\(\alpha\) (60.5 ± 4.3, \(P < 0.01\)). These responses were blocked by pretreatment with the iPLA2\(_{\beta}\)-selective inhibitor (S)-bromoenol lactone and absent in lung endothelial cells from iPLA2\(_{\beta}\)-KO mice. These data indicate that endothelial cell iPLA2\(_{\beta}\) is responsible for PAF production and adherence of E0771 cells and may play a role in cancer cell migration to distal locations.

carcinoma; cancer death; PAF; metastasis; breast cancer

CANCER DEATHS ARE MORE OFTEN CAUSED BY DISTANT METASTASES THAN BY GROWTH OF THE PRIMARY TUMOR (23). ORGAN-SPECIFIC SPREADING OF TUMOR CELLS RELIES ON HETERTYPIC AND HOMOTYPIC ADHESIVE INTERACTIONS AND ON CHEMOKINES AND THEIR RECEPTORS (16). ALTHOUGH MANY STUDIES HAVE CHARACTERIZED THE ROLE OF ADHESION MOLECULES IN TUMOR CELL EXTRAVASATION AND ENDOTHELIAL CELL ATTACHMENT (16), THE ROLE OF PLATELET-ACTIVATING FACTOR (PAF) IN THIS PROCESS HAS BEEN LESS WELL STUDIED. PAF IS ONE OF THE MOST POTENT INFLAMMATORY LIPID MEDIATORS AND PARTICIPATES IN VARIOUS PHENOMENA OF CANCER PROGRESSION, INCLUDING TUMOR GROWTH, ANGIogenesis, AND METASTASIS (10, 12, 13, 40, 41).

Several groups have demonstrated that inhibition of the interaction of PAF with its receptor suppresses experimental tumor growth and metastasis (15, 29, 35, 36, 49). PAF receptor antagonists interfere with murine melanoma cell adhesion to endothelium and with formation of lung metastases by melanoma cells (35, 36). These studies suggest that tumor cell behavior is affected by PAF produced by endothelial cells via interaction with tumor cell PAF receptors. Endothelial cell PAF production in inflammatory or hypersensitivity responses occurs primarily via a remodeling pathway that involves phospholipase A\(_{2}\) (PLA2\(_{\alpha}\))-catalyzed hydrolysis of membrane phospholipids, e.g., 1-O-hexadecanoyl, 2-arachidonoyl-glycerylphosphocholine, to generate lyso-PAF, which is then acetylated by acetyl-CoA:lyso-PAF acetyltransferase (28, 34, 38).

Three major classes of phospholipases A\(_{2}\) in mammalian cells are the secretory (sPLA2), cytosolic (cPLA2), and calcium-independent (iPLA2) enzymes (32, 33, 39). Members of each class are assigned to subgroups based on their amino acid sequences (50). The sPLA2s require millimolar Ca\(^{2+}\) concentration for catalytic activity, exhibit little specificity for fatty acid substituent side chain identity, and participate in various inflammatory conditions, e.g., rheumatoid arthritis and ulcerative colitis. The cPLA2\(_{\alpha}\) is expressed constitutively in most cells, prefers substrates with sn-2 arachidonoyl substituents, and prevents arachidonic acid for agonist-induced eicosanoid production in many cells and tissues.

We have demonstrated previously that the majority of endothelial cell PLA2 activity is attributable to iPLA2\(_{\alpha}\) (45). Racemic BEL inhibits iPLA2\(_{\alpha}\) activity at concentrations over 1,000-fold lower than those required to inhibit cPLA2 and sPLA2 enzymes (45). In addition, (S)-BEL inhibits iPLA2\(_{\beta}\) preferentially over iPLA2\(_{\gamma}\), and the converse is true for (R)-BEL (27). We have used BEL enantiomers to determine that endothelial cell PAF production appears to require iPLA2\(_{\beta}\) activity (49). However, BEL also inhibits phosphatidate phosphohydrolase (1) and serine pro tease (20). Additionally, hydrolysis of BEL by iPLA2 generates a diffusible bromomethyl keto acid that can alkylate thiol groups of susceptible neighboring enzymes, such as those with active cysteine residues (51). Such "off target" effects complicate interpretation of studies in which BEL is used as a pharmacologic inhibitor of iPLA2 and have motivated studies of genetic manipulations of iPLA2 enzymes to elucidate their roles in biological processes.

Mice that do not express iPLA2\(_{\beta}\) have been generated by homologous recombination (5), and these iPLA2\(_{\beta}\)-knockout
(iPLA$_2$-KO) mice have been used previously to identify roles for iPLA$_2$ in insulin secretion and glucose homeostasis, in macrophage functions, and in vascular myocyte biology (3, 4, 6, 44, 42, 49, 58, 59). In this study, we have used iPLA$_2$-KO mice to evaluate whether development of cancer cell metastases to distant sites involves a mechanism similar to that described for neutrophil recruitment and transmigration, which requires iPLA$_2$ activity and PAF biosynthesis.

MATERIALS AND METHODS

iPLA$_2$-KO mice. The generation of mice deficient in iPLA$_2$ has been described previously (5). Mice were housed in a pathogen-free facility, and studies were conducted under protocols approved by Saint Louis University Animal Care and Use Committee.

C57BL/6-derived breast cancer cell line E0771. The C57BL/6-derived breast cancer cell line E0771 was obtained from Dr. Rong Xiang (Scripps Research Institute, La Jolla, CA) and stably transfected with green fluorescent protein (GFP) cDNA to act as a tumor-specific marker. Cells (2 × 10$^5$) were mixed with 75% Matrigel and injected into the mammary pads of syngeneic female wild-type (WT) and iPLA$_2$-KO mice. Primary tumor growth was measured at weekly intervals using calipers. Tumor volumes (in mm$^3$) were calculated using the formula: width$^2$ × length/2, where width is the smaller of the two measurements. Mice were killed at days 14, 19, and 27, and primary tumor, blood, liver, and lungs were harvested. Mice and primary tumors were weighed after death, and final tumor volumes (in mm$^3$) were calculated using the formula: width × length × depth/2. Pieces of tumor tissue and the right apical lobe of the lung from each animal were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) for histological examination. Results were analyzed in a blinded fashion.

Endothelial cell isolation and culture. Endothelial cells were isolated from mouse lung by collagenase digestion. Diced lung tissue was incubated in a solution of collagenase (1 mg/ml, 1 h, 37°C), and the digested tissue was passed through a cell strainer. Single-cell suspension was obtained by incubating (10 min) in trypsin-EDTA. Endothelial cells were isolated by incubation with murine immunoglobulin-G to block Fc receptors and then with rat anti-mouse CD31, rat anti-mouse CD105, and biotinylated isoclot B4. Cells were washed, incubated with rat anti-mouse Ig and streptavidin-conjugated microbeads, and separated using an AutoMACs cell separator. Eluted cells were washed, resuspended in EGM-2MV cell culture medium (Lonza), and plated in 25-cm$^2$ culture flasks. Nonadherent cells were removed the next day, and cells were grown to confluence and passed at 1:3 dilution. Cells from passages 3-4 were used for experiments.

Real-time RT-PCR analysis. The right azygos lobe of the lung and a section of liver were snap frozen, and the tissue was homogenized using a rotor-stator homogenizer (Tissuemiser, Fisher Scientific, St. Louis, MO). Blood was obtained by cardiac puncture. RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was prepared using the TaqMan Reverse Transcription Gene Expression Assay kit (Applied Biosystems, Carlsbad, CA). Real-time PCR analysis of green fluorescent protein (GFP) and 18s RNA was performed using the ABI 7500 Real Time PCR System (Applied Biosystems).

Measurement of PAF production. Endothelial cells grown in 35-mm culture dishes were washed twice with Hank’s balanced salt solution containing (in mM) 135 NaCl, 0.8 MgSO$_4$, 10 HEPES (pH 7.4), 1.2 CaCl$_2$, 5.4 KCl, 0.4 KH$_2$PO$_4$, 0.3 Na$_2$HPO$_4$, and 6.6 glucose. Cells were incubated with [${}^{3}$H]acetic acid (10 µCi/ml, 20 min). After stimulation with thrombin (Sigma Chemical) or TNF-α (Calbiochem, Gibbstown, NJ), lipids were extracted from cells by the method of Bligh and Dyer (7). The chloroform layer was concentrated by evaporation under nitrogen, resuspended in 9:1 CHCl$_3$/CH$_3$OH, applied to a silica gel 60 TLC plate, and developed in chloroform-methanol-aceticacid-water (50:25:8.4 vol/vol/vol). The region corresponding to [${}^{3}$H]PAF was scraped, and radioactivity was quantified by liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by addition of a known amount of [${}^{14}$C]PAF as an internal standard.

Adherence of E0771 cells to endothelial cell monolayers. Murine medullary breast adenocarcinoma (E0771) cells were labeled by incubation (45 min, 37°C) with calcein-AM (4 µg/ml, Alexis Biochemicals, Lausen, Switzerland). After being washed three times, cells (2 × 10$^5$) were layered onto confluent endothelial cell monolayers. Medium and unbound cells were removed and discarded. Adherent E0771 cells and endothelial cells were washed with Dulbecco’s phosphate-buffered saline and lysed (0.2% Triton, 1 ml). Samples were sonicated (10 s, 550 Sonic Disembrator, Fisher Scientific, Pittsburgh, PA), and the amount of calcein-AM fluorescence was measured using a Synergy 2 microplate reader (Biotek, Winooski, VT) at an excitation wavelength of 485 nm and emission wavelength of 530 nm. The percent E0771 cell adherence was calculated from the calcein-AM fluorescence measured in 2 × 10$^5$ cells.

RESULTS

WT and iPLA$_2$-KO mice were injected in the mammary pad with 200,000 GFP-expressing E0771 cells suspended in 50% Matrigel. Mice were observed for up to 27 days, and primary tumor volume was measured at regular intervals (Fig. 1). Tumor growth in both WT and iPLA$_2$-KO mice was cally transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Richmond, CA). The blocked PVDF membrane was incubated with rabbit anti-human PAF receptor antibody (1 in 200, Cayman Chemical, Ann Arbor, MI) and horseradish peroxidase-conjugated goat anti-rabbit antibody (1 in 5,000, Sigma Chemical, St. Louis, MO). Regions of antibody binding were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL) after exposure to film (Hyperfilm, Amersham).

Fig. 1. Tumor growth in the mammary pad following injection of E0771 cells into wild-type (WT) and calcium-independent phospholipase A$_2$β (iPLA$_2$β)-knockout (KO) mice. Data represent means ± SE; n = 4–7 animals.
similar over the experimental period, with a small increase in measurable size up to 13 days followed by an exponential increase in tumor volume up to 27 days. At 19 or 27 days, mice were weighed and killed, and primary tumor, blood, liver, and lungs were harvested. No significant difference in weight was observed between WT and iPLA2-KO mice (Table 1), and there was also no significant difference in primary tumor weight or volume in tumors from WT or iPLA2-KO mice (Table 1 and Fig. 1).

Lungs, blood, and liver from WT and iPLA2-KO mice were analyzed for tumor metastasis by quantitative RT-PCR analysis of GFP, which acts as a tumor-specific marker. Table 2 shows the PCR analysis of lungs from one set of mice killed at day 19 and another set on day 27. At day 19, GFP was detected in the lungs of both WT and KO animals, but the differences were not significant. By day 27, there was more GFP expression in both WT and KO mice compared with day 19, and significantly more GFP was observed in WT compared with iPLA2-KO mice (\(\Delta C_{\text{T}} 15.8\) vs. 19.3, \(P < 0.05\)), which reflects an 11-fold difference between the number of metastatic E0771 breast cancer cells in the lungs of WT than those of iPLA2-KO mice. Table 3 shows the H&E staining evaluation of lungs from WT and KO mice and is consistent with the quantitative PCR metastasis measurements. Figure 2 depicts representative histological analyses of lungs from WT and KO mice and is consistent with the quantitative PCR metastasis measurements. Figure 2B also shows perivascular blood and the liver for evidence of tumor metastasis. In contrast to the lung, we found very low, barely detectable levels of E0771 cells at these sites in both WT and KO mice by quantitative PCR. Therefore, at the times of our analysis, we were only able to detect metastasis to the lung.

To determine whether the observed difference in lung metastases could be, at least in part, a result of decreased endothelial cell PAF production and transendothelial cell migration, we isolated endothelial cells from lungs of WT and iPLA2-KO mice by selecting cells that expressed CD31 and CD105. Confluent monolayers were stained for coagulation factor VIII to determine endothelial cell purity, and preparations consisting of >80% endothelial cells were used for subsequent studies. In previous studies, we have demonstrated increased PAF production in human lung microvascular endothelial cells upon treatment with thrombin or tryptase (47). Increased PAF production is inhibited completely by pretreatment with the iPLA2-selective inhibitor BEL (47). To verify that mouse lung endothelial cells release PAF via iPLA2 activation, we incubated WT and iPLA2-KO lung endothelial cells with thrombin or TNF-\(\alpha\) and measured PAF production (Fig. 3). Incubation of WT lung endothelial cells with thrombin (1 IU/ml, 10 min) or TNF-\(\alpha\) (10 ng/ml, 2 h) induced a fourfold rise in PAF production, and these responses were completely prevented by pretreatment of the cells with (S)-BEL (5 \(\mu\)M, 10 min, Cayman Chemical), which is consistent with the involvement of iPLA2 in the responses. Stimulation of iPLA2-KO endothelial cells with thrombin or TNF-\(\alpha\) failed to induce any increase in PAF production (Fig. 3), which is consistent with a requirement for iPLA2 in thrombin- and TNF-\(\alpha\)-stimulated PAF production by pulmonary endothelial cells. PAF expressed by endothelial cells binds to its cognate receptors on circulating inflammatory cells, and results in cell adherence to an activated endothelial cell monolayer. To determine whether E0771 cells may adhere to the endothelium via a PAF-PAF receptor interaction, we performed immunoblot analysis on E0771 cells to verify that they express the PAF receptor (Fig. 4). PAF receptor immunoprotein was detected in E0771 cells (Fig. 4, left lanes), human coronary artery endothelial cells (Fig. 4, middle lanes), and mouse atrial cardiomyocytes (HL-1, Fig. 4, right lanes). We incubated endothelial cells with E0771 breast cancer cells to determine whether increased PAF production was associated with increased cell adherence. As shown in Fig. 5, stimulation of WT lung endothelial cells with thrombin or TNF-\(\alpha\) resulted in a sixfold increase in E0771 cell adherence. E0771 cell adherence was inhibited when WT mouse lung endothelial cells were pretreated with (S)-BEL before incubation with thrombin or TNF-\(\alpha\) (Fig. 5). Pretreating E0771 cells with the PAF receptor antagonist CV3988 (10 \(\mu\)M, 10 min) before incubating them with WT endothelial cells resulted in complete inhibition of adherence, which demonstrates the requirement of the interaction of PAF with its receptor in order for cell adherence to occur (Fig. 5). In contrast, stimulation of iPLA2-KO lung endothelial cells with thrombin or TNF-\(\alpha\) failed to increase their adherence to E0771 cells (Fig. 6). These results are consistent with a requirement for iPLA2 in thrombin and

Table 1. Whole body weight and breast cancer tumor weight and volume for wild-type and iPLA2-\(\beta\)-knockout mice injected with 200,000 E0771 cells into the mammary pad

<table>
<thead>
<tr>
<th></th>
<th>Day 19</th>
<th></th>
<th>Day 27</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor Volume, (\text{mm}^3)</td>
<td>Tumor Weight, g</td>
<td>Mouse Weight, g</td>
</tr>
<tr>
<td>Wild type</td>
<td>701 ± 242</td>
<td>0.9 ± 0.4</td>
<td>23.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>((n = 8))</td>
<td>((n = 8))</td>
<td>((n = 8))</td>
</tr>
<tr>
<td>iPLA2-(\beta)-knockout</td>
<td>907 ± 230</td>
<td>1.1 ± 0.5</td>
<td>22.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>((n = 6))</td>
<td>((n = 6))</td>
<td>((n = 6))</td>
</tr>
</tbody>
</table>

Data represent means ± SE. GFP, green fluorescent protein; \(C_{\text{T}}\), cycle threshold; WT, wild type; NS, not significant.

Table 2. Real-time RT-PCR analysis of GFP and 18s RNA expression in the lungs of wild-type and iPLA2-\(\beta\)-knockout female mice at 19 and 27 days following injection of 200,000 GFP-expressing E0771 cells into the mammary pad

<table>
<thead>
<tr>
<th></th>
<th>Day 19</th>
<th></th>
<th>Day 27</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta C_{\text{T}}) vs. 18s RNA</td>
<td>Fold Change From WT</td>
<td>(\Delta C_{\text{T}}) vs. 18s RNA</td>
<td>Fold Change From WT</td>
</tr>
<tr>
<td>Wild type</td>
<td>19.4 ± 1.2</td>
<td>((n = 10))</td>
<td>15.8 ± 1.2</td>
<td>((n = 7))</td>
</tr>
<tr>
<td>iPLA2-(\beta)-knockout</td>
<td>21.8 ± 1.1</td>
<td>((n = 7))</td>
<td>-5.3</td>
<td>((n = 7))</td>
</tr>
<tr>
<td></td>
<td>((n = 7))</td>
<td>((n = 7))</td>
<td>19.3 ± 0.8</td>
<td>((P &lt; 0.05))</td>
</tr>
<tr>
<td></td>
<td>((n = 7))</td>
<td>((n = 7))</td>
<td>-11.3</td>
<td>((\text{NS}))</td>
</tr>
</tbody>
</table>

Data represent means ± SE. GFP, green fluorescent protein; \(C_{\text{T}}\), cycle threshold; WT, wild type; NS, not significant.
TNF-α-stimulated endothelial cell PAF production and inflammatory cell adherence.

DISCUSSION

Cancer is the second most prevalent cause of death, and the majority of cancer deaths are caused by distant metastases rather than by growth of the primary tumor (23). Development of new management strategies that target metastasis development are thus required to improve cancer morbidity and mortality outcomes. Here, we have demonstrated that lungs of WT mice accumulate an 11-fold greater number of metastatic breast cancer cells than do those of iPLA2-KO mice in our model, and this identifies iPLA2 as a potentially important target for pharmacologic or other interventions designed to reduce adverse outcomes from metastatic breast cancer, which is the leading cause of cancer death in US women. Our data suggest that the protection against breast cancer metastasis conferred by iPLA2 deficiency is attributable, at least in part, to decreased endothelial cell PAF production and consequently reduced breast cancer cell adherence to, and transmigration across, the endothelium. Several previous studies have investigated the role of adhesion molecules in facilitating tumor cell detachment from primary tumors to gain access to the lymphatic or blood circulatory systems, but few have demonstrated the role of endothelium-derived PAF in promoting tumor cell egress from the circulation and migration into tissue. Tumor cell extravasation employs a process similar to that involved in recruitment of inflammatory cells to the endothelium, which involves a sequence of events that include the interaction of endothelial cell adhesion molecules with corresponding receptors on inflammatory cells and the interaction of PAF with its receptor (35, 36).

In a previous study, we demonstrated an increase in human lung microvascular endothelial cell (HMVEC-L) PAF production, and polymorphonuclear leukocyte adherence in response to thrombin or tryptase (47). Subsequently, we demonstrated that HMVEC-L PAF production required iPLA2 activity, as reflected by inhibition of thrombin- or tryptase-stimulated PAF production by pretreatment with (S)-BEL (49). Endothelial cells isolated from lungs of WT mice exhibited increased PAF production upon stimulation with thrombin or tryptase, but this response was absent with iPLA2-KO lung endothelial cells (49). Murine monocyte/macrophage RAW 264.7 cells exhibited increased adherence to activated WT lung endothelial cells, and this response was blocked by pretreatment of the endothelial cells with racemic BEL (49). Here, we demonstrate that pretreatment of WT lung endothelial cells with (S)-BEL completely inhibited E0771 cell adherence in response to thrombin or TNF-α, while pretreatment with (R)-BEL had little effect (Fig. 4). Moreover, pretreatment of E0771 cells with the PAF receptor antagonist CV3988 completely inhibited their adherence to stimulated WT lung endothelial cells, and this response was blocked by pretreatment of the endothelial cells with racemic BEL (49). Here, we demonstrate that pretreatment of WT lung endothelial cells with (S)-BEL completely inhibited E0771 cell adherence in response to thrombin or TNF-α, while pretreatment with (R)-BEL had little effect (Fig. 4). Moreover, pretreatment of E0771 cells with the PAF receptor antagonist CV3988 completely inhibited their adherence to stimulated endothelial cells. These data demonstrate that iPLA2 activity and increased PAF production are involved in adherence of E0771 cells to an activated endothelium. Although these in vitro data support our hypothesis that endothelial cell iPLA2 is involved, we cannot rule out the possibility that the absence of iPLA2 in other cells is playing an instrumental role in metastasis.

E0771 cells are syngeneic to C57BL/6 mice and were originally isolated from a spontaneous medullary breast adenocarcinoma (17, 52). Subcutaneous injection of E0771 cells has been shown to result in secondary metastases in the lung that are morphologically similar to primary E0771 tumors (18, 63). At 27 days postinjection, histological examination dem-

Table 3. Histological analysis of lungs removed from WT and iPLA2β-knockout mice at 27 days following injection of 200,000 E0771 cells into the mammary pad

<table>
<thead>
<tr>
<th>Animal</th>
<th>Diagnosis</th>
<th>Distribution</th>
<th>Severity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>Carcinoma, metastatic</td>
<td>Focal</td>
<td>2</td>
<td>3 of 3 sections</td>
</tr>
<tr>
<td>WT2</td>
<td>Carcinoma, metastatic</td>
<td>Multifocal</td>
<td>2</td>
<td>1 of 3 sections</td>
</tr>
<tr>
<td>WT3</td>
<td>Carcinoma, metastatic</td>
<td>Focal</td>
<td>1</td>
<td>1 of 3 sections</td>
</tr>
<tr>
<td>WT4</td>
<td>No evidence of neoplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO1</td>
<td>No evidence of neoplasia</td>
<td>Focal</td>
<td>1</td>
<td>2 of 3 sections</td>
</tr>
<tr>
<td>KO2</td>
<td>Carcinoma, metastatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO3</td>
<td>No evidence of neoplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO4</td>
<td>No evidence of neoplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Severity graded as 1, minimal; 2, mild; 3, moderate; 4, severe. KO, iPLA2β-knockout.

Fig. 2. Hematoxylin and eosin-stained sections of mouse lung from WT and iPLA2β-KO mice. A: representative section of lungs from KO mice, with no evidence of tumor from a section of lung from knockout mouse no. 1. B: section of lung from wild-type mouse no. 2, showing focal metastasis of E0771 cells.
onstrated detectable carcinoma in 75% of WT mice, but only 25% of iPLA2β-KO mice exhibited any evidence of neoplasia (Table 3).

A reduction in development of metastases in iPLA2β-KO mice was also observed upon real-time RT-PCR evaluation of micrometastasis by GFP-expressing E0771 cells in the lung (Table 2). This suggests that iPLA2β contributes to the development of metastasis, perhaps by providing substrate for PAF production by endothelial cells.

Formation of metastases in specific organs involves adhesion molecules, chemokines, and cancer stem cells (reviewed recently in Ref. 16), but there has been relatively little examination of the role of PAF in breast cancer metastases. Several breast cancer cell lines produce PAF upon stimulation and express PAF receptors (9). Increased PAF production has been proposed to promote cell proliferation and angiogenesis in several breast cancer cell lines (9). Pretreatment of human breast cancer cells with the PAF receptor antagonist WEB-2086 has been reported to inhibit cell growth and differentiation in vitro. In the studies described here, we did not observe a significant difference in primary tumor size in WT and iPLA2β-KO mice, although there was a trend toward increased tumor development in the iPLA2β-KO mice (Fig. 1). These data suggest that the absence of iPLA2β adjacent to the injected E0771 cells did not affect primary tumor breast cancer cell growth or differentiation in vivo.

Enhanced PAF production in response to stimulation is observed in highly invasive breast cancer cells, which suggests that breast cancer PAF production may play a role in the development of metastases (9). We observed a significant decrease in metastasis formation in the lung after injection of breast cancer cells into iPLA2β-KO mice, and we propose that this is due, at least in part, to impaired endothelial cell PAF production and transendothelial cell migration from the primary tumor to the circulation, from the circulation to distal sites, or from both of those processes. We attempted to identify circulating tumor cells by analyzing blood for the presence of GFP using quantitative PCR; however, we were not able to detect tumor cells. Our study is the first of which we are aware that demonstrates reduced development of metastases after injection of breast cancer cells into mammary pads of iPLA2β-KO mice and to provide evidence that endothelial cell iPLA2β activity and PAF production are important in this process. A role for PAF in development of lung metastasis development after intravenous injection of melanoma cells has been demonstrated previously (36). Im et al. (26) demonstrated that intraperitoneal instillation of PAF resulted in increased development of lung metastases after
injection of melanoma cells, and Melnikova et al. (36) have shown that pretreating mice with the PAF receptor antagonist PCA4248 inhibited development of melanoma metastases in the lung. A recent study examining tumorigenesis and ascites formation after intraperitoneal injection of IB8 ovarian cancer cells demonstrated that these processes were much reduced in iPLA2β-KO compared with WT mice, which suggests that iPLA2β may represent a useful target for the development of antineoplastic drugs or other therapeutic interventions (30).

The importance of an inflammatory microenvironment in cancer initiation and progression offers opportunities for exploration of new approaches to cancer treatment. Inflammatory cells in the tumor microenvironment release cytokines, chemokines, and other factors that can enhance tumor growth, angiogenesis, and metastasis. Thrombin can regulate tumor cell adherence to endothelial cells and is a critical component of the microenvironment that influences tumor cell behavior (reviewed in Ref. 37). We demonstrate here that E0771 cell adherence to isolated endothelial cells is enhanced by stimulation with thrombin, which suggests that thrombin may contribute to transmigration of E0771 cell across the endothelial barrier from the primary tumor. In addition, we observed increased endothelial cell PAF production and E0771 cell adherence in response to TNF-α stimulation. Im et al. (26) have previously demonstrated that PAF mediates metastasis-enhancing activities of TNF-α and IL-1α, and we propose that at least part of this mechanism may involve PAF production by the endothelium in response to TNF-α stimulation.

Phospholipase A2 enzymes hydrolyze the sn-2 fatty acid on the glycerol backbone of membrane phospholipids to yield products that include a free fatty acid, e.g., arachidonic acid, and a 2-lysophospholipid, e.g., 1-O-hexadecanoyl-2-lyso-glycero-phosphocholine, that can act directly or be converted to myriad phospholipid-derived signaling molecules that affect tumor cell behavior and cancer progression, including PAF and various eicosanoids. Expression of sPLA2 enzymes is increased in several human cancers, including breast cancer (60–62). Similarly, cPLA2α is activated by 17β-estradiol in MCF-7 breast cancer cells and inhibition of cPLA2α suppressed MCF-7 cell growth (11). Although the role of iPLA2 in cancer has been less well characterized, in vitro studies demonstrate that both iPLA2β and iPLA2γ are expressed in several human cancers (2, 43, 53). PLA2 inhibitors have been proposed as anticancer drugs because inhibition of PLA2 could theoretically decrease production of several metabolites implicated in cancer progression. For example, free arachidonic acid can be metabolized to eicosanoids that induce cancer cell growth and proliferation in vitro (for recent reviews, see Refs. 60–62). Nonsteroidal antiinflammatory drugs may reduce breast cancer incidence by up to 20%, and this might be related to their ability to inhibit cyclooxygenase-2 or reduce serum estradiol in postmenopausal women (reviewed recently in Refs. 19, 21, and 24). Our data suggest that inhibition of endothelial cell iPLA2β could represent a therapeutically advantageous means to reduce PAF production and to prevent or retard development of metastases from the primary tumor. Since the vast majority of breast cancer-related deaths are due to metastatic disease rather than growth of the primary tumor, this represents a novel and potentially important therapeutic approach to the management of breast cancer.

**GRANTS**

This work was supported by United States Public Health Service Grants R37-DK34388, P41-RR00954, P60-DK20579, and P30-DK56341 (to J. Turk) and by a merit review grant from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, and Biomedical Laboratory Research and Development (to J. Kornbluth).

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**

PAF AND METASTASIS


