Involvement of p38α Mitogen-activated Protein Kinase in Lung Metastasis of Tumor Cells*

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To study the role of p38 mitogen-activated protein kinase (p38) activity during the process of metastasis, p38α+/− mice were subjected to an in vivo metastasis assay. The number of lung colonies of tumor cells intravenously injected in p38α+/− mice was markedly decreased compared with that in wild-type (WT) mice. On the other hand, the time-dependent increase in tumor volume after subcutaneous tumor cells transplantation was comparable between WT and p38α+/− mice. Platelets of p38α+/− mice were poorly bound to tumor cells in vitro and in vivo compared with those of WT mice. E- and P-selectin mRNAs were markedly induced in the lung after intravenous injection of tumor cells. However, the induction of these selectin mRNAs in p38α+/− mice was significantly reduced compared with that of WT mice. The number of tumor cells attached on lung endothelial cells of p38α+/− mice was poorly bound to tumor cells in vitro and in vivo compared with those of WT mice. Furthermore, the resting expression levels of E-selectin in lung endothelial cells and P-selectin in platelets of p38α+/− mice were suppressed compared with those of WT mice. The number of tumor cells attached on lung endothelial cells of p38α+/− mice was significantly reduced compared with that of WT mice. The transmigrating activity of tumor cells through lung endothelial cells of p38α+/− mice was similar to that of WT mice. These results suggest that p38α plays an important role in extravasation of tumor cells, possibly through regulating the formation of tumor-platelet aggregates and their interaction with the endothelium involved in a step of hematogenous metastasis.

Mitogen-activated protein kinases (MAPKs)3 transduce a variety of extracellular signals to the transcriptional machinery via cascades of protein phosphorylation. There are three genetically distinct MAPKs in mammals, consisting of extracellular signal-regulated kinase, c-Jun N-terminal kinase, and p38 MAPK (p38). All three members are activated by dual phosphorylation of the conserved TXY motif and then phosphorylate their respective substrates on serine or threonine residues (1–3). There are four mammalian isoforms of p38 (α, β, γ, and δ). Among them, p38α and -β are expressed relatively ubiquitously, as shown by Northern blot analysis of adult tissues (4). Although targeted disruption of the p38α gene results in homozygous embryonic lethality because of defects in erythropoiesis and placental organogenesis (5, 6), the p38α+/− mouse is a useful tool for analyzing the in vivo role of p38 in disease models (7–9).

Tumor metastasis is a significant process resulting in unexpected death in cancer patients. Recent advances in molecular cancer research have clarified a variety of therapeutic targets, some of which are being tested in clinical trials (10). Very recently, the relationship between tumor metastasis and MAPKs has been investigated (11, 12). It is reported that p38α is important for the maintenance of breast cancer with an invasive phenotype by promoting the stability of urokinase plasminogen activator and its receptor mRNA (13). It has also been demonstrated that p38 is involved in various metastatic processes (14–17). On the other hand, MKK4, a common upstream prerequisite for c-Jun N-terminal kinase and p38 activation as an MAPK kinase, is characterized as a metastasis suppressor gene in human ovarian carcinoma (18, 19). Likewise, loss of p38 activation leads to an increase in tumorigenesis because of a cell cycle defect (20). These findings clearly suggest that p38 activity in tumor cells regulates tumor progression and metastasis. However, there is no in vivo confirmation of a pathophysiological role of p38 in hosts during tumor metastasis. To elucidate this point, we used p38α+/− mice to examine the in vivo role of p38α during tumor metastasis. Here, we showed that tumor metastasis is suppressed in p38α+/− mice.

EXPERIMENTAL PROCEDURES

Experimental Animals—The use of animals in all of our experiments was in accordance with the guidelines for animal care of Chiba University and RIKEN. Female mice heterozygous for targeted disruption of the p38α gene (6) were crossed with C57BL/6J male mice (Saitama Experimental Animal Supply Co.) to generate p38α+/− and p38α+/+ (wild-type; WT) mice. Offspring (>8 generations) were genotyped by PCR analysis of tail-derived DNA. Multiplex PCR with three primers per
reaction was used. The primers were as follows: A, 5'-CCCTA-TACTCCCTCTCTGTGTAACCTTGT-3'; B, 5'-'CCCAAAC-CCCAAGAAGAAATGATG-3'; C, 5'-TTCAGTGACAGC-TCGAGCACAGCTG-3'. Using these primers for one cycle at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, with an extension step of 7 min at 72 °C at the end of the last cycle, produced 800- and 450-bp fragments from the mutant and WT alleles, respectively. 8–12-week-old the end of the last cycle, produced 800- and 450-bp fragments from the mutant and WT alleles, respectively.

In Vivo Binding of Platelets to Tumor Cells—B16 cells labeled with Hesperos-Tyroom's buffer (10 mM Hepes, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, 11.9 mM NaHCO₃, 5 mM glucose, pH 7.2). B16, LLC, and HEK293T cells were seeded on 8-well chamber slides and incubated with fluorescently labeled platelets at a density of 10⁷/ml for 1 h at 37 °C. In the case of activating platelets, 1 unit/ml thrombin and 20 μg/ml collagen were added to each well just after the application of platelets. For elucidating the effect of heparin, 20 units/ml heparin (Wako Chemicals) was added to each well just after the application of platelets. After washing the chamber slides with Hesperos-Tyrome's buffer twice, binding of platelets to tumor cells was examined by a fluorescence microscope (Axioplan; Zeiss).

In Vivo Binding of Platelets to Tumor Cells—B16 cells labeled with calcein were resuspended and used for a systemic injection into WT and p38α+/− mice. After 30 min, the lungs were dissected and fixed with 3% paraformaldehyde buffer (pH 7.4), and then the resultant sample (1 μm in thickness) was placed on poly-l-lysine-coated slides. The sections were stained with a monoclonal anti-mouse CD41 (integrin αIIb) antibody (Pharmingen) in combination with a secondary antibody (Alexa Fluor 594-labeled goat anti-rat IgG (Molecular Probes, Inc., Eugene, OR)) and observed under a fluorescence microscope (Axioplan; Zeiss). The resulting fluorescence profile was analyzed by an Intelligent Quantifier (Bio Image) to quantify the fluorescence intensity of platelets adherent to calcein-labeled tumor cells.

Reverse Transcription-PCR Detection of P- and E-selectin mRNAs—B16 cells were injected into WT and p38α+/− mice. As a negative control, B16-free PBS was injected into WT and p38α+/− mice. The lungs were taken from these mice 4 h after injection. Total RNA was prepared from the lungs using ISOGEN (Wako Chemicals) according to the manufacturer's instructions. Single strand cDNA was synthesized from prepared RNA (3 μg), with Moloney murine leukemia virus reverse transcriptase (Invitrogen) using an oligo(dT) primer (Invitrogen) in a total volume of 20 μl. The resultant cDNA sample (1 μl) was subjected to PCR for amplification of mouse P- or E-selectin cDNA using specific primers (for P-selectin, sense primer, 5'-TGCAGCTTTTCTGTGTATGAAGGC-3'; antisense primer, 5'-ATAGAGCCAACACACATCTGC-3'; for E-selectin, sense primer, 5'-GACCTTTCAAAAATGGG-TCCAG-3'; antisense primer, 5'-AGAGCATGAGGCACAGA-TGTCAGGAG-3'). As an internal control, mouse glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified using specific primers (sense primer, 5'-GACCACAGTCCATGCATC-3'; antisense primer, 5'-TCCACACCCCTCCTG-3'). The settings of the thermal cycle were 30 cycles of 30 s at 94 °C, 30 s at 59 °C, and 1 min at 72 °C for mouse E- and P-selectin and 25 cycles of 40 s at 94 °C, 1 min at 60 °C, and 1 min at 72 °C for mouse glyceraldehyde-3-phosphate dehydrogenase. For detecting P-selectin mRNA in platelets, total RNA sample (0.25 μg) was subjected to reverse transcription reaction with Moloney murine leukemia virus in a total volume of 20 μl, and then the resultant sample (1 μl) was subjected to PCR with 35 cycles. The amplified products were separated in 1.2% agarose gel and visualized with ethidium bromide staining.

In Vitro Binding of Platelets to Tumor Cells—Platelets were prepared from WT and p38α+/− mice by the method previously described (9). Then platelets were fluorescently labeled with calcein by incubation with 1 μM calcein-acetoxyethyl ester (Molecular Probes) for 30 min at 37 °C and washed twice

Cells—Mouse B16 melanoma F10 (B16) cells, mouse Lewis lung carcinoma (LLC) cells, and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% (v/v) fetal calf serum under a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. B16 cells stably expressing β-galactosidase (B16-β-gal) were generated by cotransfection of pCMVβ (BD Biosciences) and pBLAST (InvivoGen), using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Stable transfectants were selected in the medium containing 50 μg/ml blasticidin S (Funakoshi). After 2–3 weeks, colonies were isolated by limiting dilution, and β-galactosidase-expressing clones were identified by X-gal staining after fixation with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4).

Experimental Lung Metastasis Model—B16 and LLC cells were trypsinized and recovered from the medium by centrifugation. They were suspended with physiological saline to a concentration of 10⁷ cells/ml. Mice (WT and p38α+/−) were anesthetized with an intraperitoneal injection of 5% pentobarbital sodium, and briefly ventilated with a hand caliper, and tumor volume was calculated as (length × width)²/2.

In Vivo Binding of Platelets to Tumor Cells—B16 cells were injected into WT and p38α+/− mice. As a negative control, B16-free PBS was injected into WT and p38α+/− mice. The lungs were taken from these mice 4 h after injection. Total RNA was prepared from the lungs using ISOGEN (Wako Chemicals) according to the manufacturer’s instructions. Single strand cDNA was synthesized from prepared RNA (3 μg), with Moloney murine leukemia virus reverse transcriptase (Invitrogen) using an oligo(dT) primer (Invitrogen) in a total volume of 20 μl. The resultant cDNA sample (1 μl) was subjected to PCR for amplification of mouse P- or E-selectin cDNA using specific primers (for P-selectin, sense primer, 5'-TGCAGCTTTTCTGTGTATGAAGGC-3'; antisense primer, 5'-ATAGAGCCAACACACATCTGC-3'; for E-selectin, sense primer, 5'-GACCTTTCAAAAATGGG-TCCAG-3'; antisense primer, 5'-AGAGCATGAGGCACAGA-TGTCAGGAG-3'). As an internal control, mouse glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified using specific primers (sense primer, 5'-GACCACAGTCCATGCATC-3'; antisense primer, 5'-TCCACACCCCTCCTG-3'). The settings of the thermal cycle were 30 cycles of 30 s at 94 °C, 30 s at 59 °C, and 1 min at 72 °C for mouse E- and P-selectin and 25 cycles of 40 s at 94 °C, 1 min at 60 °C, and 1 min at 72 °C for mouse glyceraldehyde-3-phosphate dehydrogenase. For detecting P-selectin mRNA in platelets, total RNA sample (0.25 μg) was subjected to reverse transcription reaction with Moloney murine leukemia virus in a total volume of 20 μl, and then the resultant sample (1 μl) was subjected to PCR with 35 cycles. The amplified products were separated in 1.2% agarose gel and visualized with ethidium bromide staining.
under UV radiation. Specific amplification of the expected size (mouse E-selectin, 570 bp; P-selectin, 371 bp; and mouse glyceraldehyde-3-phosphate dehydrogenase, 453 bp) was observed.

Isolation of Lung Endothelial Cells—The lungs were dissected out from WT and p38α+/− mice and washed with ice-cold PBS three times. The tissues were cut into small pieces and incubated in Dulbecco’s modified Eagle’s medium supplemented with 2 mg/ml collagenase (Worthington), 2 mg/ml hyaluronidase (Sigma), and 1 mg/ml dispase (Invitrogen) at 37 °C for 30 min with shaking. Then the tissues were suspended well by a pipette, and the resultant suspension was passed through a 70-μm nylon mesh filter (Falcon). The cells were collected by centrifugation at 400 × g for 10 min. After washing twice with PBS, the cells suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 50 units/ml heparin were put on the top of Percoll solution (50% lower and 30% upper) layers and centrifuged at 800 g for 30 min. The resultant interface fraction was collected and washed with PBS twice. The cells were suspended in 0.1% bovine serum albumin/PBS (Type V; Sigma) and subjected to MACS separation with rat monoclonal anti-PECAM-1 (BD Biosciences) and anti-rat IgG microbeads (Miltenyi Biotec). The isolation of PECAM-1-positive endothelial cells was performed according to the manufacturer’s protocol (Miltenyi Biotec). The cells were seeded on a collagen-coated 6-well plate and maintained for 2 days in CS-C medium (Sigma) supplemented with 10% fetal bovine serum and EC growth supplement (Sigma). After confirming over 90% purity by the uptake of Dil-acetylated low density lipoprotein, the cells were used for experiments.

Western Blot Analysis—The cell lysates of lung endothelial cells (7.5 μg) and platelets (15 μg) were subjected to Western blot analysis with anti-α-catenin, anti-PECAM-1, and anti-CD41 mouse monoclonal antibodies (BD Biosciences, Sigma) and anti-p38, anti-phospho-p38, and anti-phospho-ATF2 rabbit polyclonal antibodies (Cell Signaling), anti-E-selectin rat monoclonal antibody (R & D Systems), and anti-P-selectin goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Flow Cytometry—Liver endothelial cells were isolated from WT and p38α+/− mice by the method previously described (21) with modification. Cells were cultured for 2 days, and over 90% purity was confirmed by the uptake of Dil-acetylated low density lipoprotein and immunostaining with antibodies to VE-cadherin and vascular endothelial growth factor receptor-2. Then cells were stained for P-selectin, E-selectin, or PECAM-1 (CD31), followed by fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin (BD Biosciences). The labeled cells were analyzed on a FACScan flow cytometer (BD Biosciences), and rates of positive cells were quantified using CELLQuest software 2.1.1 (BD Biosciences).

Assay for Attachment and Transmigration of Tumor Cells—Primary cultured lung endothelial cells (105 cells) were seeded on 24-well cell culture inserts (apical chamber; Falcon) with pore size of 8 μm and maintained in CS-C medium overnight. After replacing the medium with Opti-MEM I (Invitrogen), Dil-labeled B16 cells (3 × 104 cells) were added to the apical chambers. At the same time, platelets (3 × 107) freshly isolated from WT and p38α+/− mice were added to the apical chambers for determination of the effect of platelets. As a negative control, Dil-labeled B16 cells with or without platelets were added to the endothelial cell-free apical chambers. All apical chambers were washed with Opti-MEM I twice for removing nonattaching cells 30 min after applying B16 cells to the apical chambers. Some apical chambers were fixed with 4% paraformaldehyde in 0.1 m sodium phosphate buffer (pH 7.4) for counting the number of B16 cells attached on endothelial cells by a fluorescence microscope (Axioplan; Zeiss). Some other apical chambers were further incubated to determine the transendothelial migration of B16 cells. After incubation for 8 h, nonmigrating cells were removed by scraping the apical side of the apical chambers with a cotton swab. Then the apical chambers were fixed and subjected to the examination of transmigrating B16 cells by a fluorescence microscope (Axioplan; Zeiss).

RESULTS AND DISCUSSION

Resistance of p38α+/− Mice to Experimental Lung Metastasis of Tumor Cells but Not to Tumor Growth—Two transplantable tumor cell lines, B16 and LLC, were used in the present study, because both are highly metastatic to the lung (22, 23). As shown in Fig. 1, A and B, there was a significant difference in surface pulmonary metastatic foci between WT and p38α+/− mice following intravenous injection of B16 cells (WT, 91.8 ± 21.0 cells/mouse; p38α+/−, 9.3 ± 3.2 cells/mouse). Also in the case of LLC cell injection, the formation of pulmonary metastatic foci was significantly decreased in p38α+/− mice compared with WT mice (WT, 31.2 ± 5.4 cells/mouse; p38α+/−, 11.2 ± 4.3 cells/mouse). To explore in greater detail whether the significant reduction in surface pulmonary metastases in p38α+/− mice resulted from tumor growth, we established B16 cells stably expressing β-galactosidase (B16-β-gal) in order to visualize a small focus of B16 cells. By using B16-β-gal, we investigated the formation of pulmonary metastatic foci at an earlier time after injection of tumor cells. Also in this case, a significant reduction in pulmonary metastasis was observed in p38α+/− mice (Fig. 1C). The size of metastatic foci dyed blue was comparable between WT and p38α+/− mice, indicating that tumor growth is not impaired in p38α+/− mice (data not shown). To further clarify this notion, we investigated the time-dependent increase in tumor volume after subcutaneous tumor cell transplantation. Both B16 and LLC cells when injected subcutaneously formed tumors with 100% penetrance, in which the steady growth of tumors showed no difference between WT and p38α+/− mice (Fig. 2). These results suggest that host p38α affects the metastatic potential of tumor cells but neither tumor cell growth nor tumor rejection.

Angiogenesis is well known to be an important process in tumor cell growth (24, 25). The microenvironment of the local host tissue appears to be an active participant in exchanging cytokines and enzymes with tumor cells that modify the local extracellular matrix, stimulate migration, and promote tumor angiogenesis, proliferation, and survival. It has been demonstrated that p38 plays a role in angiogenesis via regulating the production of inflammatory mediators (26). Likewise, targeted disruption of the p38α gene results in homozygous embryonic lethality because of defects in placental organogenesis, in which
angio genesis plays a crucial role (5). Thus, the reduction of p38 intrinsic activity in the local host tissue by a single copy disruption of the p38α gene is thought to cause some impairment of angiogenesis. However, the comparable tumor growth between WT and p38α−/− mice suggests that angiogenesis during tumor growth may not show impairment in p38α−/− mice in the present experimental model.

Reduction of Tumor Cell-Platelet Interaction in p38α−/− Mice—Tumor cells are frequently observed in the vasculature in complexes with platelets, and this association, together with the hypercoagulable state of malignant disease, appears to be essential for successful metastasis. The ability of tumor cells to induce platelet aggregation is widespread among cancers, including colon adenocarcinoma (27), lung carcinoma (28), melanomas (29), and others. The ability of tumor cells to induce platelet aggregation is of benefit for the survival of tumor cells for the following reasons: tumor cells coated with platelets acquire the ability to evade the body’s immune system and blood flow’s high shear forces; larger tumor-platelet aggregate tends to embolize in the microvasculature; and tumor cells efficiently receive a number of growth factors from platelets (30). Thus, platelets possibly act to facilitate all of the intermediate steps of transvascular metastasis, including tumor cell retention and arrest, subendothelial interaction, and extravasation from the microvasculature. We recently demonstrated that the binding activity of activated platelets from WT mice is suppressed compared with the case of platelets from WT mice (9). This clearly indicates that the function of platelets is attenuated in p38α−/− mice compared with WT mice. Then we investigated whether p38α mediates the interaction of platelets with tumor cells in vitro and in vivo.

We labeled platelets freshly isolated from WT and p38α−/− mice with calcein and applied them to B16 and LLC cells in vitro. We used HEK293T cells as a negative control and confirmed that resting and activated platelets from WT mice did not show the activity of rosetting on the cells. As shown in Fig. 3A, resting and activated platelets from WT mice rosetted on B16 and LLC cells, and this interaction was clearly reduced in the case of p38α−/− mice. Heparin markedly inhibited the

FIGURE 1. Single copy disruption of the p38α gene reduced the metastatic potential of circulating tumor cells. A, representative examples of metastatic pulmonary foci produced 28 and 21 days after intravenous injection of B16 and LLC cells, respectively, in WT and p38α−/− littermates. B, numbers of B16 (left) and LLC (right) foci found in the lungs of WT and p38α−/− littermates. Data are mean ± S.D. (WT, n = 6; p38α−/−, n = 6). * significantly different from metastatic pulmonary foci in WT mice (p < 0.05, B16, Welch’s t test; LLC, Student’s t test). C, histochemical determination of metastatic pulmonary foci of B16-β-gal in WT and p38α−/− littermates. Welch’s t test was used for statistical analysis.

FIGURE 2. Time-dependent tumor growth of subcutaneously transplanted tumor cells. A, tumor volume after subcutaneous injection of B16 cells in WT and p38α−/− littermates (open circles, WT; closed circles, p38α−/− littermates). B, tumor volume after subcutaneous injection of LLC cells in WT and p38α−/− littermates (open circles, WT; closed circles, p38α−/− littermates). Data are mean (n = 6). No significant difference in tumor growth of both B16 and LLC cells in WT and p38α−/− littermates was found by Student’s t test.
Involvement of p38α in Expression of P- and E-selectins—It is well defined that the specific interaction of P-selectin on platelets with mucin-type glycoproteins on tumor cells regulates tumor progression and metastasis via microemboli formation and is heparin-sensitive (31, 32). It has also been clarified that endothelial P-selectin expression contributes to the development of hematogenous metastasis (33). In P-selectin-deficient mice, the interaction of platelets with injected tumor cells in the lung was rarely observed (34), which notably shows an obvious similarity to our present data (Fig. 4). Moreover, the reduction of heparin-sensitive tumor cell-platelet interaction in p38α+/− mice (Fig. 3) suggests that low expression of P-selectin could be responsible for the reduced metastatic potential of tumor cells in p38α+/− mice. On the other hand, E-selectin expressed on endothelial cells can drive the seeding of tumor cells into metastatic sites by recognizing both mucin and nonmucin ligands on tumor cells (32, 35). Likewise, rapid induction of P- and E-selectin expression in the target organ was observed in tumor-bearing mice and might affect the metastatic potential of tumor cells (36, 37). Then we investigated the expression of P- and E-selectin mRNA in the lung before and after tumor cell injection. As shown in Fig. 5, the expression

interaction of platelets from WT and p38α+/− mice with tumor cells. Quantitative analysis showed that resting and activated platelets from p38α+/− mice had significantly decreased activity of rosetting on tumor cells compared with those from WT mice. The stimulation of platelets from WT mice with thrombin and collagen increased the number of platelets rosetted on tumor cells. This increase was moderate in the case of p38α+/− mice (WT, 1.8 times; p38α+/−, 1.5 times), indicating that agonist-induced activation is reduced in platelets from p38α+/− mice. The tumor cell-platelet interaction was significantly heparin-sensitive (Fig. 3B). To examine the tumor cell-platelet interaction in vivo, we injected calcein-labeled B16 cells, and those that lodged in the capillaries of the lung were further stained with anti-CD41, a defined platelet marker. As shown in Fig. 4A, platelets almost covered B16 cells in the lung of WT mice. In contrast, platelets weakly and partly covered B16 cells in the lung of p38α+/− mice. Quantitative analysis of the ratio of red fluorescence (CD41-like immunoreactivity) versus green fluorescence (B16) showed that the interaction of B16 cells with platelets in the lung was significantly decreased in p38α+/− mice compared with WT mice (Fig. 4B). These results suggest that suppression of the interaction of platelets with tumor cells can affect the metastatic potential of tumor cells in p38α+/− mice.

**FIGURE 3. In vitro rosetting of platelets with tumor cells. A, adhesion of calcein-labeled platelets to tumor cells. Platelets freshly prepared from WT and p38α+/− littermates were labeled with calcein and applied to B16 (first and second columns) and LLC cells (third and fourth columns). Activation of platelets was performed by the stimulation with 1 unit/ml thrombin and 20 μg/ml collagen. For determination of the effect of heparin on adhesion of platelets to tumor cells, 20 units/ml heparin was used. Heparin (second and fourth rows) and thrombin plus collagen (third and fourth rows) were applied to tumor cells just after the application of platelets to tumor cells. Nuclei of tumor cells were stained with 4',6-diamidino-2-phenylindole. Bar, 20 μm. B, quantitative analysis of platelets/tumor cells adhesion. Calcein-labeled platelets adherent to tumor cells were counted in high power fields at ×630 and expressed as platelets/single tumor cell. Data are mean ± S.E. (n = 6), p < 0.05 and p < 0.01 versus WT platelets. #, significantly different from platelets without heparin under corresponding experimental conditions (p < 0.05, Welch's t test). n.s., not significant from platelets without heparin under corresponding experimental conditions.
of P- and E-selectin mRNA was markedly induced in the lung of B16-bearing WT mice. This induction was significantly suppressed in the lung of B16-bearing p38α−/− mice, indicating that the metastatic tumor cell-induced expression of P- and E-selectin mRNA could be mediated by p38α activity. Although low resting expression levels of P- and E-selectin mRNA were observed in the lung of WT mice, those in p38α−/− mice were below the detection limits in our experimental conditions. This suggests that p38α may also be involved in the regulation of basal P- and E-selectin mRNA, probably affecting spontaneous expression of their proteins on the cell surface. To elucidate this notion and further confirm the effect of p38 signal on P- and E-selectins expression, Western blot analysis by using lung endothelial cells and platelets from WT and p38α−/− mice was performed. As shown in Fig. 6A, the expression level of p38 in lung endothelial cells from p38α−/− mice was lower than that from WT mice. In accordance with this fact, basal activation of p38 and ATF-2, a specific substrate for p38, and expression of E-selectin in lung endothelial cells from p38α−/− mice were lower than those from WT mice. The expression levels of PECAM-1 and α-catenin as internal controls were comparable between WT and p38α−/− mice. The expression of P-selectin in lung endothelial cells was below the detection limits in our experiments.

**Figure 4.** In vivo interaction of platelets with tumor cells. A, typical examples of platelet clumps attached on tumor cells in the lung. WT (A–C) and p38α−/− littermates (D–F) were injected with calcein-labeled B16 cells. Lung sections were stained with monoclonal anti-mouse CD41 antibody in combination with Alexa Fluor 594-labeled secondary antibody, and B16 (A and D) and attached platelets (B and E) were determined as green fluorescence and red fluorescence, respectively, under a fluorescence microscope. Merged profiles (C and F) show the decrease of platelets attached on tumor cells in p38α−/− mice. Bar, 20 μm. B, quantitative analysis of platelet/tumor cell adhesion in the lung of WT and p38α−/− littermates. The interaction of platelets with B16 in the lung was expressed as the ratio of fluorescent intensity (red/green). Data are mean ± S.D. (n = 5). *, significantly different from WT mice (Student’s t test).

**Figure 5.** Effect of tumor cell injection on P- and E-selectin mRNA expression in lung. Total RNA was prepared from the lung of WT and p38α−/− littermates injected with or without B16 cells. PCR product samples were subjected to 1.2% agarose gel electrophoresis and visualized by staining with ethidium bromide. Similar results were confirmed in three independent experiments.

**Figure 6.** Effect of p38 signal on resting expression of E- and P-selectins in lung endothelial cells and platelets. A, endothelial cells were freshly isolated from the lung of WT and p38α−/− littermates and maintained for 2 days. Then the cell lysate was prepared, and 7.5 μg of lysate was subjected to each Western blot analysis. B, platelets were freshly prepared from WT and p38α−/− littermates. The cell lysate (15 μg) and total RNA were prepared and subjected to each Western blot analysis and reverse transcription-PCR for P-selectin mRNA, respectively. Similar results were confirmed in three independent experiments.
experimental conditions (data not shown). On the other hand, the expression of p38 and basal activation of p38 and ATF-2 were reduced in platelets from p38α+/− mice compared with those from WT mice, which showed a good parallelism to the case with lung endothelial cells. In accordance with this, the expression of P-selectin in platelets from p38α+/− mice was lower than that from WT mice. Likewise, the expression of P-selectin mRNA in platelets was detected, and its level in platelets from p38α+/− mice was lower than that from WT mice. The expression level of CD41 as internal control was comparable between WT and p38α+/− mice (Fig. 6B). The reduction in expression of E-selectin in endothelial cells from p38α+/− mice was not restricted in lung. As shown in Fig. 7, the expression level of E-selectin on liver endothelial cells from p38α+/− mice was significantly lower than that in WT mice. In the case of P-selectin, its expression was weakly detected and also reduced on liver endothelial cells from p38α+/− mice compared with that from WT mice.

It has been demonstrated that p38 regulates the expression of E-selectin in vitro (38, 39) and that high expression of both p38α and β was found in endothelial cells (40), suggesting the possibility that p38 isoforms cooperatively play a role in the regulation of E-selectin. At least in vivo, however, disruption of a single copy of the p38α gene may be sufficient to affect E-selectin mRNA expression in endothelial cells in a resting state as well as in an activated state. Moreover, also in vitro, p38α heterogeneity affects the expression of E-selectin in endothelial cells in a resting state. As a result, the decrease in expression of E-selectin could be a rate-limiting factor for tumor cell adhesion to the endothelium in p38α+/− mice. Indeed, the number of B16 cells lodged in the capillaries of the lung was decreased in p38α+/− mice compared with WT mice (WT, 18.7 ± 10 colonies/0.16 mm²; p38α+/−, 5.0 ± 2.4 colonies/0.16 mm²; 50 slides of lung sections prepared from calcine-labeled B16-bearing mice were examined). On the other hand, it was reported that tumor necrosis factor-α and lipopolysaccharide, which are known to be p38 activators, induce expression of the murine P-selectin gene in endothelial cells (41). Furthermore, in mice lacking ATF-2, a specific substrate for p38, lipopolysaccharide-induced P-selectin mRNA expression in the lung was markedly attenuated (42). These reports give reliability to our present data that the resting expression of P-selectin reduced in platelets from p38α+/− mice correlates with the level of phosphorylated ATF-2. However, further study is needed to elucidate whether ATF-2 directly activates the P-selectin promoter. The question arises of whether the induction of P-selectin mRNA by metastatic tumor cells is restricted to endothelial cells. Circulating platelets do not have nuclei, and P-selectin constitutively stored in the α granules of platelets can be functionally expressed on the membrane surface by the rapid redistribution in response to stimuli (43). However, circulating platelets retain mRNA for certain molecules from megakaryocyte (44). In fact, P-selectin mRNA was detected, and its level in platelets from p38α+/− mice was lower than that from WT mice. Thus, the possibility that P-selectin mRNA derived from platelets interacting with tumor cells may accumulate in accordance with the number of tumor cells lodged in the capillaries of the lung is not ruled out.

**Reduction of Tumor Cell-Lung Endothelial Cell Interaction in p38α+/− Mice**—Our present data suggest that the reduction of E- and P-selectins expression in endothelial cells and platelets, respectively, may lead the low metastatic potential of tumor cells for lung in p38α+/− mice. To further elucidate this notion, attachment and transmigration of Dil-labeled B16 for lung endothelial cells in the absence and presence of platelets were investigated. As shown in Fig. 8A, attachment of B16 cells on endothelial cells from WT mice was increased by the addition of platelets from WT mice, and B16 cells formed large colonies (Fig. 8B). These profiles were not typical in case of using endothelial cells and platelets from p38α+/− mice (Fig. 8D). Quantitative analysis showed that the numbers of B16 cells attached on endothelial cells with or without platelets from p38α+/− mice were significantly decreased compared with those from WT mice, respectively. The application of platelets from WT mice significantly increased the attaching activity of B16 cells on endothelial cells from WT mice. This increase was significant but slight in the case of p38α+/− mice (Fig. 8B). These results clearly support the fact that the number of B16 cells lodged in the capillaries of the lung was decreased in p38α+/− mice compared with WT mice. As shown in Fig. 8C, the transmigration of B16 cells was comparable over all experimental conditions, indicating that a barrier function of lung endothelial cells from p38α+/− mice is similar to that from WT mice. Transendothelial migration of tumor cells is a crucial step for cancer metastasis (45). However, these results suggest that the resistance of p38α+/− mice to experimental lung metastasis of tumor cells does not result from the change in barrier function of lung endothelial cells in p38α+/− mice.

In conclusion, the present study demonstrated that p38α plays an important role in a step of tumor cell metastasis, pos-
sibly through regulating the formation of tumor-platelet aggregates and their interaction with the endothelium accompanied by selectin expression.

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