SUCCESSFUL INVASION and metastasis of tumor cells is a complex, multistep process. The malignant cell must evade the host immune response and produce proteases that enable it to degrade extracellular matrix and invade local blood vessels. A variety of proteolytic enzymes may be involved in tumor cell invasion and metastasis, such as those in the plasminogen activation/plasmin system and the matrix metalloproteinases. Urokinase (uPA), a plasminogen activator that converts the proenzyme plasminogen to its more active two chain form, was shown to increase reactive serine protease, plasmin, is expressed at high levels in the plasminogen activator (tPA) is the major physiologic regulator of plasminogen activator activity. To test the role of host PAI-1 in the invasive and metastatic capacity of B16 melanoma cells we analyzed local tumor growth and pulmonary metastases in the same model. 

Plasminogen activator inhibitor-1 (PAI-1) is a rapidly inhibitory of both uPA and tissue-type plasminogen activator (tPA) is a rapid inhibitor of both uPA and tissue-type plasminogen activator and is considered a critical element for the invasive phenotype of metastasizing cells. The B16 melanoma cell line has been widely used as a model for the invasive and metastatic capacity of these murine melanoma cells in vivo. Plasminogen activator inhibitor-1 (PAI-1), a rapid inhibitor of both uPA and tissue-type plasminogen activator (tPA) is a rapid inhibitor of both uPA and tissue-type plasminogen activator. To test the role of host PAI-1 in the invasive and metastatic capacity of B16 melanoma cells we analyzed local tumor growth and pulmonary metastases in the same model.

Plasminogen activator inhibitor-1 (PAI-1) is a 50-kD member of the serine protease inhibitor (serpin) family that constitutes the major inhibitor of plasminogen activators in plasma. PAI-1 rapidly inhibits both uPA and tPA by forming 1:1 protease-inhibitor complexes that are enzymatically inactive, and plays a major role in the regulation of plasmin activity. Complete deficiency of PAI-1 in humans results in abnormal bleeding and mice that are deficient in PAI-1 display enhanced fibrinolytic activity. Conversely, elevated levels of PAI-1 are associated with thrombotic events. PAI-1 also inhibits uPA bound to the cellular uPA receptor and studies using murine melanoma cells that overexpress PAI-1 have demonstrated marked attenuation of matrix degradation, presumably by inhibition of surface uPA activity. Furthermore, transgenic mice that overexpress human PAI-1 have been reported to experience significantly fewer spontaneous lung metastases in a Lewis lung carcinoma model, and mice injected with human melanoma cells that overexpress PAI-2 also experience fewer spontaneous lung metastases. Most human clinical studies, however, have shown a correlation of tumor-associated PAI-1 with a poor prognosis. To further examine the role of host PAI-1 in tumor invasion and metastasis, we analyzed genetically altered mice that overexpress murine PAI-1 and mice deficient in PAI-1 in two metastatic models using the B16 melanoma cell line. Our results show that host PAI-1 status does not affect primary tumor size, metastatic potential, or survival in this murine model system.

MATERIALS AND METHODS

Mice. Female, 6- to 8-week-old mice were maintained in an approved University of Michigan animal housing unit. PAI-1 deficient mice, generated by homologous recombination as previously described, were a gift of P. Carmeliet and D. Collen. Transgenic mice overexpressing a murine PAI-1 minigene under the direction of the CMV promoter were previously generated (manuscript in preparation; T. Shen, D. Ginsburg). Plasma levels of PAI-1 antigen measured by ELISA in mice carrying the PAI-1 transgene were 108 ± 17 ng/mL versus 52.0 ± 0.3 ng/mL in littermate controls. Plasma levels of active PAI-1 measured by a tPA binding assay were 59 ± 12 versus 0.9 ± 0.3, respectively (manuscript in preparation; T. Shen, D. Ginsburg). PAI-1 levels in lung homogenates of transgenic mice were 1,448 ± 320 ng/g wet tissue compared with 376 ± 38 ng/g in littermate controls. For the mice overexpressing PAI-1, mice carrying the PAI-1 transgene were used in the experimental group, whereas littermates not carrying the transgene were used as controls. All mice used were the first generation offspring of the PAI-1 transgenic founder animal (C57BL/6 × SJL/J hybrid) and a C57-
BL6/I mate. The presence of the PAI-1 transgene in individual mice was determined using polymerase chain reaction analysis of tail DNA specimens obtained at 3 weeks of age. Primers designed to anneal to base pairs 1263-1286 (5') and 1473-1496 (3') (across intron H) of the PAI-1 cDNA sequence were used to recognize the PAI-1 sequence. Two products of different sizes were generated in the presence of transgene—a 496 base pair product from the RAI-1 heterozygotes (PAI-1 polymeric chain reaction using primers designed to anneal to base pair 1007-1031 (5') and 1503-1532 (3') of the neomycin resistance gene were used to recognize the PAI-1 null allele; the primers described above were used to amplify the native PAI-1 allele. Thus, PAI-1-deficient mice (PAI-1-) only display an amplified portion of the neomycin resistance gene (520 base pairs), wild-type mice (PAI-1-) only display an amplified portion of the native PAI-1 allele (496 base pairs), and heterozygotes (PAI-1+) yield both PCR products.

Melanoma cell line. The B16-BL6 melanoma, a tumor of spontaneous origin that has been extensively characterized, was a gift of Dr Suyu Shu. This tumor is derived from a C57BL6/I mouse line of spontaneous origin that has been extensively characterized, was a gift of Dr Suyu Shu. This tumor is derived from a C57BL6/I mouse line. A9 clone was produced and maintained as previously described. For the pulmonary metastasis analysis, 1007-1031 (5') and 1503-1532 (3') of the neomycin resistance gene (520 base pairs), wild-type mice (PAI-1-) only display an amplified portion of the native PAI-1 allele (496 base pairs), and heterozygotes (PAI-1+) yield both PCR products.

Tumorigenic assays. B16 melanoma cells were grown and harvested as previously described. For the pulmonary metastasis model, doses of melanoma cells varying from 1 × 10⁵ to 1 × 10⁶ cells were injected into the lateral tail vein in 0.5 mL Hanks’ balanced salt solution (HBSS). On day 14 following injection, mice were killed; their lungs were infused with Fekete’s Solution and removed. Surface pulmonary nodules were counted manually, with the examiner blinded to the animal’s genetic status. For survival analysis, 1 × 10⁶ melanoma cells were injected in the right foot pad and tumor size was assessed each week by measuring the widest tumor dimension with calipers. On day 21, a hip disarticulation was performed under ketamine anesthesia, as previously described. Animals were then followed for survival. Mice that died within 36 hours of hip disarticulation were excluded from the analysis.

Statistical analysis. The statistical significance of differences between groups was determined by the Wilcoxon rank-sum test or Student’s t-test. Two-sided P values of <.05 were considered statistically significant.

RESULTS

Effect of PAI-1 status on primary tumor size. To determine if establishment and growth of a primary tumor focus is affected by PAI-1 status, mean tumor size ± SEM was calculated for each of the four groups at day 21 following foot pad injection. No significant differences were observed (PAI-1 control mice = 0.83 ± .1 cm v PAI-1-deficient mice = 0.75 ± 0.1 cm (P > .1); PAI-1 littermate control mice = 0.9 ± 0.01 cm v PAI-1 overexpressers = 0.78 ± 0.01 cm (P > .1). In a separate experiment, primary foot pad tumor size was measured at earlier time points (day number 10 and 17) to determine rate of growth. At day 10 (PAI-1 control mice = 0.25 ± .03 cm v PAI-1-deficient mice = 0.28 ± 0.05 cm, P > 0.1; PAI-1 littermate control mice = 0.3 ± 0.01 cm v PAI-1 overexpressers = 0.25 ± 0.3 cm, P > .1). At day 17 (PAI-1 control mice = 0.65 ± 0.06 cm v PAI-1-deficient mice = 0.68 ± 0.08 cm, P > .1; PAI-1 littermate control mice = 0.58 ± 0.05 cm v PAI-1 overexpressers = 0.50 ± 0.08 cm, P > .1, Fig 1).

Effect of PAI-1 status on pulmonary metastases and survival. To determine the effect of PAI-1 status on pulmonary metastases following intravenous injection of melanoma cells, surface pulmonary nodules were counted 14 days following injection. The number of pulmonary nodules observed did not differ significantly between wild-type (mean ± SEM = 167 ± 8) and PAI-1 overexpressers (mean ± SEM = 170 ± 35, P > .1, Fig 2A). For the PAI-1-deficient mice and controls, three different doses of melanoma cells were used to evaluate the effect of varying tumor burdens. The number of lung nodules were nearly identical between the PAI-1-deficient mice and their controls at each dose tested (1 × 10⁵ cells = 50 v 65 nodules; 2 × 10⁵ cells = 110 v 120 nodules, and 4 × 10⁵ cells = 190 v 180 nodules, Fig 2B). In addition, no significant difference in lung nodule size was observed between the different groups of mice. Survival following the removal of a primary foot pad tumor was assessed in each group. No significant difference in mean survival days was observed (PAI-1-deficient mice = 32 ± 3 v PAI-1 control mice = 29 ± 2; P > .1, PAI-1 overexpressing mice = 32 ± 4 v PAI-1 control mice = 30 ± 2; P > .1) Survival curves were also similar between the
Fig 2. Effect of PAI-1 status on pulmonary metastases. (A) PAI-1 overexpression. Littermate control mice without transgene (control, n = 4) and mice carrying the PAI-1 transgene (PAI-1 TG+, n = 4) underwent tail vein injection with 3 x 10⁵ melanoma cells in 0.5 mL buffer solution. Fourteen days later, mice were killed and their lungs were retrieved for enumeration of pulmonary nodules. No significant differences were observed in the number of nodules (P = NS). (B) PAI-1 deficiency. Mice deficient in PAI-1 (PAI-1 -/-, n = 3) and control mice (PAI-1 +/+, n = 3) with both PAI-1 alleles present underwent tail vein injection with varying doses of melanoma cells (1 to 4 x 10⁵ cells) in 0.5 mL HBSS followed by enumeration of pulmonary nodules at 14 days following injection. ●, PAI-1 deficient mice; ○, control mice.

One mouse in the PAI-1-deficient group and 1 mouse in the PAI-1 control group died in the perioperative period following hip disarticulation. No early deaths were observed in the PAI-1 overexpressors or wild-type littermates.

DISCUSSION

Urokinase binds to the cellular receptor of a variety of monocyte and neoplastic cell lines and potentiates plasminogen activation in the presence of cell bound plasminogen. While cell-associated plasmin activity is protected from inhibition by α-2-antiplasmin, regulation of cellular plasmin activity could occur at the level of plasminogen activation, as PAI-1, the most efficient plasminogen activator inhibitor known, is capable of regulating cell-surface proteolytic activity. Therefore, it has been postulated that pericellular proteolysis, a required activity for invasion and metastasis of tumor cells, is mediated by cell-bound uPA⁴ and regulated by PAI-1. This hypothesis is supported by previous B16 melanoma studies that have demonstrated enhancement or attenuation of invasive and metastatic properties by modulating cell surface uPA activity.⁴

Studies with various human malignancies have frequently demonstrated the presence of tumor-associated PAI-1. However, although in vitro data suggest that the expression of PAI-1 should limit the invasive potential of tumor cells by blocking uPA activity, most recent clinical studies have shown a correlation of tumor associated PAI-1 expression with a more invasive phenotype and worse prognosis.²¹⁻²⁵,³¹⁻³³ One potential explanation for this paradox could be marked increases in expression of uPA coincident with the observed elevation in PAI-1 resulting in a net excess of proteolytic activity, perhaps focused at particular sites of cell/matrix contact. Indeed, tumor expression of uPA is also correlated with a poor prognosis. Alternatively, PAI-1 may potentiate tumor cell migration by promoting uPA turnover from its receptor, with subsequent attachment of the uPA receptor to the extracellular matrix protein, vitronectin.²⁵ Finally, PAI-1 expression by some tumor types may simply represent an indirect marker of malignant transformation, with no direct role in tumor invasion or metastasis.
PAL-1 IN MURINE METASTATIC MELANOMA

In contrast to these previous studies, our results suggest that the PAI-1/plasminogen activator balance may not significantly influence the tumorigenicity or metastatic potential of melanoma cells in this well-established murine model. However, a major difference between the current and previous studies of neoplastic cells and the plasmin/plasminogen proteolytic system is the source of the modulating factor. Prior studies have generally manipulated the tumor cells directly, whereas we chose to alter the genetic background of the host, eliminating the potential contribution of tumor cell clonal variation. Future experiments using tumors derived from PAI-1 transgenic mice should aid in dissecting the role of tumor versus host PAI-1.

Although our results suggest that prior reports may overestimate the general role of the plasminogen activation system in tumor invasion and metastasis, application of these results to other model systems should be taken with caution. Considerable heterogeneity is likely to exist among different neoplastic cell lines and among mice with various genetic backgrounds. In addition, we cannot exclude small differences in tumor growth or metastases that might be revealed by a larger sample size nor can we exclude a critical role for PAI-1 in other tumor models. Of particular note, a preliminary report of Lewis lung carcinoma cells injected into mice overexpressing human PAI-1 showed attenuation of lung metastasis.19

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Lack of plasminogen activator inhibitor-1 effect in a transgenic mouse model of metastatic melanoma

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