

Plasminogen Activator Inhibitor-1 Accelerates Lung Metastasis Formation of Human Fibrosarcoma Cells*

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Abstract. We have studied the effects of PAI-1 on the formation of metastasis using human fibrosarcoma cells and DNA transfection. A clone (1-3C), which shows low constitutive expression of PAI-1 and low metastatic potential to the lung, was selected from human fibrosarcoma cell line HT-1080. Newly-derived clones transfected with human PAI-1 cDNA showed a 3-5 fold increase in the antigen level of PAI-1. Further, these clones demonstrated a significant increase in both the number and incidence of lung metastases when inoculated into the tail vein of athymic mice. PAI-1 could be a prognostic marker and a target for understanding the physiology of metastasis, as well as for the treatment and prevention of hematogenous lung metastasis.

There are several important phases in the formation of metastases: shedding of tumor cells from the primary lesion, migration of tumor cells to distant sites, and their attachment and growth at the metastatic sites. Essentially, augmentation of any phase enhances metastatic formation. In the relationship between metastasis and fibrinolysis, the serine protease urokinase-type plasminogen activator (uPA) plays a key role in tumor associated proteolysis resulting in the invasion and dissemination of tumor cells (1). Recently there have been reports suggesting that the expression of plasminogen activator inhibitor-1 (PAI-1), which is an inhibitor of uPA and tissue-type PA (tPA), correlates with poor prognosis in gastric cancer (2,3) and breast cancer (4).

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These data may indicate a relationship between PAI-1 and metastasis. Although the role of PAI-1 in the formation of metastases has not been clarified, PAI-1 is expected to have a role in hematogenous metastasis to distant organs for some tumors by facilitating the lodging of tumor cells in vessels. We have reported a positive correlation between PAI-1 expression and pulmonary metastatic potential in human fibrosarcoma cells (HT-1080) (5,6). When *in vivo*-selected HT-1080 subpopulations selected by repeated passages through lung metastases were tested for their ability to form lung metastases, the number of pulmonary metastases increased with passage number. Their PAI-1 antigen levels revealed a correlation between the number of metastatic colonies and the PAI-1 expression of these cells. In contrast to PAI-1, tPA and uPA decreased in highly metastatic subpopulations. mRNA levels were also compatible with the changes in antigen levels. Furthermore, *in vitro*-selected high PAI-1 clones also exhibited a high metastatic potential. No significant difference was found among these subpopulations or clones with various metastatic ability in adhesion or invasion assays (5,6).

In this current study, we directly demonstrate that increased PAI-1 expression enhances the formation of pulmonary metastases in human fibrosarcoma utilizing transfection of PAI-1 cDNA.

Materials and Methods

Cloning and ELISA. A clone (1-3C) which shows low constitutive expression of PAI-1 and low metastatic potential to the lung was selected from the human fibrosarcoma cell line HT-1080 by the limiting dilution method (7) and subsequent screening for the level of PAI-1 antigen using an enzyme linked immunosorbent assay (ELISA). PAI-1 and uPA antigens in the conditioned media were determined using commercially available ELISAs based on monoclonal antibodies (Technoclones Inc., Vienna, Austria).

Transfection. PAI-1 cDNA (8) was isolated from the plasmid pUC-PAI-1 as an EcoRI-XbaI fragment and purified by agarose gel electrophoresis. pCDNA1neo was digested with EcoRI and XbaI, and the resulting 1237-

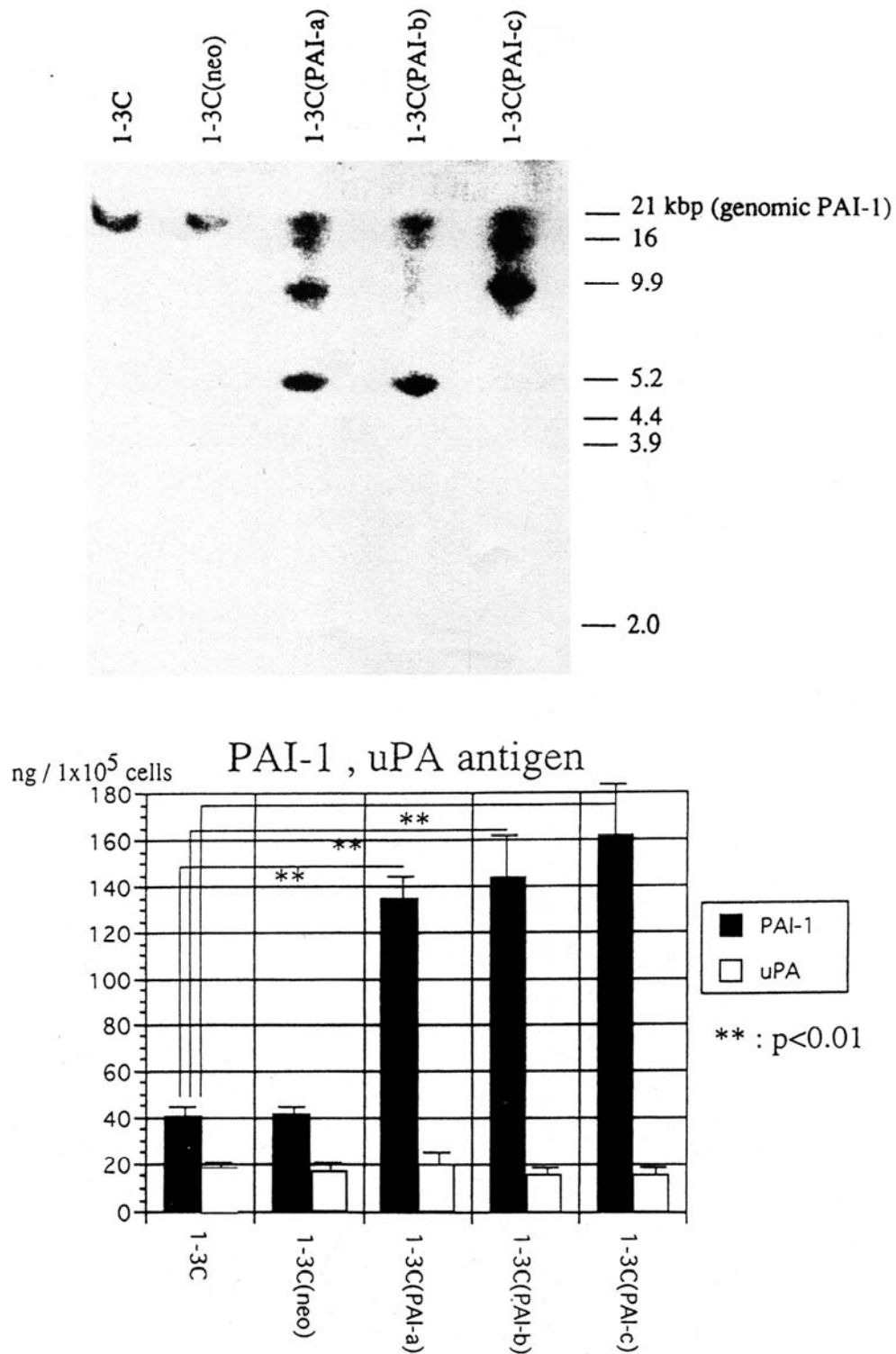


Figure 1. Expression of PAI-1 in human fibrosarcoma clones. A, Southern blot of each clone transfected with PAI-1 cDNA. Molecular size markers are shown on the right. The bands of genomic PAI-1 were detected at 21 kbp. B, Levels of PAI-1 and uPA antigen in the media conditioned by each clone. (**p<0.01, Mann-Whitney U-test)

Table I. Lung metastasis formation of human fibrosarcoma clones transfected with PAI-1.

| Clone | No. (mean±SD) | Incidence | Range |
|-------------|------------------------------------|-----------|-------|
| 1-3C | 1.7±4.2 | 1/7 | 0-12 |
| 1-3C(neo) | 0 | 0/7 | 0 |
| 1-3C(PAI-a) | 21.3 ± 11.6 (p=0.004) [#] | 7/7 | 8-43 |
| 1-3C(PAI-b) | 13.1 ± 10.5 (p=0.018) [#] | 6/7 | 0-27 |
| 1-3C(PAI-c) | 28.0±8.7 (p=0.0017) [#] | 7/7 | 18-46 |

[#] P-values from Mann-Whitney U-test in comparison with 1-3C. 1-3C(PAI-a,b,c) indicate individually transfected clones. The mean number is the mean of all the mice in the group.

bp and 5687-bp fragments were purified. The 1237-bp fragment, which contains the SV40 polyadenylation and RSV LTR regions, was ligated to PAI-1 cDNA using the XbaI site. pcDNAneo/PAI-1 plasmid was constructed by ligating the fused fragment with the dephosphorylated 5687-bp fragment from pcDNAneo. The pcDNAneo/PAI-1 plasmid used for the lipid-mediated DNA transfection (9) into 1-3C cells contained the cDNA insert in the sense orientation, and the configuration was confirmed by restriction mapping. Expression of transfected PAI-1 was detected by Southern blot analysis (10).

Lung metastasis assay. Athymic nude mice (BALB/c *nu/nu*, 4- to 6-week-old females) were injected *via* the lateral tail vein with 1×10^5 cells of clones exhibiting high expression of PAI-1 antigen. Three weeks after the injection the mice were killed, and the number of metastatic colonies was counted under a dissecting microscope after India ink staining and fixation with Fekete's solution (11). Seven mice were used for each experiment. Metastatic colonies on the lung surface of more than 50 μ m in diameter were counted.

Results

Human PAI-1 cDNA (a 1426-bp EcoRI-BglIII fragment of polymerase chain reaction amplified cDNA), containing the entire coding region, 75 nucleotides of the 5'-untranslated region, and 142 nucleotides downstream of the stop codon, was transfected into a human fibrosarcoma cell line HT-1080 clone (1-3C) selected for low expression of PAI-1 and low metastatic potential to the lung. In Southern blot analysis, the bands of genomic PAI-1 were seen at 21 kbp and the bands of transfected PAI-1 at various levels of bp (Figure 1A).

Antigen levels of PAI-1 and uPA in the conditioned media of each clone were measured by ELISA with monoclonal antibodies (Technoclone Inc., Vienna, Austria). A 3- to 4-fold increase in PAI-1 antigen was noted in the clones (1-3C(PAI-a,b,c)) transfected with PAI-1 cDNA compared to the parental clone, while the expression of uPA in these clones was not influenced (Figure 1B).

When the clones with high expression of PAI-1 antigen were analyzed for their ability to form lung metastases upon

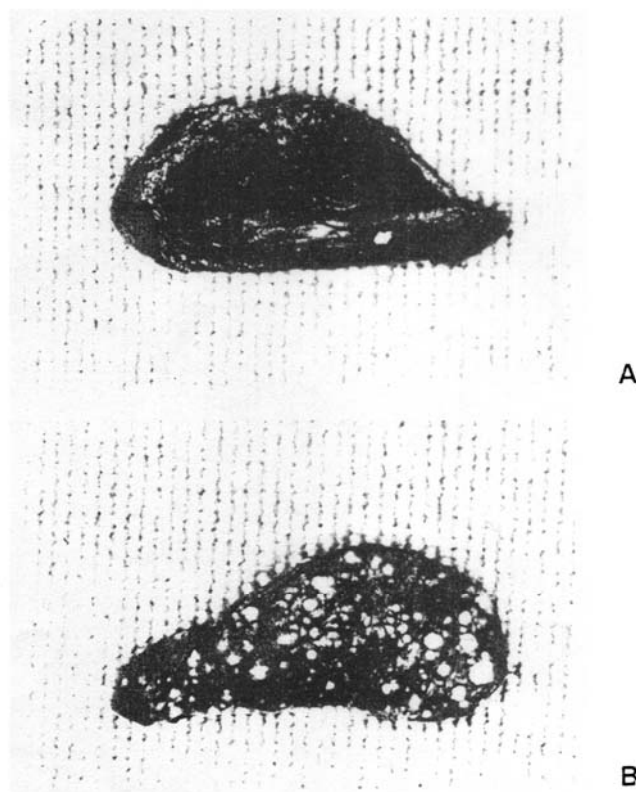


Figure 2. Photographs of lung metastases. Clone 1-3C formed lung metastases in one out of seven mice and the clone transfected with pcDNAneo alone formed no lung metastases (A). The 1-3C(PAI-a) clone transfected with PAI-1 cDNA showed the increase of lung metastases in the number and incidence compared with the parental clone 1-3C (B).

injection of tumor cells into the tail vein of BALB/c *nu/nu* athymic mice, the clones with high PAI-1 expression showed a significant increase in both the number and incidence of lung metastases (Table I, Figure 2). No metastases were detected in liver or bones. There was no difference in the growth rate seen when these clones and parental cell lines were injected subcutaneously into the backs of athymic mice. Tumor doubling time was 39.2 to 42.8 hours in the clones transfected with PAI-1 cDNA and 40.8 hours in the parental clone 1-3C.

Discussion

Little attention has been paid to the role of PAI-1 in the formation of metastases. PAI-1 is an inhibitor of uPA, which activates plasminogen to plasmin and indirectly promotes tumor cell invasion *via* the degradation of extracellular matrix proteins (1). However, ascites tumor cells with low fibrinolytic activity produced more metastatic nodules in the lung after intravenous injection compared to tumor cells with high fibrinolytic activity (12). Furthermore, while metasta-

sizing primary tumors showed greater fibrinolytic activity, the metastases themselves had lower activity (13) and in melanoma cell lines the production not only of uPA but also of PAI-1 correlated with the frequency of formation of spontaneous lung metastases after subcutaneous inoculation (14). These data are compatible with the view that fibrinolysis in the primary tumor is associated with tumor cell shedding, while low fibrinolytic activity and/or high antifibrinolytic activity in the metastases helps to retain the microthrombus formation necessary for tumor cell lodgement. The fact that only very few of the circulating cancer cells, and essentially those with a low fibrinolytic potential, are able to form metastases (15) would be consistent with the importance of PAI-1 for the formation of metastases.

We have found that PAI-1 accelerates the lung metastases of human fibrosarcoma in athymic mice. Possibly the promotion and maintenance of tumor cell lodgement is dependent on sustained inhibition of the degradation of previously formed fibrin. That is, while tPA secreted from endothelial cells generally acts to remove tumor emboli, PAI-1 produced by tumor cells may enhance lodgement in vessels, thereby promoting the establishment of metastases in the lung. Our system is artificial in that since we injected tumor cells directly into the bloodstream, the initial step with the need for invasion by a primary tumor was bypassed. Since it appears that the metastatic potential of a primary tumor is enhanced by a high fibrinolytic state, but lodgement is enhanced by a low fibrinolytic/high anti-fibrinolytic state, the next step might be to study what happens to the cells during migration to enable them to switch from one state to the other.

Historically, even when local control was achieved by surgery, more than 50% of patients with soft-tissue sarcoma and 80% of patients with osteosarcoma eventually developed distant metastases and died, usually within two years. In most high-grade bone and soft-tissue sarcomas, hematogenous micrometastasis in the lung which leads to death exists from an early stage of the disease (16-19). It is only by the elimination of hematogenously borne micrometastases that further improvements in survival will occur. Our data indicate that much more attention should be paid to PAI-1 as a prognostic marker and a target for understanding the physiology of metastasis, as well as for the treatment and prevention of hematogenous lung metastasis. Although we have preliminarily suppressed pulmonary metastases of human fibrosarcoma cells in athymic mice by the injection of mouse anti-human PAI-1 antibody (20), the introduction of anti-PAI-1 antibodies with greater specificity or an antisense PAI-1 gene could be useful in the treatment of some cancer metastases.

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