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Platelet Factor 4 Gene Transfection into Tumor Cells Inhibits Angiogenesis, Tumor Growth and Metastasis

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Abstract. *Tumor growth and metastasis depend on angiogenesis, which is triggered by a chemical signal from the tumor cells to resting endothelial cells which then enter into a phase of rapid growth. Platelet Factor 4 (PF4) inhibits endothelial proliferation in vitro and angiogenesis in vivo. PF4 also inhibits tumor growth, however, as with other angiogenesis inhibitors, sustained tumor growth inhibition requires prolonged exposure to the recombinant protein. In this study, Lewis lung carcinoma (LLH) cells were transfected with the human PF4 via mammalian expression vectors and the ability of the transfected cells to form tumors and metastasis in vivo was evaluated. To evaluate the tumor growth rate of PF4-transfected (LLH/PF4) or control (LLH/neo) cells in vivo, we injected LLH/PF4 or LLH/neo cells subcutaneously (s.c.) or intravenously (i.v.). In the s.c. assay, LLH/PF4 had no significant effect on tumor growth. Conversely, in the i.v. assay, PF4 significantly reduced the number of lung metastasis (p=0.019) and weight (p=0.056). The inhibition of lung metastasis suggests that PF4 may inhibit tumor-associated neovascularization, and may prevent the affinity of tumor cells for the normal lung tissue.*

The regulation of angiogenesis is fundamental to a variety of physiological and pathological processes, including inflammation and tumorgenicity. Although a number of factors are involved in neovascularization, it is becoming increasingly apparent that endogenous angiostatic factors may play an important role in the regulation of angiogenesis during wound repair, chronic inflammation and growth of solid tumors. Tumor growth and metastasis depend on angiogenesis, which is initiated by a chemical signal from the tumor cells to resting endothelial cells which then enter into a phase of rapid growth (1). Platelet factor 4 (PF-4), a member of the CXC subgroup of the chemokine family, is released in high concentrations from activated platelets (2). In addition, chemokines are involved in hematopoiesis, cell proliferation, angiogenesis and glycosaminoglycan binding (3-6). Members of the chemokine gene superfamily of cytokines share homologous sequences and a highly conserved cysteine motif. Chemokines can be divided into four types, depending on whether the first two cysteines are separated (CXC) or not (CC) by an intervening amino acid (7), whether the second cysteine is missing (C), or whether the first pair of cysteines are separated by three intervening amino acids (CXXXC). Interleukin-8 (IL-8) has been found in various human inflammatory diseases. It is a member of the CXC chemokines, and has been found to be an angiogenic factor (8,9). In contrast, PF4, another CXC chemokine, has been shown to have anti-angiogenic properties (10). It is interesting that the major structural difference between IL-8 and PF4 is the absence in PF4 of the NH_2-terminal ELR (Glu-Leu-Arg) motif that precedes the first cysteine amino acid residue in IL-8. Due to this difference, PF4 does not share certain pro-inflammatory properties of other CXC family members (11).

A number of observations have indicated that PF4 is an inhibitor of angiogenesis. First, PF4 inhibits endothelial cell proliferation, migration, and angiogenesis *in vitro* and *in vivo* (12-14). Second, PF4 is found *in vivo* in endothelial cells that undergo active angiogenesis (15). Third, tumor growth *in vivo* is inhibited by PF4 through an angiogenesis-dependent mechanism. Recombinant human PF4 inhibits tumor angiogenesis and the growth of melanoma cells or HCT 116 colon carcinoma cells (16). In addition, human glioma cells transfected with PF4 cDNA grew slowly and formed only hypovascular tumors *in vivo* (17).

The mechanism of PF4 action is incompletely understood and controversial. Sato *et al.* (18) have reported that PF4

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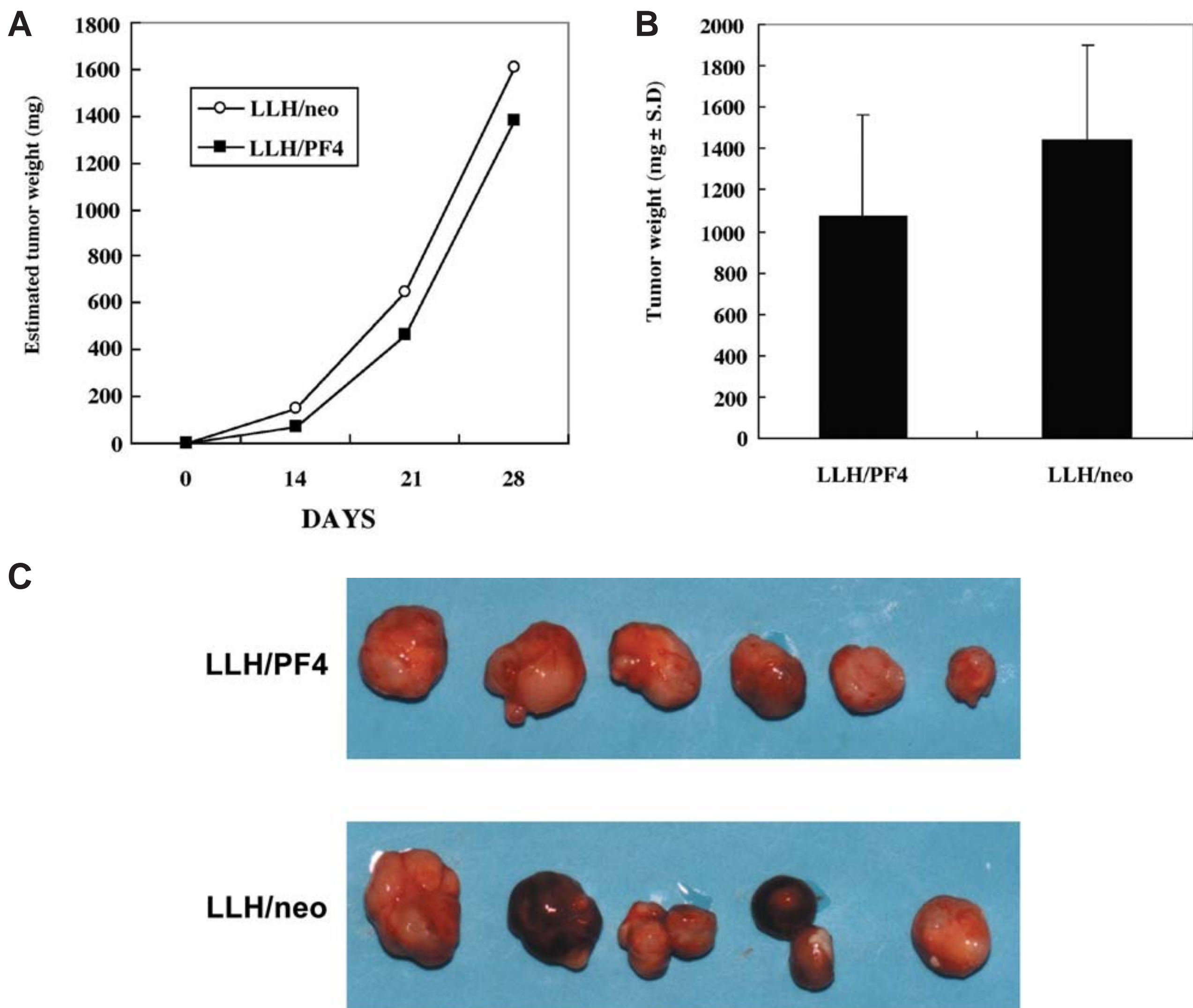

Figure 1. *A. Comparison of the estimated tumor weight of PF4-transfected LLH clones (LLH/PF4) and control (LLH/neo). B. The final tumor weight (mg±S.D) as assayed at 28 days after transplantation. The mean tumor weight in LLH/PF4 and LLH/neo were 1073.8±488.1mg and 1438.9±463.2mg, respectively (N.S). C. Gross anatomy of subcutaneous tumors from different treatment groups including LLH/PF4 (upper) and LLH/neo (lower) at 28 days after transplantation.*

inhibits the binding of the basic fibroblast growth factor (bFGF) to low-affinity binding sites and high-affinity receptors of NIH3T3 fibroblasts. Furthermore, Gengriowitch *et al.* (13) reported that vascular endothelial growth factor (VEGF) binding to endothelial cell VEGF receptors was inhibited by PF4. Finally, Gupta and Singh (10) showed that PF4 intervenes at a specific point in the cell cycle by blocking the progression of endothelial cells in S-phase.

In this study, Lewis lung carcinoma (LLH) cells were transfected with the human PF4 gene *via* mammalian expression vectors, and the ability of the transfected cells (LLH/PF4) to form tumors and metastasis *in vivo* was evaluated.

Materials and Methods

Expression plasmids. PF4 cDNA was isolated by the polymerase chain reaction (PCR) method using a human cDNA library. The following primers were used: 5'-CAC AAG CTT CCG CAG GAT GAG CTC CGC AGC-3', 5'-GTG GGA TCC GCA AAT GCA CAC ACG TAG GCA GC. These primers were designed to introduce *Hind*III and *Bam*HI restriction sites at the 5' and 3' extremities of the PCR product, respectively. The human PF4 cDNA was finally excised from the pUC119 vector by digestion with *Hind*III- *Bam*HI, and subcloned in-flame into the mammalian expression vector *pHβ3pneo* (19), between the *Hind*III and *Bam*HI restriction sites. Correct in-flame insertion of human PF4 cDNA was confirmed by automated DNA sequencing.

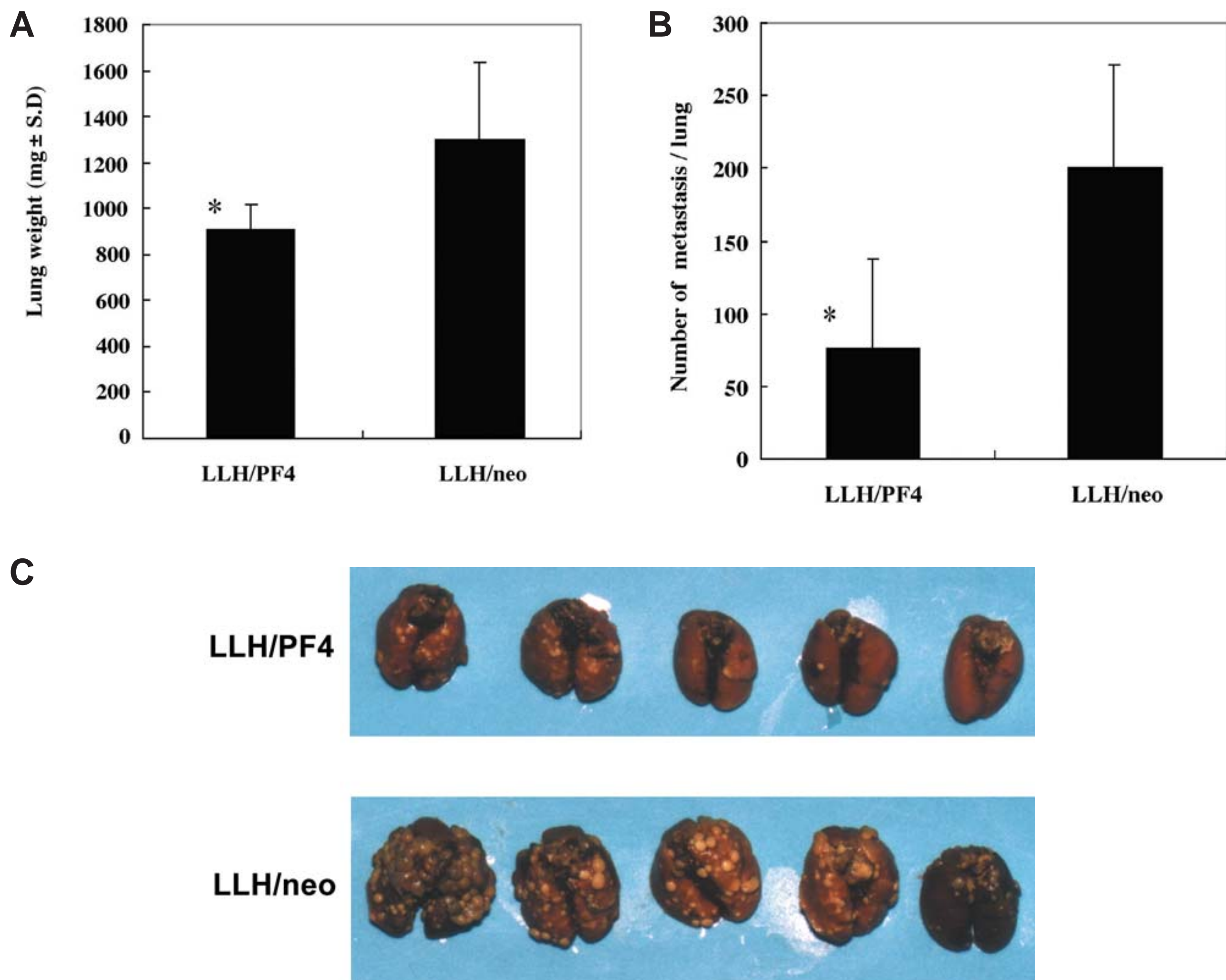

Figure 2. *A. The lung weight as assayed at 28 days after intravenous injection. The mean lung weight (±S.D) in LLH/PF4 and LLH/neo was 1073.8±488.1mg and 1299.6±336.9mg, respectively. *p=0.056 vs. LLH/neo. B. The number of lung metastases as assayed at 28 days after intravenous injection. The mean number of lung metastases (±S.D) in LLH/PF4 and LLH/neo were 79.6±61.9 and 199.8±71.1, respectively. *p=0.019 vs. LLH/neo. C. Gross anatomy of lungs from different treatment groups including LLH/PF4 (upper) and LLH/neo (lower) at 28 days after transplantation.*

DNA transfection. LLH cells were maintained in RPMI 1640 medium supplemented with 100 units/ml of penicillin G, 100 mg/ml of streptomycin, and 10% fetal calf serum (FCS) (Hyclone Lab., Logan, VT, USA). Subconfluent cultures in 100-mm petri dishes were transfected with chemokine expression plasmids or vector alone in lipofectin (GIBCO BRL, Bethesda, MD, USA). After 48h, G418 (Geneticin: GIBCO BRL) at 600 μg/ml (active form) was added to the cells. G418-resistant clones were randomly selected, isolated and expanded individually.

Northern blot analysis. Total RNA was prepared by GIT (guanidinium isothiocyanate) lysis followed by CsCl gradient ultracentrifugation. RNA (10 μg) from transfectants was electrophoresed in a formaldehyde-containing 1.0% agarose gel and transferred to a nylon membrane filter (Boehringer Mannheim, Germany). Filters were prehybridized for 16h at 42˚C in Hybrisol 1 (ONCOR, Gaithersburg, MD, USA) and hybridized with ^{32}P-labelled PF4 cDNA at 42˚C in the presence of 50% formamide for 24h, washed twice in 2 x saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by two washes in 0.2 x SSC, 0.1% SDS at 65˚C for 30 min and then exposed to Kodak X-OMAT, AR X-ray film (Eastman Kodak, Rochester, NY, USA) with intensifier screens at -70˚C for 24h.

Animal studies. Seven-week-old male BALB/c nu/nu mice were purchased from Nihon Crea (Atsugi, Japan). The transplantation assay for LLH/PF4 (PF4 transfectant) was performed in combination with LLH/neo (vector alone) as a control. Tumor cell injections were carried out using freshly prepared suspensions at a density of 2 x 10^7 and 5 x 10^6 cells/ml.

Subcutaneous injection assay. To evaluate the tumor growth rate of PF4-transfected (LLH/PF4) or control (LLH/neo) cells *in vivo*, cells were injected subcutaneously (*s.c.*). The total number of tumor cells injected per animal was 2 x 10^6 (0.1ml of 2 x 10^7 cells/ml). All injections were performed *s.c.* in the right lower abdominal quadrant *via* 27-gauge needles. Tumor volumes were measured in cubic millimeters with a vernier caliper and recorded by the formula (a x $b^2/2$), where "a" is the larger and "b" is the smaller of the two dimensions. Twenty-eight days after the implantation of LLH/PF4 or LLH/neo, animals were sacrificed and final tumor size and weight were assessed.

Intravenous injection assay. To evaluate the ability for lung metastasis, LLH/PF4 or LLH/neo cells were injected into the vessels. The total number of tumor cells injected per animal was 1 x 10^6 (0.2ml of 5 x 10^6 cells/ml). All injections were performed *i.v.* in the tale vein *via* 27-gauge needles. Animals were sacrificed on day 28 after injection of the tumor cells, and both lungs were surgically removed to measure lung weight and number of metastatic nodules.

Statistical analysis. Results were analyzed statistically using the unpaired Student's *t*-test . All *p*-values presented are two-sided and differences were considered significant at $p<0.05$.

Results

Production of PF4 in transfectants. Twelve independent G-418-resistant colonies from each transfection of PF4 expression plasmid were isolated and expanded. One clone, showing the highest level of PF4 mRNA expression (LLH/PF4), was selected for further study by the RNA blotting method. The LLH cells transfected with vector alone (LLH/neo; control) did not express cross hybridizing endogenous PF4 gene (data not shown).

Inhibition of tumor growth in vivo. The transfection and expression of PF4 by LLH cells did not alter their growth properties *in vitro,* as assayed by doubling time or morphology (data not shown). The transplantation assay for LLH/PF4 was performed in combination with LLH/neo as a control.

i) Subcutaneous injection assay: Figure 1A shows the results of the *in vivo* growth rate of LLH/PF4 and LLH/neo after *s.c.* implantation. LLH/PF4 showed almost the same growth rate as LLH/neo *in vivo*. Figure 1B shows the final tumor weight as assayed at 28 days after transplantation. The final tumor weight was not remarkably different between LLH/PF4 and LLH/neo. The mean tumor weights in LLH/PF4 and LLH/neo were 1073.8 mg and 1438.9 mg, respectively (N.S). Figure 1C shows the gross anatomy of tumors from different treatment groups, at 28 days after transplantation.

ii) Intravenous injection assay: Figure 2A and Figure 2B show the results of lung weights and number of lung metastases, as assayed at 28 days after intravenously injection. LLH/PF4 grew significantly more slowly than LLH/neo. The mean lung weights in LLH/PF4 and LLH/neo were 908.6 mg and 1299.6 mg ($p=0.056$) and number of lung metastases were 75.6 nodules/mouse and 199.8 nodules/mouse, respectively ($p=0.019$). Figure 2C shows the gross anatomy of lungs from different treatment groups at 28 days after transplantation.

Discussion

The study of the formation of new blood vessels was greatly encouraged by the recognition of the role of angiogenesis for tumor growth, and the effects of growth factors on endothelial cells (20). Popular models of angiogenesis are the neovascularization of the cornea or the chick chorioallantoic membrane. Angiogenic factors are applied locally, and angiostatic substances may also be injected systemically. Enhancement or inhibition of endothelial cell proliferation and/or *in vitro* migration are considered as predictive of angiogenic or angiostatic activity, respectively.

A possible involvement of chemokines in the regulation of angiogenesis was originally suggested by studies showing that PF4 has angiostatic (21) and potential anti-tumor activity (16). Similar effects were observed with other cationic proteins, and recently it was shown that PF4 and IP10 share binding sites on heparan sulfate and inhibit the proliferation of endothelial cells, presumably by displacing growth factors (22). The opposite effect, angiogenesis, was reported for IL-8 and several other CXC chemokines with the NH_2-terminal ELR-motif (11, 23). Modification of the ELR motif reportedly confers angiostatic properties to IL-8, while introduction of the ELR motif converts the chemokine, Mig, from angiostatic to angiogenic.

PF4 is one such agent that inhibits endothelial proliferation *in vitro* and angiogenesis *in vivo*. PF4 also inhibits tumor growth, however, as with other angiogenesis inhibitors, sustained tumor growth inhibition requires prolonged exposure to the recombinant protein. Peptide and protein angiostatic agents (*e.g.*, PF4, thorombospondin, angiostatin), in particular, present difficult pharmacological problems with regard to continuous drug delivery. PF4, for example, is rapidly cleared from the circulation following intravenous administration secondary to binding of the protein to vascular endothelium, thereby making it highly unlikely that cytostatic intratumoral concentrations of PF4 could be continuously maintained following systemic administration. Thus, for locally invasive tumors, direct transduction of the tumor and surrounding normal tissue by anti-angiogenesis peptide-encoding cDNA may represent a more efficient method of achieving constant intratumoral concentrations of the peptide.

To test the strategy of anti-angiogenic gene transfer, we chose the LLH cell line because it has been well characterized for its metastatic potential. In the subcutaneous injection assay, the final tumor weight was not remarkably different between LLH/PF4 and LLH/neo cells. Conversely, in the intravenous assay, PF4 significantly reduced the number of lung metastasis and lung weight. This finding is similar to the previously reported inhibition of murine melanoma lung metastasis formation by concurrent systemic administration of melanoma

cells and PF4 (24). The inhibition of lung metastasis suggests that PF4 may inhibit tumor-associated neovascularization.

Lung metastasis occurs through hematogenous spread of the tumor cells. Most studies have concluded that PF4 inhibited tumor angiogenesis, which resulted in decreased vascular access for the tumor cells (16, 24). In our "experimental lung metastatic model" without a primary tumor, it may be presumed that PF4 blocked the installation of the tumor cells in the normal lung tissue. Soncin *et al.* (25) showed that PF4 reduced HT-29 cell adhesion onto human angiogenin in a dose-dependent manner. There is a possibility that PF4 may prevent the affinity of the tumor cells for the normal lung tissue.

Numerous naturally occurring angiostatic molecules have been characterized, including thrombospondin 1, angiostatin endostatin and PF4. However, the molecular mechanism of their action is still unknown. An understanding of the molecular action of naturally occurring angiostatic agents could lead to the development of targeted therapies effective in various diseases associated with dysregulated angiogenesis.

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