Desmopressin Reduces Melanoma Lung Metastasis in Transgenic Mice Overexpressing Tissue Inhibitor of Metalloproteinases-1

GISELLE V. RIPOLL[1], HERNÁN G. FARINA[1], HITOSHI YOSHIJI[2],
DANIEL E. GOMEZ[1] and DANIEL F. ALONSO[1]

[1]Laboratory of Molecular Oncology, Department of Science and Technology,
Quilmes National University, Bernal, Buenos Aires, Argentina;
[2]Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan

Abstract. *Desmopressin (DDAVP) is a synthetic vasopressin analog capable of inducing an increase in the plasma levels of von Willebrand factor and coagulation factor VIII. DDAVP has been used during surgery to prevent bleeding in patients with coagulation defects. We have previously demonstrated that adjuvant perioperative DDAVP therapy inhibits lung and lymph node metastasis in a breast cancer model. Here the effect of DDAVP on experimental lung colonization of B16 melanoma cells was investigated in a transgenic mice model with high levels of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the systemic circulation. Transgenic C57BL/6j-CBA mice overexpressing human TIMP-1 in the liver under the control of the mouse albumin promoter/enhancer were employed. Treatment with DDAVP (2 μg/kg/dose) at the time of intravenous injection of B16 cells significantly inhibited the formation of lung metastases in TIMP-1 transgenic animals (p=0.021), while no significant effect was obtained in control hybrid mice. The inhibition was not due to direct cytotoxic effects of DDAVP on tumor cells and no expression of vasopressin receptors was detected in B16 cells. Our data indicate that DDAVP therapy may impair successful implantation of circulating melanoma cells and suggest that high levels of circulating TIMP-1 display a cooperative role in the antitumor activity of the compound.*

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP), the synthetic analog of the antidiuretic hormone, is a well-tolerated and convenient haemostatic drug that can be used

Correspondence to: Dr. Daniel F. Alonso, Laboratorio de Oncología Molecular, Universidad Nacional de Quilmes, R. Sáenz Peña 352, Bernal B1876BXD Buenos Aires, Argentina. Fax: +54 11 4365 7132, e-mail: dfalonso@unq.edu.ar

during surgery in patients with bleeding diathesis (1, 2). The compound increases the plasma levels of coagulation factor VIII, von Willebrand factor (VWF) and tissue-type plasminogen activator, and also enhances platelet adhesion to the vessel wall (3). DDAVP is a selective agonist for the vasopressin V2 membrane receptor, which is expressed in endothelial cells and in the kidney collecting duct, mediating hemostatic and antidiuretic effects of the peptide, respectively (3). Interestingly, the presence of vasopressin receptors was also documented in several tumor variants, including breast and lung cancer (4).

Several years ago, we reported for the first time that DDAVP strongly inhibited blood-borne lung colonization by aggressive breast cancer cells (5). The DDAVP effect was exerted in the early stages of metastasis, possibly limiting the formation of tumor cell emboli, as well as altering the interaction of cancer cells with the endothelium at the target organ. The antitumor properties of DDAVP were not associated with direct cytotoxicity to tumor cells suggesting that the compound modulates a selective effect on the host which influences tumor metastasis. We further demonstrated that peri-operative DDAVP treatment dramatically reduced lymph node and lung metastasis in a mouse model of mammary tumor manipulation and surgical excision (6).

Matrix metalloproteinases (MMPs) are involved in physiological cellular processes and pathological situations, such as tumor invasion and metastasis. MMPs have been considered as promising targets for cancer therapy and a number of different synthetic and natural MMP inhibitors have been identified (7). Tissue inhibitor of MMPs-1 (TIMP-1) is a multifunctional glycoprotein that plays an important role modulating cell morphology, extracellular matrix remodeling and angiogenesis (8). The growth and spread of melanoma cells was examined in a TIMP-1 transgenic mouse model with targeted expression of the transgene in the liver. High levels of TIMP-1 in the peripheral blood was able to reduce lung metastasis,

although paradoxical effects on vascularization and growth of primary tumors were observed (9).

In the present study, our goal was to investigate the effect of DDAVP on experimental lung colonization of metastatic melanoma cells in TIMP-1 transgenic mice, in the search for a potential cooperative role of the protease inhibitor in the antitumor activity of DDAVP.

Materials and Methods

Animals. Transgenic C57BL/6j-CBA mice overexpressing human TIMP-1 (hTIMP-1) in the liver under the control of the mouse albumin promoter/enhancer were employed (9). Because TIMP-1 possesses the secretion signal peptide sequence, high levels of the inhibitor can be seen in the liver and peripheral blood. hTIMP-1 concentration in the liver ranged from 86 to 92 ng/mg protein and the plasma levels were from 486 to 548 ng/ml. TIMP-1 activity in the liver of transgenic mice was significantly higher than that of control animals, indicating that hTIMP-1 contributed to the MMP inhibitory activity (10). We employed wild-type C57BL/6j-CBA hybrid mice, with the same genetic background as the control group. Animals with an age of 12-18 weeks and an average weight of about 25 g were used and maintained with water and food *ad libitum*. All procedures were performed in accordance with recommendations for the proper care of laboratory animals.

Tumor cells and culture conditions. The parental melanoma cell line B16-F0, syngeneic for the C57BL/6 mouse strain, was used. B16 cells were maintained as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) from Gibco (Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. For harvesting, cells were trypsinized using standard procedures.

Tumor cell inoculation. To assess the formation of experimental lung metastases, B16 cells (10^5 viable cells/0.3 ml DMEM) were injected into the lateral tail vein of unanesthetized mice. Three weeks later, animals were sacrificed by cervical dislocation, lungs were fixed in Bouin's solution and the surface lung nodules were counted, as previously described (9). In addition, to examine primary tumor growth, mice were inoculated subcutaneously in the right flank with B16 cells ($5x10^4$ viable cells/0.2 ml DMEM). Tumor development was monitored by palpation and tumor diameters were measured with a caliper twice a week.

DDAVP treatment. DDAVP acetate from Ferring AB (Malmö, Sweden) was injected *i.v.* at doses of 2 μg/kg body weight (50 ng in 0.3 ml DMEM per mouse). Two DDAVP doses were administered, the first co-injected at the time of tumor cell inoculation and the second 24 hours later. In some experiments, daily DDAVP treatment was maintained for 3 additional days.

Cell survival and in vitro cytotoxicity. B16 cell suspensions were incubated at 37°C in serum-free DMEM with appropriate concentrations of DDAVP (50-250 ng/ml). After 1-5 h, cell viability was assessed using the trypan blue exclusion technique. DDAVP was also assayed on semiconfluent tumor cell monolayers for 24 or 48 h in serum-free DMEM or DMEM plus 5% FBS, respectively. Monolayers were washed, fixed with formalin, stained with

toluidine blue and solubilized with 1% SDS. The number of cells was estimated by measuring the absorbance at 595 nm.

Vasopressin receptor expression. The expression of V2 receptors in B16 melanoma cells was examined by reverse transcription-polymerase chain reaction (RT-PCR), as described by Oksche *et al.* (11). Primers corresponding to V2 receptor cDNA sequences (GenBank accession no. NM000054; gi: 4895106) 902-928 (5'-GCC TGC CAG GTG CTC ATC TTC CGG GAG-3') and 1,179-1,207 (5'-GAT CCA GGG GTT GGT GCA GCT GTT GAG GC-3'), which are highly conserved among the different species, including mouse and human were used. Mouse kidney samples were used as a control for positive V2 receptor expression.

Zymography. For evaluation of gelatinolytic MMP activity in lung tissue and plasma from both transgenic and control mice, zymographic analysis was performed using SDS-polyacrilamide gels copolymerized with gelatin, as reported previously (12). Frozen lung tissue samples were homogenized at 4°C in an extraction buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40 and 100 μg/ml trypsin inhibitor, centrifuged at 21,000 xg at 4°C and stored at –20°C until assayed. Arterial blood plasma samples were obtained in the presence of 0.38% sodium citrate as described (13). Plasma aliquots were stored at –20°C and used only once after thawing. Lung and blood samples were also obtained 30 min after *i.v.* administration of DDAVP, as indicated above.

Statistical analysis. Comparison of lung metastasis was done using the unpaired *t*-test. Differences between control and experimental conditions *in vitro* were assessed by one-way ANOVA. $P<0.05$ was considered significant.

Results

Inhibition of experimental lung colonization of B16 melanoma cells by DDAVP in TIMP-1 transgenic mice. Administration of DDAVP at the time of *i.v.* inoculation of B16 melanoma cells significantly inhibited the formation of experimental lung metastases in TIMP-1 transgenic mice. The number of pulmonary nodules was reduced by approximately 60% in transgenic animals treated with 2 *i.v.* doses of 2 μg/kg of DDAVP, administering the first dose during tumor cell injection and the second 24 h later (Figure 1). Similar results were obtained with 5 daily doses of DDAVP, beginning at the time of B16 cell inoculation. No sex-dependent differences in the antimetastatic properties of DDAVP were detected in TIMP-1 transgenic mice. Control wild-type hybrid mice treated with DDAVP had a mild, non-significant reduction of about 20% in lung metastasis (Figure 1). We also explored the effects of DDAVP on primary tumor growth; no antitumor effects of DDAVP against subcutaneous B16 melanoma tumors were observed using either transgenic or wild-type mice (data not shown).

Lack of in vitro effects of DDAVP on B16 melanoma cells. In vitro exposure of melanoma cell suspensions to DDAVP did not affect cell viability at the range of concentrations
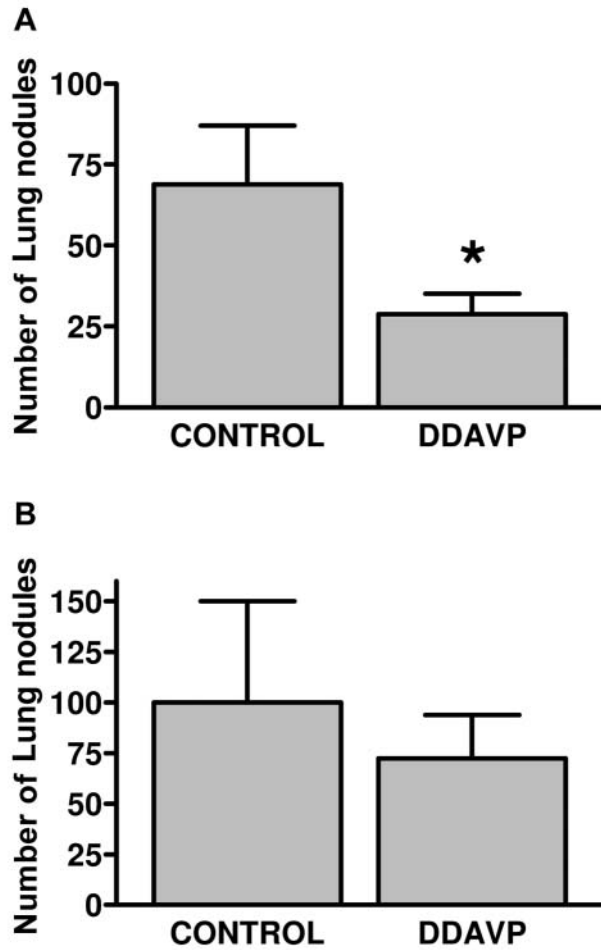
Figure 1. *Effect of DDAVP on B16 melanoma lung-colonizing ability in TIMP-1 transgenic mice (A) and wild-type hybrids (B). Two intravenous DDAVP doses were administered (2 μg/kg/dose), the first co-injected at the time of tumor cell inoculation and the second 24 h later. The results of two independent experiments are presented together. Values represent means±S.E. of at least 8 animals per group. *p=0.021, t-test.*

Figure 2. *Tumor cell survival after in vitro DDAVP exposure: (A) cell viability of B16 melanoma cells after 1-5 h in the presence of DDAVP; (B) DDAVP effect on cultured B16 monolayers for 24 h (open bars) or 48 h (solid bars). (C) RT-PCR for vasopressin V2 receptor expression in B16 melanoma cells with kidney tissue as a positive control. Beta-actin was used as internal control for gene expression.*

employed in the *in vivo* treatment (Figure 2A). Similarly, semiconfluent monolayers were not affected by incubation for 24-48 h in the presence of DDAVP (Figure 2B). Vasopressin V2 receptor expression was not detected in B16 melanoma cells using RT-PCR, while mouse kidney was positive for the expected fragment of 305 bp (Figure 2C).

MMP activity in samples from TIMP-1 transgenic mice. A zymographic analysis of MMP activity in plasma samples from TIMP-1 transgenic mice and wild-type controls was performed. Main gelatinolytic activities of about 70 and 105 kDa were detected, corresponding to MMP-2 (gelatinase A) and MMP-9 (gelatinase B), respectively. Overexpression of hTIMP-1 in the blood of transgenic animals was associated with a slight decrease of MMP-2 and an increase of MMP-9
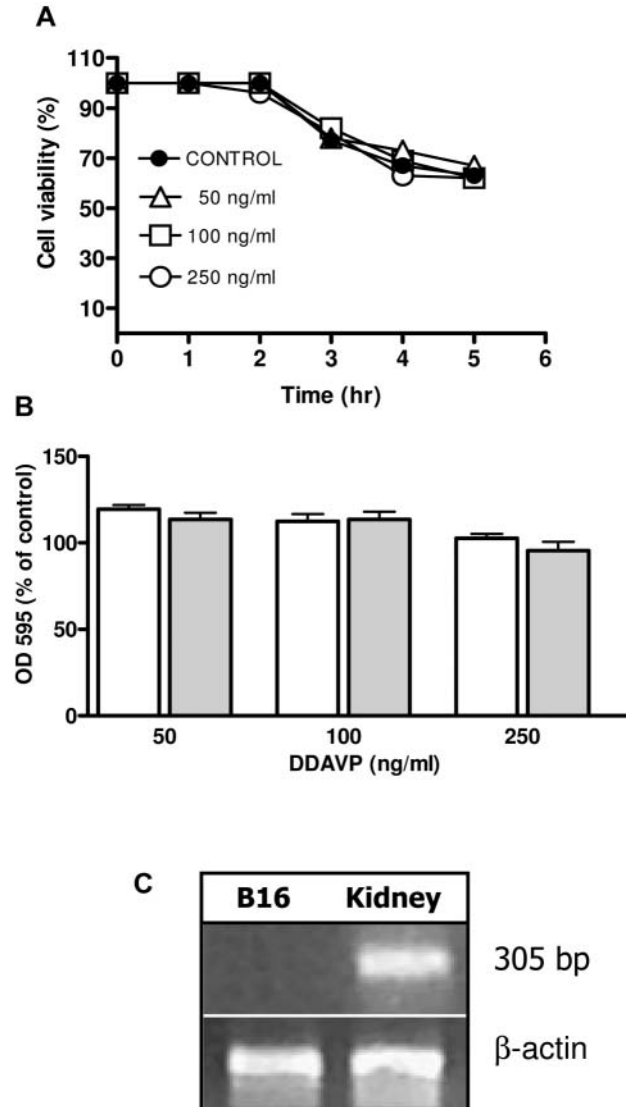
in plasma samples. Lung tissue homogenates showed a major band corresponding to MMP-2, that was weaker in samples from TIMP-1 transgenic mice (Figure 3). No relevant effects on MMP activity were observed after administration of a single dose of DDAVP (data not shown).

Discussion

The aggressive behavior of melanoma has been attributed to a lineage-specific phenotype expressed in melanocytes, indicating
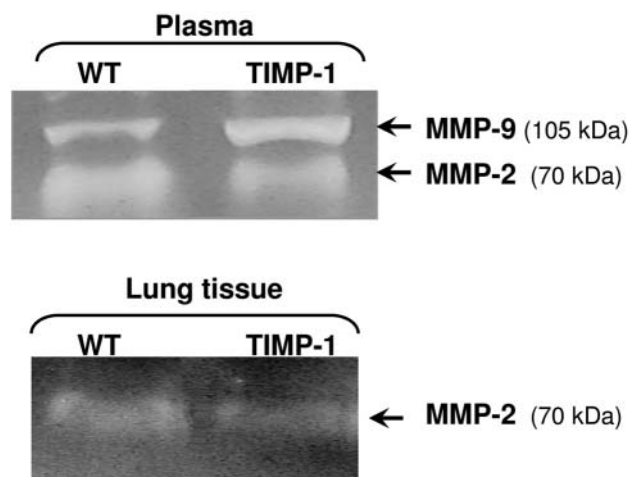
Figure 3. *Zymographic analysis of MMP activity contained in plasma and lung tissue from TIMP-1 transgenic mice (TIMP-1) and wild-type hybrids (WT). In all cases, 3 μg of protein per lane was loaded and gelatinolytic activity was fully inhibited by 15 mM EDTA. Molecular weights were determined using prestained standards (Bio-Rad, Hercules, CA, USA).*

that factors present before neoplastic transformation can have a pivotal role in the metastatic propensity (14). During blood-borne metastasis, intravasated tumor cells localize at the target site *via* a series of processes involving adhesion to the endothelium and invasion through the basal membrane. Platelet aggregation, plasminogen activation and some factors involved in coagulation may affect melanoma metastasis (15).

We have previously demonstrated the antitumor properties of DDAVP, a synthetic derivative of the peptide hormone vasopressin, using a breast cancer model in syngeneic mice (5, 6). To the best of our knowledge, this is the first report of the inhibition of lung colonization of metastatic melanoma cells by DDAVP. Our data indicate that the effect of DDAVP is exerted in the early stages of lung metastasis, possibly limiting the survival of tumor cell emboli, as well as altering the interaction of melanoma cells with endothelium at the target organ. It is clear that DDAVP was not mediating direct antitumor effects on B16 melanoma, since the compound did not affect viability of either B16 cell suspensions or semiconfluent monolayers, nor did tumor cells express the V2 vasopressin receptor in the present animal model.

Recently, Terraube *et al.* (16) showed that VWF plays a protective role against tumor cell dissemination in a VWF-deficient mutant mice model. An increased metastatic potential of B16 melanoma cells was observed in VWF-null mice. Restoration of VWF plasma levels in mutant mice by coinjection of tumor cells with human recombinant VWF reduced melanoma lung metastasis. VWF is synthesized by both endothelial cells and megakaryocytes, but plasma VWF appears to be mainly of endothelial origin. Intravenous injection of DDAVP induces the release of highly

multimerized forms of VWF, with a time to peak levels of about 60 min and a plasma half-life of 8-10 h (1, 3). VWF might participate in the interaction of tumor cells with platelets and the subendothelium, and appears to impede metastasis by reducing the sustained adherence and/or survival of melanoma cells in lung microvasculature (16).

However, other mechanisms of action on host tissues could also be contributing to the antitumor properties of DDAVP. The compound may modify tumor cell attachment by altering P-selectin expression on endothelial cells (17) or platelets (18). DDAVP may also alter the hemodynamics of blood flow or induce lysis of metastatic cells through the production of nitric oxide from the vasculature (19, 20).

The complexity of the metastatic process has complicated the full understanding of the development of life-threatening cancer lesions. Recent advances in gene expression profiling have contributed to identifing inherited metastasis risk factors, suggesting that the host genetic background influences metastatic progression (21). Transgenic models are valuable tools in investigating whether a certain genetic profile impacts on the metastatic capacity of aggressive cancer cells.

Administration of DDAVP at the time of *i.v.* inoculation of B16 melanoma cells reduced about 60% of lung nodules in TIMP-1 transgenic mice, while no significant antimetastatic effects were obtained in wild-type controls. These results strongly suggest a cooperative role of TIMP-1 overexpression in the antimetastatic activity of DDAVP. High levels of hTIMP-1 in the circulation were important in decreasing the ability of melanoma cells to colonize the lung after intravenous injection (9), and systemic administration of recombinant TIMP-1 suppressed B16 melanoma metastasis (22). In addition, the antimetastatic effect of TIMP-1 has been well-documented in other tumor variants. Host TIMP-1 overexpression conferred resistance to experimental brain metastasis of fibrosarcoma cells (23). Transfectants of a bladder carcinoma overexpressing TIMP-1 or TIMP-2 showed inhibition of extravasation at metastatic distant sites (24).

Based on the role of MMPs in VWF proteolytic processing, an altered VWF activity may be expected after sustained MMP inhibition. The MMP known as ADAMTS13 (a disintegrin-like metalloprotease with thrombospondin type I repeats 13) cleaves the large VWF multimers after their release from the endothelium (25). Defects in the assembly of VWF multimers or exaggerated degradation by ADAMTS13 can cause coagulation disorders in humans (25, 26). In our model, TIMP-1 transgenic mice showed a reduction of MMP-2 activity in lung tissue samples and plasma, as well as an increase of MMP-9 as a result of a compensatory production of proteases in response to MMP inhibition (9). Thus, DDAVP may induce an enhanced VWF-dependent antitumor effect in TIMP-1 transgenic mice. However, the precise mechanism mediated by MMP inhibition in VWF processing remains to be investigated.

The potential role of DDAVP and VWF in melanoma, limiting the spread of metastastic cells, warrants further investigation. Pharmacological modulation of proteolysis and coagulation is an attractive antitumor strategy. Amino acid substitutions in the DDAVP peptide sequence may generate novel vasopressin analogs with improved antimetastatic effects to be tested at a preclinical level in appropriate animal models (27).

Acknowledgements

References

1 Mannucci PM: Desmopressin (DDAVP) in the treatment of bleeding disorders, the first 20 years. Blood *90*: 2515-2521, 1997.

2 Gomez DE, Ripoll GV, Giron S and Alonso DF: Desmopressin and other synthetic vasopressin analogues in cancer treatment. Bull Cancer *93*: E7-12, 2006.

3 Kaufmann JE and Vischer UM: Cellular mechanisms of the hemostatic effects of demopressin (DDAVP). J Thromb Haemos *1*: 6682-689, 2003.

4 North WG: Gene regulation of vasopressin and vasopressin receptors in cancer. Exp Physiol *85*: 27S-40S, 2000.

5 Alonso DF, Skilton G, Farias EF, Bal de Kier Joffe E and Gomez DE: Antimetastatic effect of desmopressin in a mouse mammary tumor model. Breast Cancer Res Treat *57*: 271-275, 1999.

6 Giron S, Tejera AM, Ripoll GV, Gomez DE and Alonso DF: Desmopressin inhibits lung and lymph node metastasis in a mouse mammary carcinoma model of surgical manipulation. J Surg Oncol *81*: 38-44, 2002.

7 Mannello F, Tonti G and Papa S: Matrix metalloproteinase inhibitors as anticancer therapeutics. Curr Cancer Drug Targets *5*: 285-298, 2005.

8 Gomez DE, Alonso DF, Yoshiji H and Thorgeirsson UP: Tissue inhibitors of metalloproteinases; structure, regulation and biological functions. Eur J Cell Biol *74*: 111-122, 1997.

9 De Lorenzo MS, Ripoll GV, Yoshiji H, Yamazaki M, Thorgeirsson UP, Alonso DF and Gomez DE: Altered tumor angiogenesis and metastasis of B16 melanoma in transgenic mice overexpressing tissue inhibitor of metalloproteinases-1. In Vivo *17*: 45-50, 2003.

10 Yoshiji H, Kuriyama S, Miyamoto Y, Thorgeirsson UP, Gomez DE, Kawata M, Yoshii J, Ikenaka Y, Noguchi R, Tsujinoue H, Nakatani T, Thorgeirsson SS and Fukui H: Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. Hepatology *32*: 1248-1254, 2000.

11 Oksche A, Leder G, Valet S, Platzer M, Hasse K, Geist S, Krause G, Rosenthal A and Rosenthal W: Variant amino acids in the extracellular loops of murine and human vasopressin V2 receptors account for differences in cell surface expression and ligand affinity. Mol Endocrinol *16*: 799-813, 2002.

12 Gabri MR, Lorenzano Menna P, Scursoni AM, Gomez DE and Alonso DF: Role of tumor-derived granulocyte-macrophage colony-stimulating factor in mice bearing a highly invasive and metastatic mammary carcinoma. Pathobiology *67*: 180-185, 1999.

13 Alonso DF, Farias EF and Bal de Kier Joffe E: Impairment of fibrinolysis during the growth of two murine mammary adenocarcinomas. Cancer Lett *70*: 181-187, 1993.

14 Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW, Naber SP and Weinberg RA: The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. Nat Genet *37*: 1047-1054, 2005.

15 Im JH, Fu W, Wang H, Bhatia SK, Hammer DA, Kowalska MA and Muschel RJ: Coagulation facilitates tumor cell spreading in the pulmonary vasculature during early metastatic colony formation. Cancer Res *64*: 8613-8619, 2004.

16 Terraube V, Pendu R, Baruch D, Gebbink MF, Meyer D, Lenting PJ and Denis CV: Increased metastatic potential of tumor cells in von Willebrand factor-deficient mice. J Thromb Haemost *4*: 519-526, 2006.

17 Kanwar S, Woodman RC, Poon MC, Murohara T, Lefer AM, Davenpeck KL and Kubes P: Desmopressin induces endothelial P-selectin expression and leukocyte rolling in postcapillary venules. Blood *86*: 2760-2766, 1995.

18 Wun T, Paglieroni TG and Lachant NA: Desmopressin stimulates the expression of P-selectin on human platelets *in vitro*. J Lab Clin Med *126*: 401-409, 1995.

19 Kaufmann JE, Iezzi M and Vischer UM: Desmopressin (DDAVP) induces NO production in human endothelial cells *via* V2 receptor- and cAMP-mediated signaling. J Thromb Haemost *1(4)*: 821-828, 2003.

20 Hirano S: *In vitro* and *in vivo* cytotoxic effects of nitric oxide on metastatic cells. Cancer Lett *115*: 57-62, 1997.

21 Hunter K: Host genetics influence tumour metastasis. Nat Rev Cancer *6*: 141-146, 2006.

22 Schultz RM, Silberman S, Persky B, Bajkowski AS and Carmichael DF: Inhibition by recombinant human tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res *48*: 5539-5545, 1988.

23 Kruger A, Sanchez-Sweatman OH, Martin DC, Fata JE, Ho AT, Orr FW, Ruther U and Khokha R: Host TIMP-1 overexpression confers resistance to experimental brain metastasis of a fibrosarcoma cell line. Oncogene *16*: 2419-2423, 1998.

24 Kawamata H, Kawai K, Kameyama S, Johnson MD, Stetler Stevenson WG and Oyasu R: Overexpression of tissue inhibitor of matrix metalloproteinases (TIMP1 and TIMP2) suppresses extravasation of pulmonary metastasis of a rat bladder carcinoma. Int J Cancer *63*: 680-687, 1995.

25 Chauhan AK, Motto DG, Lamb CB, Bergmeier W, Dockal M, Plaimauer B, Scheiflinger F, Ginsburg D and Wagner DD: Systemic antithrombotic effects of ADAMTS13. J Exp Med *203*: 767-776, 2006.

26 Sadler JE: von Willebrand factor: two sides of a coin. J Thromb Haemost *3*: 1702-1709, 2005.

27 Gomez DE, Ripoll GV, Giron S and Alonso DF: Desmopressin and other synthetic vasopressin analogues in cancer treatment. Bull Cancer *93*: E7-E12, 2006.