

Insight into the Effect of the Vasopressin Analog Desmopressin on Lung Colonization by Mammary Carcinoma Cells in BALB/c Mice

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Abstract. *Background/Aim:* Desmopressin (dDAVP) is a synthetic peptide analog of vasopressin with antidiuretic and hemostatic properties. Recent experimental evidence have suggested that dDAVP can inhibit metastasis formation by agonist action on V2 vasopressin receptors present in both tumor and endothelial cells. We have examined the kinetics of dDAVP effect during metastatic colonization and its potential association with hemostasis. *Materials and Methods:* The experimental metastasis assay was performed by injecting F3II mammary carcinoma cells into the lateral tail vein of syngeneic female BALB/c mice. *Results:* Clinically relevant doses of dDAVP (0.3 to 2 µg/kg intravenously (i.v.)) produced a dose-dependent inhibition in the formation of lung nodules when administered during the first 24 hours after F3II tumor cell injection. The hemostatic agent tranexamic acid (10 mg/kg, i.v.) had no effect on metastasis formation in the same experimental conditions, while the anticoagulant enoxaparin (1 mg/kg, subcutaneously (s.c.)) did not modify the antimetastatic action of dDAVP. *In vitro*, dDAVP had a strong inhibitory effect on F3II cell colony formation. *Conclusion:* dDAVP interferes with early metastatic disease, and direct association of this effect with hemostatic mechanisms is unlikely.

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Key Words: Desmopressin, vasopressin, peptide analog, hemostasis, metastasis, breast cancer.

Desmopressin (dDAVP, 1-deamino-8-D-arginine vasopressin), a synthetic peptide analog of vasopressin, was initially described in the late 1960s (1). Desmopressin is a selective agonist for the V2 vasopressin membrane receptor (V2r) mediating the well-known antidiuretic action of the compound, as well as a safe hemostatic effect associated with release of von Willebrand factor (vWF), coagulation factor VIII and tissue-type plasminogen activator from microvascular endothelia (2). Interestingly, V2r expression was detected in several tumor variants (3) and selective agonist effect on V2r displayed by dDAVP could induce growth inhibition of human breast cancer cells (4, 5).

Previously, we reported for the first time that dDAVP was capable of inhibiting lung colonization by blood-borne breast cancer cells in an aggressive experimental mouse model (6, 7). Furthermore, perioperative administration of dDAVP significantly prolonged survival in dogs with locally advanced mammary cancer (8, 9). The aim of the present work was to examine the kinetics of dDAVP effect during metastatic lung colonization by F3II mammary carcinoma cells and its potential association with hemostasis.

Materials and Methods

Tumor cells and culture conditions. The F3II cell line is a highly aggressive variant derived from a clone of a spontaneous BALB/c mouse mammary tumor, which expresses the V2r on the cell surface (5). F3II cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine and 80 µg/ml gentamycin. For harvesting, tumor cells were trypsinized using standard procedures.

Animals. Syngeneic, pathogen-free BALB/c mice were purchased from the School of Veterinary Sciences-UNLP (La Plata, Argentina) and kept at our animal house facility according to an institutionally approved protocol. Food and water were provided *ad libitum* and general health status of the animals was monitored daily. Adult female mice with a weight of 20-25 g were used.

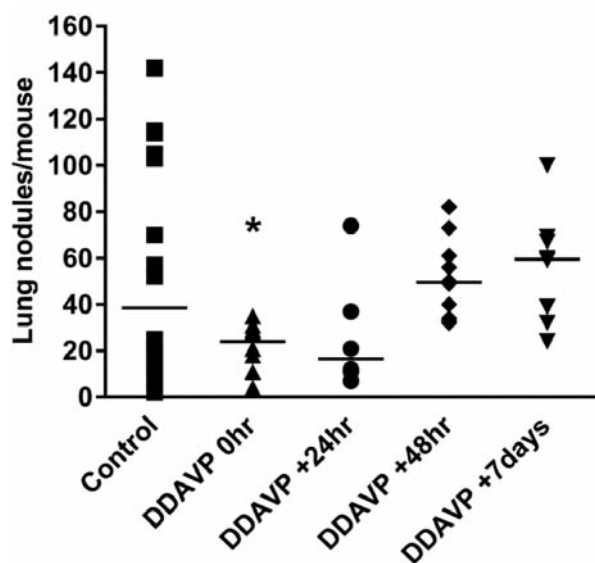


Figure 1. Kinetics of dDAVP on lung colonization by F3II cells. Two hundred thousand F3II cells per mouse were injected into the lateral tail vein of BALB/c mice. A dose of 1 µg/kg of dDAVP was administered at time zero, 24 h, 48 h or 7 days after tumor cell inoculation. Controls received saline vehicle. Data points represent individual mice and horizontal lines indicate the median values from two independent experiments. * $p < 0.05$ versus control by the Kruskal-Wallis plus Dunn's test (at least 6 animals per group were used).

Experimental metastasis assay. F3II cells at a concentration of 2×10^5 viable cells/0.3 ml DMEM/mouse were injected into the lateral tail vein of unanesthetized mice. After 3 weeks, animals were sacrificed by cervical dislocation and necropsied. Lungs were removed, fixed in Bouin's solution and the number of surface lung nodules was determined under a dissecting microscope. Selected organs were further processed for paraffin embedding and sectioning for histopathological analysis.

Administration of dDAVP. Groups of at least 6 animals received dDAVP from Ferring Pharmaceuticals (Malmö, Sweden) by the intravenous (*i.v.*) route in saline solution. The compound was administered at the dosage of 0.3-2 µg/kg of body weight. These doses are clinically relevant with proven hemostatic effects and safety in humans (2). To investigate the kinetics of dDAVP effect during metastatic lung colonization, treatment was given at time zero, 24 hours, 48 hours or 7 days after tumor cell inoculation. Controls received only the saline vehicle.

Administration of tranexamic acid and enoxaparin. The hemostatic agent tranexamic acid (Ariston, Buenos Aires, Argentina) was administered intravenously at a dose of 10 mg/kg (10) at time zero and/or 24 h after tumor cell inoculation. The anticoagulant enoxaparin (Sanofi Winthrop, Le Trait, France) was given by daily subcutaneous (*s.c.*) injection at a dose of 1 mg/kg (11) for 7 days, starting at the moment of tumor cell inoculation.

In vitro studies. Cytostatic effects of dDAVP were studied at low cell density by colony formation assay, as described (5). F3II cells

Table I. Dose-dependent effects of dDAVP on lung colonization by F3II mammary carcinoma cells. Mice were injected with 2×10^5 F3II cells into the lateral tail vein, and sacrificed 3 weeks later and necropsied.

Treatment ^a	Percent reduction of lung nodules ^b
dDAVP 0.3 µg/kg	32%*
dDAVP 1 µg/kg	64%**
dDAVP 2 µg/kg	70%**

^aTwo dDAVP administrations were performed for each dose level, the first at time zero and the second 24 h after tumor cell injection. ^bLung nodules were counted under a dissecting microscope and percent reduction of median values with respect to controls was calculated. * $p < 0.05$ and ** $p < 0.01$ versus control, by the Kruskal-Wallis with Dunn's test.

were plated at 6×10^2 cells/well in 24-well plates and grown for 7 days in complete medium with or without the compound. Cultures were then stained with crystal violet and the concentration producing 50% inhibition (IC_{50}) was determined by plotting the percentage of cell colonies versus dDAVP concentration. Additionally, the effects on slowly-growing, subconfluent F3II cultures were assayed.

Statistical analysis. Differences were evaluated with the GraphPad Prism 4.0 software package (Graphpad Software, La Jolla, California, USA). *p*-Values less than 0.05 were considered statistically significant.

Results

We first analyzed the kinetics of dDAVP effect during lung colonization by metastatic F3II mammary carcinoma cells. As shown in Figure 1, dDAVP reduced about 50-60% the formation of lung nodules when administered at time zero or 24 hours after tumor cell inoculation, while later dDAVP administration had no effect. Representative sections of lungs from control and treated animals are depicted in Figure 2. Multiple subpleural and intrapulmonary nodules were visualized in controls. In contrast, lungs from animals receiving early dDAVP treatment showed a few peripheral nodules. We have also examined dose dependency for the antimetastatic effect. Following the data from the kinetics experiment, mice received two dDAVP intravenous administrations, the first coinjecting at the time of tumor cell inoculation and the second 24 hours later. We observed a clear dose-dependent effect on metastasis formation, showing an inhibition of 70% in the higher dDAVP dose of 2 µg/kg (Table I). As expected, similar doses administered by the subcutaneous route were not effective (data not shown).

In order to explore the potential role of hemostatic mechanisms in early metastasis formation as well as in the antimetastatic activity of dDAVP, we evaluated hemostatic and anticoagulant drugs. Under the same experimental conditions of effective dDAVP treatment, the hemostatic

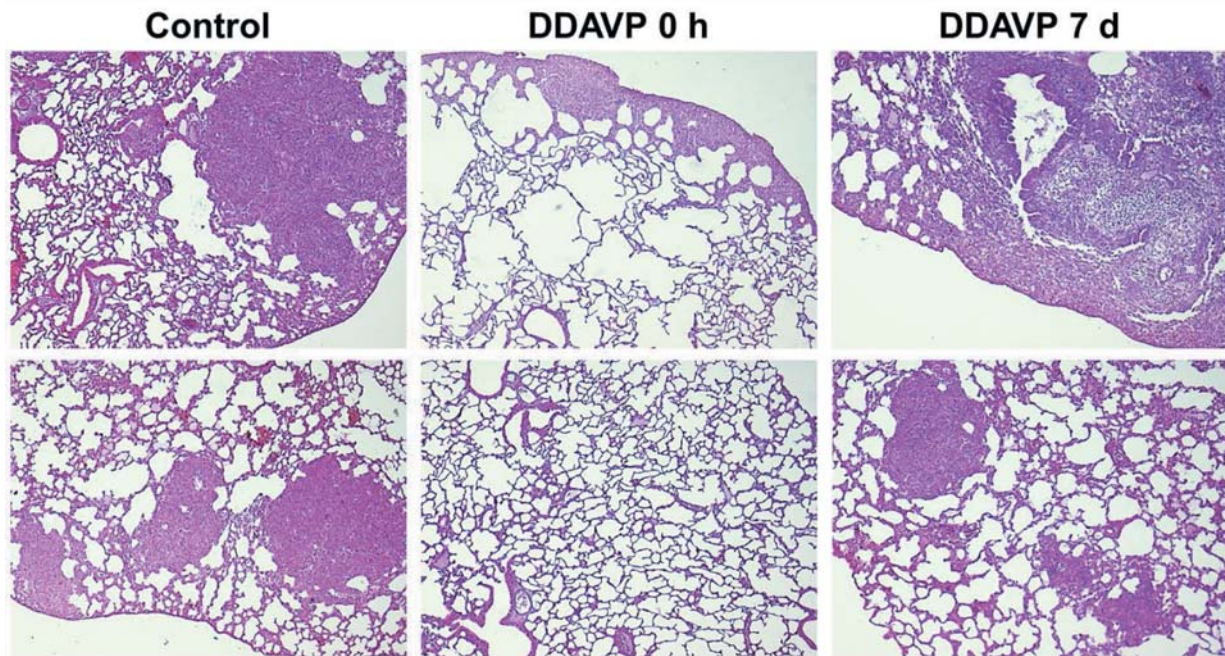


Figure 2. Representative microphotographs of lungs from control and treated mice. Representative microphotographs of lungs from control mice receiving saline solution and mice treated with dDAVP (1 $\mu\text{g/kg}$) at time zero (dDAVP 0h) or 7 days (dDAVP 7d) after inoculation of F3II mammary carcinoma cells. Staining by hematoxylin and eosin, original magnification $\times 40$.

agent tranexamic acid had no effect on the formation of lung metastasis by F3II cells when administered at time zero and 24 hours after tumor cell injection (Figure 3A). Similarly, daily treatment with the anticoagulant enoxaparin for 7 days, beginning the day of tumor cell injection, did not modify the antimetastatic action of dDAVP (Figure 3B).

As illustrated in Figure 4A, dDAVP had a strong cytostatic effect on *in vitro* colony formation, with an IC_{50} value of about 0.7 μM against F3II cells, confirming previous results with murine and human breast cancer cells (5, 12). On the other hand, slowly-growing, subconfluent F3II cultures were not significantly affected by micromolar concentrations of dDAVP (Figure 4B).

Discussion

The synthetic peptide dDAVP is a selective agonist of V2r present on both endothelial and breast cancer cells. Recent evidence indicated that dDAVP promotes tumor-mediated production of angiostatin (5) and also activates endothelial release of vWF, which may affect survival of micrometastatic cells (13). Previous experimental data showed that dDAVP is able to inhibit development of metastasis in the aggressive F3II mouse mammary cancer model. Here, we demonstrated that dDAVP is effective when treatment is applied during the early metastatic events. As expected, control animales

showed multiple subpleural and intrapulmonary metastatic nodules, while animals receiving dDAVP displayed only a few peripheral nodules. On the other hand, dDAVP showed a marked dose-dependency in inhibition of metastasis, with highest effects at clinical relevant doses of 1-2 $\mu\text{g/kg}$.

The systemic release of hemostatic and profibrinolytic factors induced by dDAVP could act modulating biological mechanisms of metastasis development. It is well known that dDAVP stimulates endothelial cells to release large multimeric forms of vWF, provoking a rapid increase of this factor in the circulation (14). Von Willebrand factor has been classically described as a hemostatic effector molecule. However, recent studies have related this protein with a more complex role since it has been implicated as a regulator of angiogenesis, tumor metastasis and also as inductor of apoptosis in cancer cells (15). As demonstrated by Terraube *et al.* (13) vWF could play a protective role against tumor cell dissemination *in vivo* inducing the early death of metastatic cells in the microvasculature of the target organ by limiting adherence of the tumor cell-endothelial cell. Moreover, vWF induced apoptosis of highly aggressive carcinoma cells mainly in lung blood vessels (16). Inactivation of proapoptotic vWF by ADAM 28 (a disintegrin and metalloproteinase 28) improved survival of cancer cells with a high level expression of ADAM28 at the metastatic site.

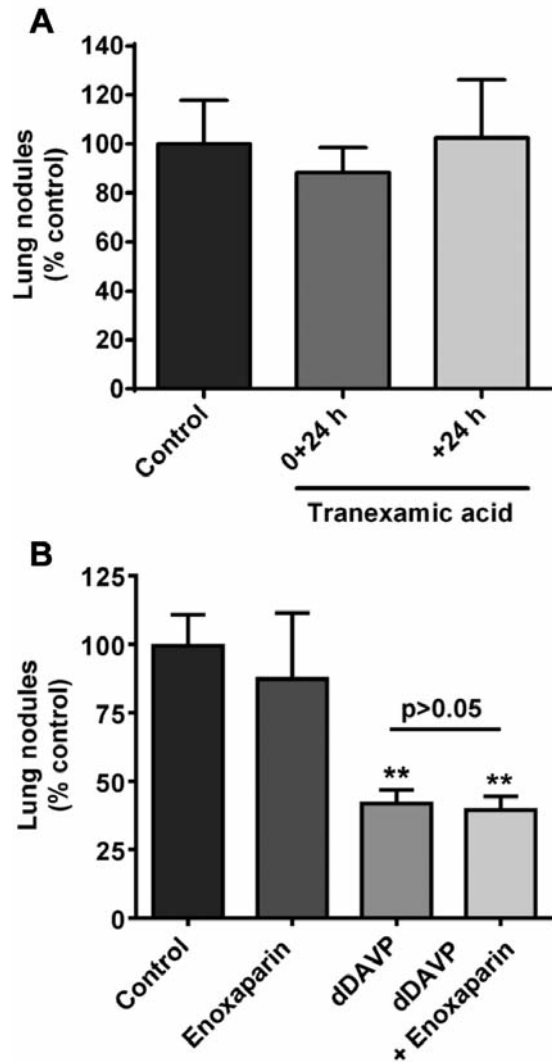


Figure 3. Effect of treatment of tranexamic acid and enoxaparin on metastatic dissemination. Effect of treatment of 10 mg/kg i.v. of tranexamic acid (A) and 1 mg/kg/day s.c. of the anticoagulant agent enoxaparin (B) on metastatic dissemination. Results are expressed as mean \pm standard error from two independent experiments. ** $p < 0.05$ versus control by the Kruskal-Wallis plus Dunn's test.

The antimetastatic effect displayed by dDAVP could not be reproduced by other hemostatic agents like tranexamic acid neither was affected by concomitant treatment of the anticoagulant enoxaparin. Tranexamic acid is a potent competitive inhibitor of plasminogen activation and, at higher concentrations, a non-competitive inhibitor of plasmin (17). It has been used for years in the management of peri- and postoperative bleeding and blood disorders. Particularly, tranexamic acid administration is a safe practice in surgical resection of tumor in cancer patients (18). Enoxaparin is a powerful anticoagulant that interferes with the transformation

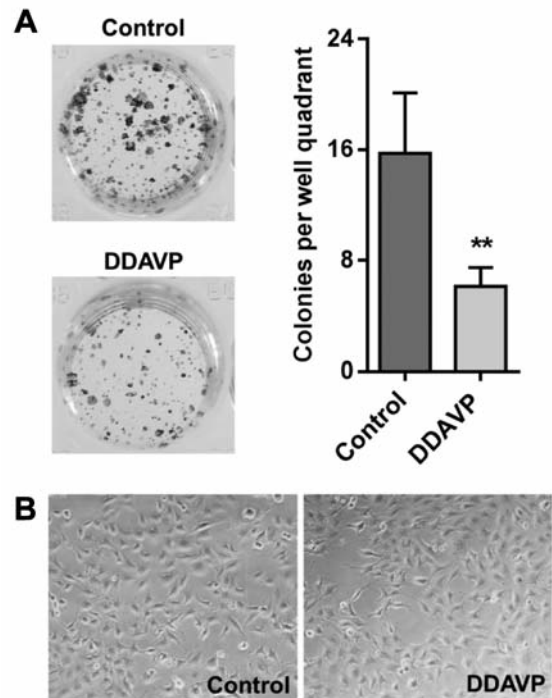


Figure 4. Antiproliferative effect of dDAVP on F3II cells. (A) Representative microphotographs of the effect of seven-day treatment of dDAVP on the colony formation assay at low density. Quantitative analysis (right) of number of colonies per quadrant are also shown. Values represent mean \pm standard error. Results are representative of three independent experiments. *** $p < 0.001$, t-test. (B) Representative microphotographs of subconfluent F3II cultures treated or not with 1 μ M of dDAVP. No direct effect was observed with 24 h incubation of dDAVP (original magnification $\times 100$).

of fibrinogen into fibrin preventing production of fibrin clot, the last step in the coagulation cascade. Thus, the mechanism of action of dDAVP would not be directly related with coagulation effectors that are at the end of the cascade.

Surgery for malignant disease carries a risk of deep vein thrombosis and pulmonary embolization, particularly in pancreatic cancer. Interestingly, enoxaparin is safe and effective preventing venous thromboembolism in patients undergoing major elective surgery for malignancies (19). In this regard, combination of perioperative dDAVP and enoxaparin may result in greater beneficial effects.

We have also explored the antiproliferative effects of dDAVP on low density F3II cultured cells. Under this condition, dDAVP displayed a strong effect on the ability of F3II cells to form colonies. The cytostatic mechanism of dDAVP was not associated with direct cytotoxicity since slowly-growing F3II cultures were not affected by high concentrations of dDAVP.

Our findings suggested that the dDAVP effect would not depend on a hemostatic direct action but other mechanisms could be playing a role in the antitumor final effect.

The specific interaction of dDAVP with V2r triggers antiproliferative mechanisms activating adenylate cyclase followed by intracellular cAMP elevation (20). Besides, dDAVP stimulates tumor-mediated production of angiostatin, a strong angiogenesis inhibitor (5). The compound induces secretion of urokinase-type plasminogen activator, favoring angiostatin generation by the proteolytic cleavage of plasminogen (21). The endothelial release of vWF by dDAVP as well, would be acting as a cooperative mechanism that helps to obstruct metastatic spread.

The biological effects of dDAVP administration on both endothelial and V2r-expressing cancer cells are complex and further investigations are required. Although a hemostatic mediator such as vWF may be involved, among other factors, the compound seems to induce antimetastatic effects not directly associated with coagulation mechanisms.

Acknowledgements

We would like to thank Technician Mariela Crubellatti for expert histological preparations. This work was supported by the R&D Grant Program 53/1004 from the National University of Quilmes, and by the National Agency of Scientific and Technological Promotion (ANPCyT). The support of Chemo-Romikim is also acknowledged. J.G. is a research fellow and D.E.G., D.F.A. and G.V.R. are members of the National Research Council (CONICET).

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Received June 9, 2014

Revised July 7, 2014

Accepted July 8, 2014