Effects of the Synthetic Vasopressin Analog Desmopressin in a Mouse Model of Colon Cancer

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Abstract. *Experimental and clinical data indicated that perioperative administration of the hemostatic peptide desmopressin (DDAVP) can inhibit progression of residual metastatic cells. The compound seems to act by inducing an agonist effect on specific V2 vasopressin membrane receptors present in both tumor cells and endothelial cells. Here we explored the antitumor effects of DDAVP in cultured colon carcinoma cells and in a syngeneic Balb/c mouse model. Both human Colo-205 and mouse CT-26 colon carcinoma cell lines expressed the V2 receptor, as revealed by immunofluorescence. DDAVP (at doses ranging from 100 ng/ml to 1 μg/ml) exerted a modest but significant antiproliferative effect on cultured CT-26 and Colo-205 cells. In vivo, DDAVP (2 intravenous doses of 2 μg/kg) reduced accumulation of ascites and formation of intestinal tumor nodules in mice intraperitoneally inoculated with CT-26 cells. Perioperative administration of DDAVP significantly inhibited tumor progression in animals surgically implanted in the spleen with CT-26 cells, and caused some reduction in liver metastasis. Although DDAVP and 5-fluorouracil demonstrated additive cytostatic effects in vitro, no antitumor effects were observed in this study in mice receiving a single cycle of chemotherapy (25 mg/kg) in combination with the peptide. Our data suggest that DDAVP may be potentially used to minimize spread or survival of residual malignant cells during surgical procedures for colon and other gastrointestinal tumors.*

The peptide desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is a synthetic analog of the antidiuretic hormone vasopressin, first described by Zaoral *et al*. in the late 1960s (1). In contrast to the natural hormone, which binds to different vasopressin receptors, DDAVP is a selective agonist for the V2 membrane receptor (2). This receptor subtype is expressed in the kidney collecting duct, mediating the antidiuretic action, and is also present in endothelial cells, mediating most of the non-renal effects of DDAVP, including a potent hemostatic effect (3). In this regard, it is a well-tolerated hemostatic compound that can be used during surgery in patients with bleeding diathesis (4). Among other actions, DDAVP causes release of von Willebrand factor (VWF), coagulation factor VIII and tissue-type plasminogen activator from microvascular endothelial stores (3, 4).

The presence of vasopressin receptors, and particularly of V2 receptor, was reported in transformed epithelial cells, as well as in a wide panel of human tumor cell lines (5, 6). Vasopressin expression is a feature of breast cancer, and products of this expression are attractive as potential targets for therapy (7). Interestingly, DDAVP reduced *in vitro* growth in cultures of V2 receptor-expressing human breast carcinoma cells. Such a cytostatic effect was blocked by a selective V2 receptor antagonist (8, 9).

A decade ago, we reported that DDAVP was able to inhibit lung colonization by blood-borne breast cancer cells in an experimental murine model (10). Moreover, we demonstrated that DDAVP dramatically reduced lymph node and lung metastasis in a model of mammary tumor manipulation and surgical excision in mice when administered perioperatively (11), and also exerted antitumor effects in combination with chemotherapy (12). A veterinary clinical study showed that perioperative DDAVP significantly prolonged survival in surgically treated bitches with aggressive mammary cancer (13).

More recently, Monstein *et al*. presented a complete characterization of vasopressin receptor expression in the human gastrointestinal tract and surrounding tissues (14). They demonstrated that V2 receptor is widely expressed in biopsy samples from colon (ascendens, transversum and sigmoideum), rectum, ileum, duodenum, stomach, esophagus and gallbladder. In addition, expression was confirmed in commercially available colon tumor samples (14). The

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Key Words: Desmopressin, vasopressin, peptide analog, chemotherapy, metastasis, colon cancer.

central aim of the present study was to explore antitumor effects of DDAVP on human colon carcinoma cells and in a syngeneic mouse model of colon cancer.

Materials and Methods

Tumor cell lines and culture conditions. Human Colo-205 and mouse CT-26 colon carcinoma cell lines were used. Tumor 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.

Immunofluorescence detection of V2 vasopressin receptor. Colon cancer cells were seeded on glass coverslips, washed with cold phosphate-buffered saline (PBS), and then fixed with 3% paraformaldehyde for 15 min. Cells were washed with cold PBS, incubated with 50 mM NH_4Cl for 5 min, again washed with PBS, and incubated with 3% FBS as blocking agent for 30 min. Cells were then incubated with a rabbit anti-human V2 vasopressin receptor primary antibody (4 μg/ml in 0.1 % FBS) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 37˚C. This antibody recognizes a conserved epitope of the receptor, present in humans, rats and mice. Receptor-bound antibodies were detected with a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:400 in 0.1% FBS) (Chemicon International Inc., Temecula, CA, USA) and nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) using Vectashield fluorescent mounting medium (Vector Laboratories Inc., Burlingame, CA, USA). Samples were examined by standard fluorescence microscopy using a Nikon TE-2000 fluorescence microscope, and images were processed using Nikon NIS-Elements software (Nikon, Tokyo, Japan). Cultures of MCF-7 human breast carcinoma cells were used as a positive control for V2 receptor expression, as reported elsewhere (8). Negative controls consisted of omission of the primary antibody and were consistently negative.

Antitumor compounds. DDAVP acetate was obtained from Ferring Pharmaceuticals (Malmö, Sweden) and diluted in physiological saline solution. The chemotherapeutic drug 5-fluorouracil (5-FU) was provided by Bristol-Myers Squibb (Princeton, NJ, USA).

In vitro studies. Tumor cells were seeded on 96-well plates (2.5×10^3 cells/well) in DMEM plus 5% FBS. After 24 h, a range of concentrations of DDAVP (50 ng/ml-1 μg/ml) or 5-FU (15 nM-5 μM) was added and culture was continued for 72 h at 37˚C in a humidified 5% CO_2 atmosphere. The effect of compounds was tested by the 3,(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay and the concentration producing 50% inhibition (IC_{50}) was determined by plotting the percentage of cell survival *versus* drug concentration.

In vivo studies. Syngeneic adult male Balb/c mice (School of Veterinary Sciences, UNLP, La Plata, Argentina.) with a weight of 20-25 g were inoculated intraperitoneally ($1.5\text{-}3\times10^5$ cells) or intrasplenically ($1\text{-}1.5\times10^4$ cells) with CT-26 colon cancer cells. For splenic inoculation, mice were anesthetized, followed by left upper quadrant laparotomy and splenic exteriorization, as described in detail elsewher (15). Animals were monitored for water consumption, weight and general behavioral status, and sacrificed by cervical dislocation 21 days after CT-26 cell inoculation. To investigate the presence of metastases, mice were necropsied and the liver, intestines and spleen were removed and fixed in Bouin's solution. Surface hepatic and intestinal nodules were counted under a dissecting microscope, and lesions were further confirmed by histopathology. Mice were maintained under standard conditions with food and water provided *ad libitum*.

Administration of DDAVP and chemotherapy. Groups of at least 8 animals were administered DDAVP in 2 doses, 30 min before and 24 h after intraperitoneal or intraesplenic tumor inoculation. In all cases, mice received DDAVP by an intravenous route in saline solution at a final dose of 2 μg/kg body weight (50 ng in 0.3 ml saline per dose), as reported elsewhere (11). Controls received only the saline vehicle. In some experiments, a week after tumor inoculation, mice were intraperitoneally administered 5-FU at a dose of 25 mg/kg.

Statistical analysis. Differences between groups were assessed using Prism 4 statistical software (GraphPad, Inc. CA, USA). For multiple group comparisons *in vitro* one-way ANOVA was performed, followed by Dunnet or Tukey post-test. Comparison of spleen weight was done using unpaired *t*-test. The effects of DDAVP treatment on accumulation of ascites and formation of tumor nodules were evaluated with the non-parametric Mann-Whitney *U*-test. Results were considered significant at $p<0.05$.

Results

We first checked vasopressin receptor expression in human colon cancer cells by immunofluorescence. As shown in Figure 1, Colo-205 cells brightly expressed the V2 receptor on the cell surface to the same extent as in MCF-7, a human breast carcinoma cell line known to display normal forms of all vasopressin membrane receptors plus an abnormal V2 receptor (8). DDAVP significantly reduced proliferation in Colo-205 cell cultures by about 20% at a dose of 1 μg/ml (Figure 2).

We then tested the cytostatic effect of DDAVP on the CT-26 cell line, a proper syngeneic mouse colon cancer model in the Balb/c strain (11). CT-26 cells also expressed the V2 receptor (see also Figure 1), although to a lesser extent than Colo-205 cells. DDAVP significantly reduced proliferation of CT-26 cells at a dose of 100 ng/ml or higher (Figure 3A). Additionally, we explored the effect of DDAVP in combination with 5-FU, a chemotherapeutic agent commonly used for the treatment of colorectal cancer. The IC_{50} value for 5-FU was found to be 1.1 μM after a 72-h exposure of log-phase growing CT-26 cells. The combined treatment of DDAVP (1 μg/ml) and a suboptimal dose of 5-FU (250 nM) resulted in a significant increase in the growth-inhibitory effect, as compared to treatment with each compound alone (Figure 3B).

In vivo, syngeneic Balb/c mice were inoculated intraperitoneally with a high tumor burden of 3×10^5 CT-26 colon carcinoma cells. After 15-20 days, animals developed an aggressive disease with invasive tumor nodules in the bowel wall and progressive abdominal distention due to accumulation

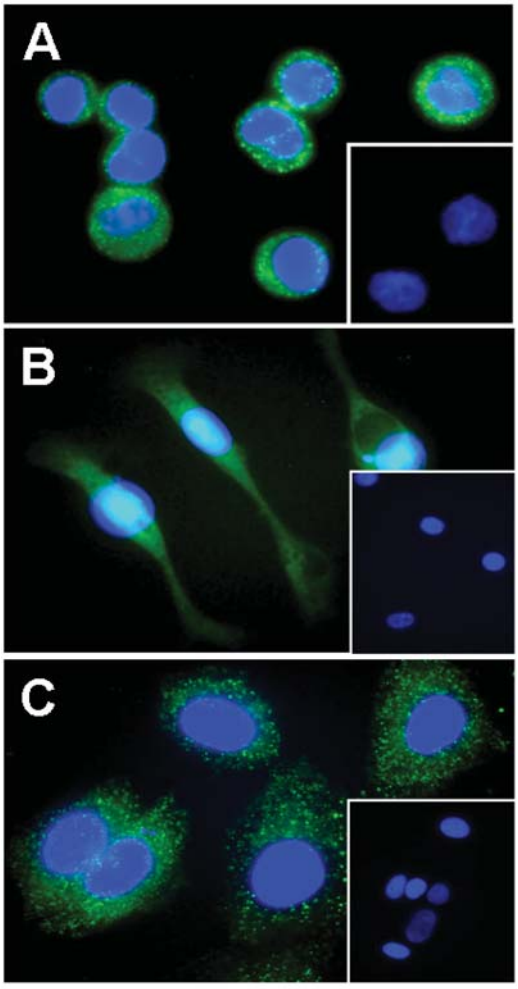

Figure 1. *Immunofluorescence detection of vasopressin receptor in colon cancer cells. Vasopressin V2 receptor expression was detected using a specific anti-V2 antibody and a secondary antibody labelled with fluorescein. A: Colo-205 human colon carcinoma cells. B: CT-26 mouse colon carcinoma cells. C: MCF-7 human breast carcinoma (positive control). Insets, no detection was found on omission of the primary antibody (negative control). Original magnification, ×1000.*

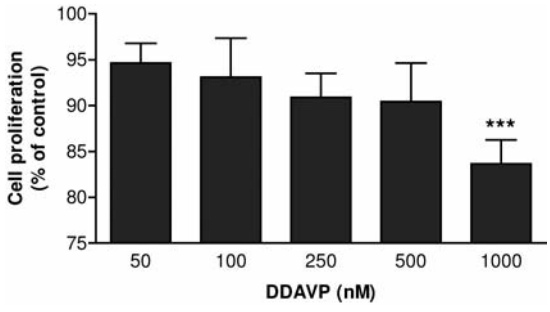

Figure 2. *Antiproliferative effect of DDAVP on human colon cancer cells. Colo-205 cells were grown on 96-well plates in the presence of appropriate concentrations of DDAVP for 72 h and then tested by the MTT assay. Values represent means±SEM from two independent experiments. ***p<0.001 versus control (ANOVA, contrasted with Dunnet test).*

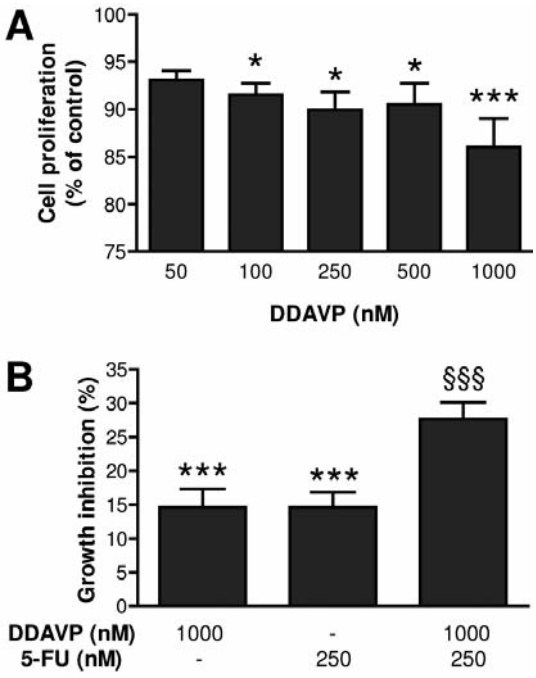

Figure 3. *Effect of DDAVP and 5-FU on in vitro growth of mouse colon carcinoma cells. CT-26 cells were grown on 96-well plates in the presence of appropriate concentrations of DDAVP and/or 5-FU for 72 h and then tested by the MTT assay. A: Dose-dependent antiproliferative effect of DDAVP. *p<0.05, ***p<0.001 versus control (ANOVA, contrasted with Dunnet test). B: Additive effect of 5-FU and DDAVP. Values represent means±SEM from three independent experiments. ***p<0.001 versus control; §§§p<0.001 versus DDAVP or 5-FU alone (ANOVA, contrasted with Tukey test).*

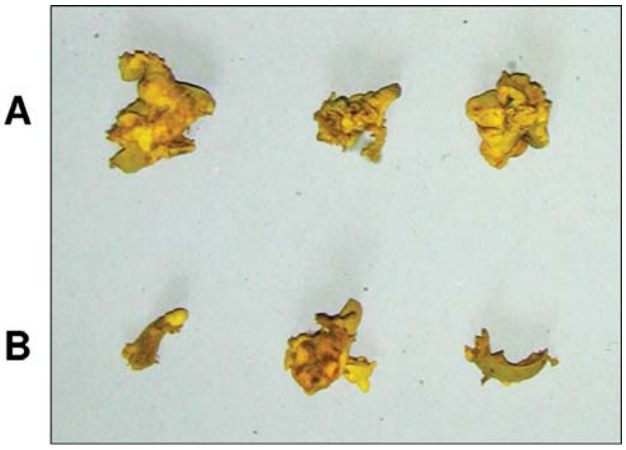

Figure 4. *Effect of DDAVP on spleen tumor development in mice inoculated with syngeneic colon carcinoma cells. DDAVP, at a dose of 2 μg/kg, was administered i.v. 30 min before and 24 h after intrasplenical inoculation of CT-26 cells. Representative spleens from untreated (A) and DDAVP-treated (B) tumor-bearing animals are shown.*

Table I. *Effect of DDAVP on the spread of colon cancer in mice inoculated intraperitoneally with CT-26 cells. Mice were inoculated intraperitoneally with 1.5×10^5 CT-26 colon carcinoma cells and DDAVP was administered i.v. 30 min before and 24 h after tumor inoculation at a dose of 2 μg/kg. The number of nodules per mouse are expressed as the median (range).*

	Intestinal tumor nodules[a]				Hepatic tumor nodules	
	Incidence (+/total)	Total	Small	Large	Incidence (+/total)	Total
Control	9/9 (100%)	43 (36-60)	14 (10-23)	29 (20-43)	5/9 (55%)	1 (0-12)
DDAVP	10/10 (100%)	37 (17-54)	11 (9-15)*	26 (8-41)	5/10 (50%)	0.5 (0-4)

[a]Tumor nodules were classified as small or large, according to the major diameter (≤1 mm or >1 mm, respectively). *p<0.05, Mann-Whitney U-test.

Table II. *Effect of DDAVP on the growth and spread of colon cancer in mice inoculated intrasplenically with CT-26 cells. Mice were inoculated intrasplenically with 1-1.5×10^4 CT-26 colon carcinoma cells and DDAVP was administered i.v. 30 min before and 24 h after tumor inoculation at a dose of 2 μg/kg. The number of nodules per mouse are expressed as the median (range). Two independent experiments are presented together.*

	Splenic tumor disease		Hepatic tumor nodules	
	Incidence (+/total)	Spleen weight (g)	Incidence (+/total)	Total
Control	18/18 (100%)	1.09±0.12	15/18 (83%)	4.5 (0-47)
DDAVP	18/18 (100%)	0.64±0.07**	12/18 (66%)	2 (0-55)

**p<0.01, unpaired t-test.

of ascites. At day 17, in controls we collected 1.24±0.75 ml per mouse (n=9) of ascites fluid, with a range of 0.3 to 2.3 ml. Mice treated with two intravenous doses of DDAVP (2 μg/kg/dose) at the time of tumor inoculation exhibited a reduction of ascites accumulation of 45%, with a volume of 0.68±0.62 ml per mouse (n=8), and a range of 0.0 to 1.9 ml. This difference, however, was not quite significant (p=0.09, Mann-Whitney U-test). We also explored the effects of DDAVP on abdominal disease progression using a lower tumor burden of 1.5×10^5 of CT-26 cells. As shown in Table I, the compound produced a modest reduction in the formation of intestinal tumor nodules, only significant when analyzing small nodules (1 mm in diameter or less). DDAVP treatment also caused a trend towards a decrease of metastatic tumor nodules in the liver.

Mice receiving surgical intrasplenic inoculation of CT-26 colon cancer cells and perioperatively administered DDAVP showed a remarkable inhibition of spleen tumor progression (Figure 4). In treated animals, a significant reduction of 41% was observed in spleen weight, as a gross measure of tumor burden (Table II). Similarly, the incidence and number of surface liver metastases were slightly lower in mice treated with DDAVP (see also Table II).

Treatment with a cycle of 5-FU one week after CT-26 inoculation did not exert relevant antitumor effects in any case under the present experimental conditions, neither alone nor in combination with DDAVP. Administration of DDAVP was not associated with overt toxic effects and no significant differences in mouse body weight or water consumption were found when compared to the control group throughout the experiment.

Discussion

Many studies have demonstrated that DDAVP can contribute to the control of locoregional disease and also reduces distant metastasis when administered perioperatively at the time of primary tumor surgery for aggressive mammary cancer, both in experimental models (10, 11) and in a veterinary clinical trial (13). To our knowledge, the present study is the first to demonstrate antitumor properties of DDAVP in a colon cancer model. Recent work reported a wide expression of vasopressin receptors in human gastrointestinal tract, while its biological significance in normal or neoplastic tissue remains to be investigated (14).

Our *in vitro* results showed moderate antiproliferative effects of DDAVP on human Colo-205 and mouse CT-26 colon carcinoma cells, and clearly confirmed the expression of V2 vasopressin receptors in these cell lines. Similarly, a mild cytostatic effect of DDAVP was previously reported on V2 receptor-positive human MCF-7 breast cancer cells (9) and also

on F3II mouse mammary carcinoma cells (12). As the compound is a selective agonist for the V2 membrane receptor, antiproliferative action is likely to be mediated through V2 receptor signaling, involving activation of adenylate cyclase followed by intracellular cAMP elevation (9, 16).

In syngeneic mice intraperitoneally inoculated with CT-26 cells, DDAVP (2 doses of 2 μg/kg) reduced accumulation of ascites and formation of intestinal tumor nodules. Furthermore, perioperative administration of DDAVP significantly inhibited tumor progression in animals surgically implanted in the spleen with CT-26 cells and caused some reduction in liver metastasis. However, no antitumor effects were observed in this study when mice received a single cycle of 5-FU (25 mg/kg), either alone or in combination with the peptide.

In addition to the antiproliferative effects of DDAVP on V2 receptor-positive tumor cells, intravenous injection of DDAVP induces a rapid release of multimeric forms of VWF from endothelial cells (4). It is known that abrupt release of VWF from the microvasculature may favor the collapse of early metastatic foci (17-19). Terraube *et al*. showed that VWF plays a protective role against tumor cell dissemination in a mouse model (18). VWF might participate in the interaction of tumor cells with the subendothelium, and appears to obstruct metastasis by reducing sustained adherence of malignant cells at the target organ. Furthermore, VWF was shown to directly induce apoptosis of tumor cells *in vitro* and caused death of metastatic cells arrested in the lungs (19). Nevertheless, we cannot exclude other *in vivo* mechanisms of antitumor action of DDAVP. For instance, preliminary results indicated that treatment of tumor cell monolayers with the compound, in the presence of proper concentrations of plasminogen, is able to induce the formation of angiostatin, a natural tumor-derived inhibitor of angiogenesis (20).

It has been suggested that surgical trauma can promote metastasis in gastrointestinal cancer. Many studies in colorectal cancer patients undergoing colonoscopy or endoscopic insertion of colonic stents demonstrated that mechanical force causes liberation of cancer cells (21, 22). Esophageal cancer operation resulted in a significant increase of circulating tumor cells, as measured in blood samples by quantitative reverse transcription-polymerase chain reaction, resulting in further development of metastases (23).

In summary, DDAVP can reduce tumor progression in an experimental colon cancer model, probably acting on specific membrane receptors present in tumor cells, and also in endothelia. It seems that a safe hemostatic agent, such as DDAVP, may be potentially used to minimize spread and survival of residual malignant cells during the perioperative period. Proper chemotherapy regimens for colon cancer in combination with DDAVP remain to be explored in more depth in preclinical models.

Acknowledgements

This work was supported by R&D Grant Program 53-1004 from Quilmes National University and by the National Agency of Scientific and Technological Promotion (ANPCyT). The support of Chemo-Romikin is also acknowledged. J.G. and G.A.H. are research fellows, and G.V.R., D.E.G. and D.F.A. are members of the National Research Council (CONICET).

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Received August 31, 2010
Revised November 3, 2010
Accepted November 5, 2010