

## Featured Article

# Depleted Dopamine in Gastric Cancer Tissues: Dopamine Treatment Retards Growth of Gastric Cancer by Inhibiting Angiogenesis

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## ABSTRACT

**Purpose:** It has been recently shown that the catecholamine neurotransmitter dopamine (DA) strongly and selectively inhibits vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)-induced angiogenesis. Gastric cancer is highly angiogenic and is dependent on VEGF for its growth and progression. Because substantial amounts of DA present in normal stomach tissues has been implicated in several gastric functions, we therefore investigated the role, if any, of this neurotransmitter in the growth and progression of gastric cancer.

**Experimental Design:** Initially, the status of DA and tyrosine hydroxylase, the rate-limiting enzyme required for DA synthesis, were determined in human gastric cancer tissues and in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric cancer tissues of rats. On the basis of our observation of inverse correlation between stomach DA and gastric cancer growth, we determined the effect of pharmacological dose of DA on the angiogenesis and growth of MNNG induced gastric cancer in rats and Hs746T human gastric cancer in nude mice.

**Results:** DA and tyrosine hydroxylase were absent in both human and rat gastric cancer tissues. On the contrary, a low nontoxic pharmacological dose of DA significantly retarded tumor angiogenesis by inhibiting VEGFR-2 phosphorylation in tumor endothelial cells, which expressed DA D<sub>2</sub> receptors. This action of DA was associated with the growth inhibition of both MNNG-induced rat malignant

gastric tumors and xenotransplanted human gastric cancer in nude mice.

**Conclusions:** Our study demonstrates that there is an inverse correlation between endogenous stomach DA and gastric cancer and indicates that DA already in clinical use for other purposes might have a role as an antiangiogenic agent in the treatment of gastric cancer.

## INTRODUCTION

Besides being a predominant neurotransmitter in the brain, the physiological significance of dopamine (DA) in the periphery has now been emphasized (1). In addition to its role in renal and cardiovascular systems (1), DA also has a wide spectrum of physiological actions in the stomach (2–5). These actions of DA are mediated through DA receptors present in the stomach (1, 3, 4).

Tumor angiogenesis is essential for the growth and progression of solid tumors, including gastric cancer and vascular endothelial growth factor (VEGF) is thought to be the single most important angiogenic cytokine in malignancy (6–10). As substantial amounts of dopamine is produced by the stomach (3–5, 11, 12) and we (S. Basu and P. Dasgupta) have recently shown that VEGF-mediated angiogenesis is inhibited by DA (13), we therefore reasoned that endogenous dopamine present in stomach tissue might have a role on the growth and progression of gastric cancer *in vivo*. Accordingly, we investigated the status of DA in human gastric cancer tissues. As *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric cancer in rats is an established *in vivo* model for human gastric adenocarcinoma (14), we also investigated the status of DA concentration in the gastric cancer tissues of these animals. We report here for the first time that unlike normal stomach tissues, there is depletion of DA in cancer tissues of the stomach in both humans and rats. Furthermore, treatment with a low nontoxic dose of DA inhibits the growth of MNNG-induced gastric cancer in rats and human gastric cancer xenografted in nude mice.

## MATERIALS AND METHODS

### Materials

MNNG, domperidone, ethylene glycol tetra acetic acid, and reduced glutathione were obtained from Sigma (St. Louis, MO). DA was from TTK Healthcare (Chennai, India). OCT compound was from Miles Diagnostics (Elkhart, IN). Collagenase and DNase were from Roche Diagnostics Corporation (Indianapolis, IN). VEGFR-2, CD16/CD32, CD31 and CD34 monoclonal antibodies for flow cytometry analysis were from BD Biosciences PharMingen (San Diego, CA). CD31 monoclonal antibody for immunohistochemistry was from BD Biosciences PharMingen. DA D<sub>2</sub> receptor, VEGFR-2, and tyrosine hydroxylase antibodies for immunoblot were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY), and

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the ABC kit was from Vector Lab (Burlingame, CA). Mouse VEGF quantikine ELISA kit and tumor TACS *in situ* apoptosis detection kit (TA 5411) were from R&D Systems (Minneapolis, MN). 5-Bromo-2'-deoxyuridine (BrdUrd) labeling and detection kit was from Roche Diagnostics Corporation. Hybond-enhanced chemiluminescence nitrocellulose membrane and enhanced chemiluminescence were from Amersham (Piscataway, NJ). DMEM and fetal bovine serum were purchased from the American Type Culture Collection (Manassas, VA).

### Patients

Gastric cancer patients of both sexes with a median age of 45 years were selected. These patients did not receive any prior treatment, including medicines, and they were also free from disorders that could alter the status of DA in the stomach. Malignant gastric tumor tissue specimens were taken during surgery from different cancer patients. Similarly, benign control tissues were collected from the biopsy specimens of patients suffering from benign adenomatous polyps of the stomach, and normal tissues were collected from the histologically normal portions of the biopsy specimens not invaded by the tumors. Tissues were frozen at  $-80^{\circ}\text{C}$  until assayed for DA concentration. The protocol to obtain tissue samples for this study was approved by the Institutional Review Board, and all samples were confirmed histologically.

### Animals and Cell Line

**Animals.** Eight-week-old male Sprague Dawley rats were supplied from the institutional animal facility, and 4–6-week-old male nude mice were purchased from National Centre for Laboratory Animal Science (Hyderabad, India). All procedures in animals were approved by the Institutional Animal Care and Use Committee.

**Cell Line.** Human gastric carcinoma cell line Hs746T was purchased from the American Type Culture Collection and were maintained in culture as recommended by American Type Culture Collection. Briefly, the cells were cultured in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, and 10% fetal bovine serum. The cultures were maintained at  $37^{\circ}\text{C}$  in an atmosphere of 5%  $\text{CO}_2/95\%$  air. Cells were grown to  $\sim 80\%$  confluence before removal for xenografting. These cells were recently reported to secrete VEGF (15).

### Rats and Tumor Induction

Eight-week-old male Sprague Dawley rats were divided into two groups: control and MNNG treatment groups. The MNNG treatment group was given MNNG dissolved in drinking water at a concentration of 80 mg/liter for the first 28 weeks (16). After 28 weeks, MNNG feeding was stopped, and these animals were given drinking water without MNNG for another 24 weeks (total of 52 weeks). The control group was given drinking water without MNNG for 52 weeks. At the 53<sup>rd</sup> week of the experiment, all rats were sacrificed. As these tumors are flat, we therefore measured the tumor diameters by microcalipers to determine their growth, and this is the standard procedure for determining the growth of these tumors (16, 17). The stomach was then removed, and then the stomach specimens were examined grossly and their histology was evaluated.

### Mice and Tumor Transplantation

To obtain tumor donor animals, three male nude mice received s.c. injections of a 0.2-ml cell suspension containing  $10^6$  cells. The well developed tumors were dissected and minced, and 2- $\text{mm}^3$  pieces of tumor tissue were transplanted s.c. to both flank areas of the experimental animals (18). When tumors became measurable, experimental groups were formed with about equal average tumor sizes, and the control and treatment groups were selected at random. Tumor growth was monitored weekly, and perpendicular tumor diameters were measured using Vernier scale calipers, and the tumor volume was calculated using the formula  $[(x^2 \times y)/2]$  for an ellipsoid (19).

### DA Treatment

**Rats.** At the beginning of the 50<sup>th</sup> week, rats with MNNG-induced gastric cancer verified to have tumor diameters of 1–2 mm in laparotomy were selected for the study. Then these tumor-bearing rats were randomly divided into three groups. The first group was injected i.p. with DA at a low nontoxic dose of 50 mg/kg, corresponding to  $\sim 5\%$  of the median lethal dose ( $\text{LD}_{50}$ ) in rodents (20), beginning 50<sup>th</sup> week after MNNG feeding, and was continued for 10 days. This i.p. dose of DA raises the level of DA in rats and mice to  $2.1 \pm 0.04$  and  $1.2 \pm 0.01$  nmol/ml, respectively, in plasma 1 min after DA injection (the normal plasma level of DA in rats and mice were  $2.4 \pm 0.01$  and  $1.5 \pm 0.01$  pmol/ml, respectively).<sup>5</sup> A specific peripheral  $\text{D}_2$  DA receptor antagonist, domperidone, was injected i.p. (10 mg/kg) to the second group of rats before DA treatment. The third group received the vehicle only. After completion of DA treatment, rats from all of the three groups were sacrificed.

**Mice.** The treatment of the mice bearing Hs746T tumors was started 30 days after transplantation, when the mean tumor volume was 100  $\text{mm}^3$ . These tumor-bearing mice were randomly divided into three groups. First group was injected with DA i.p. at a dose of 50 mg/kg/day for 10 continuous days. A specific peripheral  $\text{D}_2$  DA receptor antagonist domperidone was injected i.p. (10 mg/kg) to the second group of tumor-bearing mice before DA treatment. The third group received 0.9% NaCl, i.p., for 10 consecutive days. After the completion of the treatment, all of the mice were sacrificed, tumors were excised and processed for further experiment.

### Assay of DA by High-Performance Liquid Chromatography

Samples were processed and assayed for DA by reverse-phase high-performance liquid chromatography with electrochemical detection. In short, tissue was weighed and then homogenized in 10.2 ml of ice-chilled 0.6 M perchloric acid containing 1.7 mg/ml ethylene glycol tetra acetic acid and 1.1 mg/ml reduced glutathione. After centrifugation for 15 min at  $2500 \times g$  at  $0^{\circ}\text{C}$ , 1.0 ml of the supernatant was adjusted to pH 8.6 with 6 M potassium hydroxide and then processed for de-

<sup>5</sup> S. Basu and P. S. Dasgupta, unpublished data.

termination of DA concentration after alumina-batch extraction. Twenty-five  $\mu\text{l}$  of 3 M potassium chloride were added to 200  $\mu\text{l}$  of the 0.2 M perchloric acid eluate. After centrifugation, 50  $\mu\text{l}$  of the supernatant were injected into the high-performance liquid chromatography system. The limit of sensitivity in the supernatant, defined as two times baseline noise, was 0.01 ng/ml for DA. If a concentration was lower than the limit of sensitivity, the concentration of the DA was assigned the value 0 (21).

### Flow Cytometry Analysis

A suspension of Hs746T-malignant human gastric tumors was made by passage of viable tissue through sieve and treatment with collagenase and DNase. The cells were washed, and the RBCs were lysed with PharM Lyse (BD PharMingen). The cell pellets were then resuspended in fluorescence-activated cell sorting buffer ( $1\times$  PBS plus 1% BSA), preblocked with a Fc block (CD16/CD32), and then incubated with primary antibody on ice: phycoerythrin-conjugated anti-VEGF receptor-2 (anti-VEGFR-2), 1:100; CD31, 1:100; and CD34, 1:100. One million positive tumor endothelial cells were collected by fluorescence-activated cell sorting (22, 23).

### Immunoprecipitation and Western Blot Analysis

Human and rat tissues were homogenized in 0.2% Triton in PBS (pH 7.5), assayed for protein content, and stored at  $-80^{\circ}\text{C}$ . Either 20  $\mu\text{g}$  of protein from human or rat stomach tissues or 2  $\mu\text{g}$  of protein from rat adrenals was size fractionated by SDS-PAGE and then electroblotted onto Hybond-enhanced chemiluminescence nitrocellulose membrane at 30 V for 18 h in transfer buffer (20% methanol, 25 mM Tris, and 192 mM glycine). Blots were preblocked in 5% nonfat dry milk in Tris-buffered PBS and incubated with anti-tyrosine hydroxylase antibodies (1:500) for 1 h at  $22^{\circ}\text{C}$ . Blots were then incubated with horseradish peroxidase-conjugated mouse antigoat IgG (1:1000) for 1 h at  $22^{\circ}\text{C}$  and developed using enhanced chemiluminescence (5). Immunoprecipitation and Western blot analysis using antibodies against DA  $D_2$  receptors (1:100) were also performed on Hs746T human gastric cancer cells and tumor endothelial cells isolated from Hs746T tumors. A similar experiment was performed on the same tumor endothelial cells using antibodies against VEGFR-2 (1:100) and phosphotyrosine (1:100).

### Plasma and Tumor Tissue VEGF Assays

The blood was collected from the rats in EDTA containing tubes on ice by cardiac puncture. The blood samples were centrifuged at 800 rpm for 30 min at  $4^{\circ}\text{C}$ , followed by 10 min at 3000 rpm. The plasma was collected and stored at  $-80^{\circ}\text{C}$  until analysis. Plasma and tissue VEGF were then determined by ELISA following the protocol provided by the manufacturer (R&D Systems) that has been shown to recognize both rodent and murine VEGF (24, 25).

### Immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde, rinsed in PBS, transferred to 30% sucrose in PBS at  $4^{\circ}\text{C}$ , and frozen in OCT compound. Immunohistochemistry was then performed on frozen tissue sections using antibody against CD31 (1:50). Mi-

crovessel density was quantitated by analyzing 10 random fields/section (26).

### Detection of Apoptotic Tumor Cells

Tumor cell apoptosis was assessed by measuring DNA fragmentation in a standard terminal deoxynucleotidyl transferase-mediated nick end labeling assay according to the instruction of the manufacturer (R&D Systems). The number of apoptotic cells was expressed as an apoptotic index (8).

### Cell Proliferation Index

The cellular kinetics of the tumor specimens was determined by immunohistochemical staining of BrdUrd. One h before sacrificing the animals, 20 mg/kg body weight of BrdUrd were injected i.p. into each animal. Subsequently, the animals were sacrificed, and tumors were excised and fixed in buffered formalin and embedded in paraffin. Cross-sections of the tissues were cut 4- $\mu\text{m}$  thick. Tissue sections were then immunostained by using BrdUrd labeling and detection kit. The percentage of stained cells was counted and expressed as labeling index (16).

### Statistics

Mean and SE were calculated. Differences among groups were evaluated by ANOVA and the unpaired Student's test or Dunn's multiple comparison tests.

## RESULTS

### MNNG-Induced Intestinal Type of Gastric Adenocarcinoma

MNNG-treated rats developed a single (Fig. 1A) intestinal type of adenocarcinoma of the stomach similar to human gastric cancers (Fig. 1B), and our results are in conformity with other studies (14, 27).

### Absence of DA and Tyrosine Hydroxylase in Gastric Carcinoma

In normal stomach tissues of humans and rats substantial amounts of DA (Table 1) and tyrosine hydroxylase (Fig. 2, A and B), the rate-limiting enzyme required for DA synthesis (5) was observed. On the contrary, there was total absence of endogenous DA (Table 1) and tyrosine hydroxylase (Fig. 2, A and B) in malignant tumor tissues of the stomach collected from humans and rats.

### Significant Secretion of VEGF and Striking Angiogenesis in Malignant Gastric Tissues

MNNG induced gastric cancer tissues showed striking angiogenic response as evident from CD31 expression (Fig. 3A). In contrast, we found CD31 expression in normal rat gastric tissues to be very low or insignificant (20–30/10 randomly chosen high power fields), and our results are in conformity with a recent study that indicated similar results (28). Furthermore, these malignant tumor tissues also showed a significantly higher concentration of VEGF in comparison to normal controls (Table 2;  $6.7 \pm 0.8$  versus  $0.7 \pm 0.02$  ng/g tissue, mean  $\pm$  SE,  $P < 0.05$ ). The plasma VEGF levels were also significantly higher in the malignant tumor-bearing rats when compared with normal controls (Table 2;  $25.7 \pm 1.6$  versus  $8.8 \pm 1.0$  pg/ml, mean  $\pm$



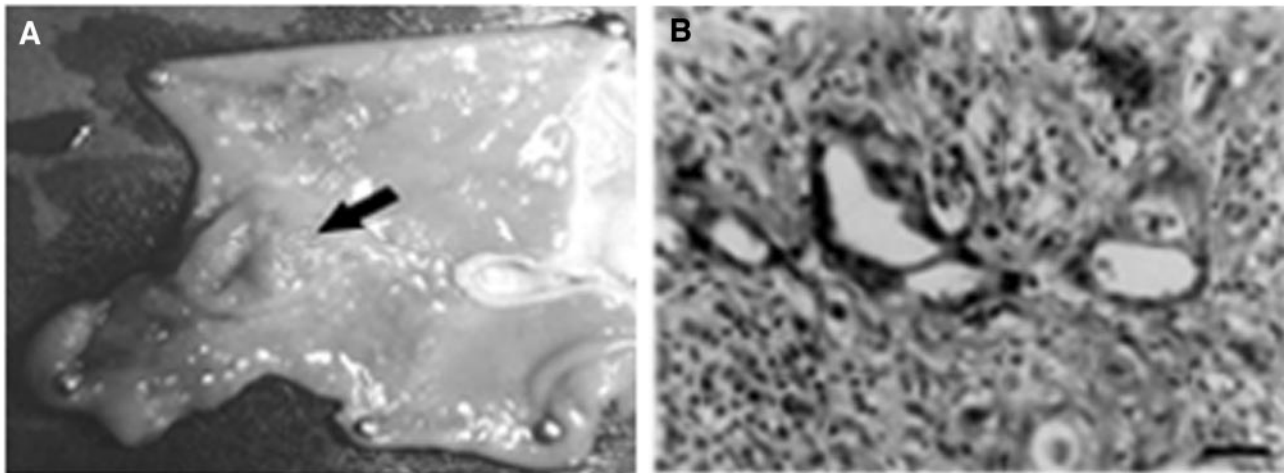


Fig. 1 *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induced gastric adenocarcinoma in a rat. Gross (A) and H&E staining (B) show intestinal type of adenocarcinoma of the stomach. B, scale bar: 75  $\mu$ m.

Table 1 DA concentration in human and rat gastric tissues<sup>a</sup>  
Results are mean  $\pm$  SE.

	No. of humans/rats	Normal/Benign tissue (ng/g tissue) <sup>b</sup>	Tumor tissue (ng/g tissue)
Human			
Normal	22	40.0 $\pm$ 3.4	
Adenomatous Polyp	6	41.7 $\pm$ 5.9	
Adenocarcinoma	22		Not detected
Rat			
Normal	24	21.4 $\pm$ 1.7	
MNNG-induced adenocarcinoma-bearing rats	18		Not detected

<sup>a</sup> MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DA, dopamine.

<sup>b</sup>  $P < 0.05$  when compared with adenocarcinoma tissues. Tissue DA was measured by high-pressure liquid chromatography with electrochemical detection.

SE,  $P < 0.05$ ). These results corroborate the striking angiogenesis observed in these tumors and, to our knowledge, is the first article that indicates that MNNG-induced malignant gastric tumors in rats also secrete VEGF.

#### DA Treatment Decreased Tumor Diameter and Angiogenesis and Increased Tumor Cell Apoptosis

**Rats.** DA when administered to gastric adenocarcinoma-bearing rats showed 3-fold decrease ( $P < 0.5$ ) in tumor diameter than control animals (Table 3). This was also associated with significant inhibition of tumor cell proliferation as evident from reduction of BrdUrd incorporation in malignant stomach tissues after DA treatment in both rats and mice (Table 4). These DA-treated animals with decreased tumor diameter also showed significant inhibition of tumor angiogenic response than untreated controls as evidenced from decreased frequency of CD31<sup>+</sup> cells in DA-treated tumor tissues (Fig. 3, A–D). Decreased angiogenic response in tumor tissues after DA treatment

was also associated with increased apoptosis of tumor cells when compared with controls (Fig. 4, A–D). The increase in apoptosis of tumor cells after DA treatment are in conformity with other studies that indicate that inhibition of angiogenesis results in apoptosis of tumor cells (8, 26). In contrast, DA did not inhibit tumor growth (Table 3) in rats that received prior treatment of domperidone (10 mg/kg i.p.), a specific peripheral D<sub>2</sub> DA receptor antagonist, thereby confirming that the action of DA is through D<sub>2</sub> DA receptor. The other two catecholamines, epinephrine and norepinephrine, did not inhibit either angiogenesis or tumor growth (data not shown).

**Mice.** Similarly DA when administered to Hs746T-bearing mice also showed 3-fold decrease ( $P < 0.5$ ) in tumor volume than control animals (Table 5). This was also associated with significant inhibition of tumor cell proliferation as evident from reduction of BrdUrd incorporation in malignant stomach

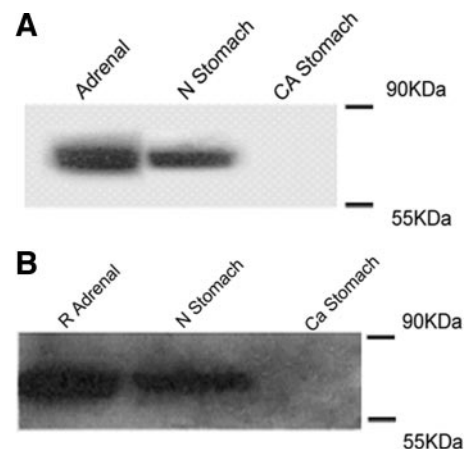
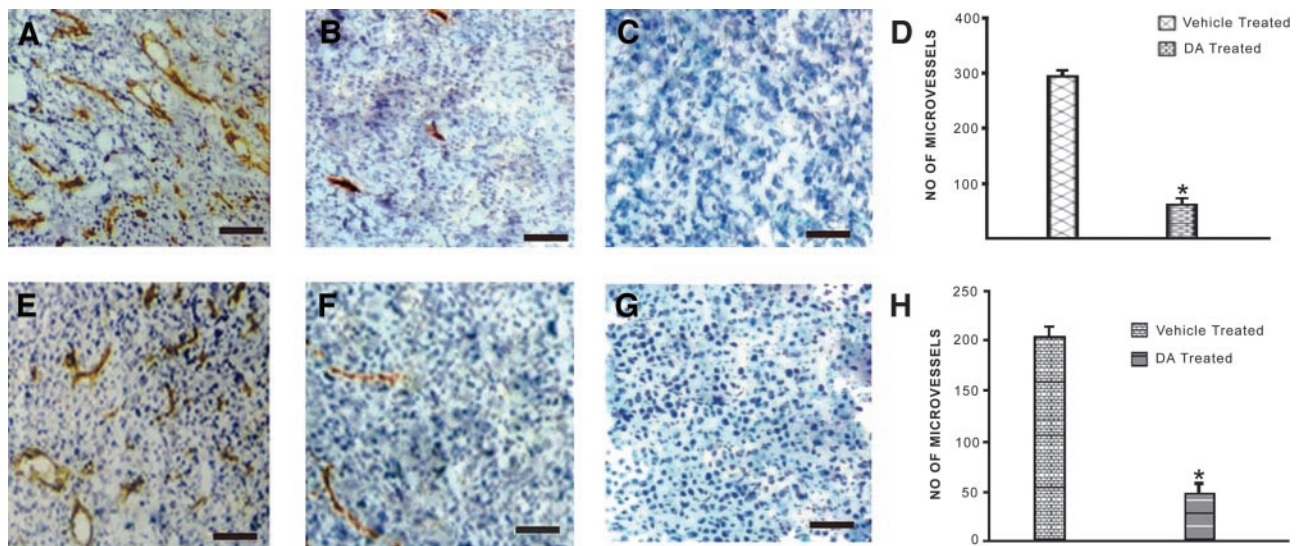


Fig. 2 Western blotting of tyrosine hydroxylase in normal and malignant tumor tissue of stomach in humans (A) and in rats (B). Tyrosine hydroxylase is present in normal stomach but absent from tumor tissue in both cases. The figures are representatives of six separate experiments with similar results.



**Fig. 3** Evaluation of microvessel density by immunohistochemical analysis of CD31<sup>+</sup> in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced rat malignant gastric tumor (A–D) and in Hs746T gastric cancer-bearing nude mice (E–H). Significant expression of CD31<sup>+</sup> cells in vehicle-treated tumor tissue (A, E, D, and H). Dopamine (DA)-treated tumor tissue showed marked reduction in CD31<sup>+</sup> cells (B, F, D, and H). Negative control consisted of sections in which primary antibody was replaced with nonimmune serum of the same IgG subclass (C and G). Microvessel density was measured by counting the number of microvessels in 10 randomly chosen high power microscopic fields within the sections showed that microvessel density was significantly reduced (\*,  $P < 0.05$ ) in DA-administered tumor-bearing animals (B and F) when compared with vehicle-treated tumor-bearing animals (A and E). A, B, C, E, F, G, scale bars: 50  $\mu$ m.

tissues after DA treatment in mice (Table 4). Decreased angiogenic response in tumor tissues after DA treatment was also associated with increased apoptosis of tumor cells when compared with controls (Fig. 5). The increase in apoptosis of tumor cells after DA treatment are in conformity with other studies that indicate that inhibition of angiogenesis results in apoptosis of tumor cells (8, 19). These DA-treated animals with decreased tumor volume also showed significant inhibition of tumor angiogenic response than untreated controls as evidenced from decreased frequency of CD31<sup>+</sup> cells in DA-treated tumor tissues (Fig. 3, E–H). In contrast, DA did not inhibit tumor growth (Table 5) in mice that received prior treatment of domperidone (10 mg/kg i.p.), a specific peripheral D<sub>2</sub> DA receptor antagonist, thereby confirming that the action of DA is through D<sub>2</sub> DA receptor. This result was additionally strengthened as we found

**Table 2** VEGF concentrations in plasma and stomach tissue of normal and MNNG-induced gastric adenocarcinoma-bearing rats<sup>a</sup>

Results are mean  $\pm$  SE. Plasma and tissue VEGF concentrations were assayed using sandwich ELISA assay kit.

	No. of animals	VEGF concentration in plasma	VEGF concentration in tissue (ng/gm tissue)
Normal	22	8.8 $\pm$ 1.0	0.7 $\pm$ 0.02
Vehicle-treated tumor-bearing rats	18	25.7 $\pm$ 1.6 <sup>b</sup>	6.7 $\pm$ 0.8 <sup>b</sup>
Dopamine treated tumor-bearing rats	18	23.2 $\pm$ 1.2 <sup>b</sup>	6.4 $\pm$ 0.6 <sup>b</sup>

<sup>a</sup> VEGF, vascular endothelial growth factor; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

<sup>b</sup>  $P < 0.05$  when compared with normal control.

**Table 3** Effect of dopamine administration on the growth of MNNG induced gastric adenocarcinoma in rats<sup>a</sup>

Results are mean  $\pm$  SE.

	No. of animals	Tumor diameter (mm)
Vehicle treated	18	4.4 $\pm$ 0.02
DA treated	18	1.3 $\pm$ 0.01 <sup>b</sup>
Domperidone + DA	18	4.1 $\pm$ 0.04

<sup>a</sup> MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DA, dopamine.

<sup>b</sup>  $P < 0.05$  when compared with vehicle-treated control. DA treatment started when the tumor diameter was 1.2  $\pm$  0.01 (mm) and continued for consecutive 10 days at a dose of 50 mg/kg/day i.p. Domperidone (10 mg/kg/day) was administered before DA treatment everyday. Tumor diameter was measured 24 h after the completion of treatment.

**Table 4** BrdUrd incorporation in MNNG induced rat and Hs746T human gastric tumor tissues<sup>a</sup>

Results are mean  $\pm$  SE.

	No. of animals	Cell proliferation index
Rat		
Normal control	24	9.12 $\pm$ 0.8
Vehicle-treated tumor-bearing rats	24	23.41 $\pm$ 1.1 <sup>b</sup>
DA-treated tumor-bearing rats	24	12.33 $\pm$ 0.7 <sup>c</sup>
Nude mice		
Vehicle-treated Hs746T-bearing mice	12	19 $\pm$ 3.7
Dopamine treated Hs746T-bearing mice	12	10 $\pm$ 2.1 <sup>b</sup>

<sup>a</sup> BrdUrd, 5-bromo-2'-deoxyuridine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DA, dopamine.

<sup>b</sup>  $P < 0.05$  when compared with normal control.

<sup>c</sup>  $P < 0.05$  when compared with vehicle-treated tumor-bearing rats. Cell proliferation index was determined by counting the percentage of BrdUrd-positive cells.

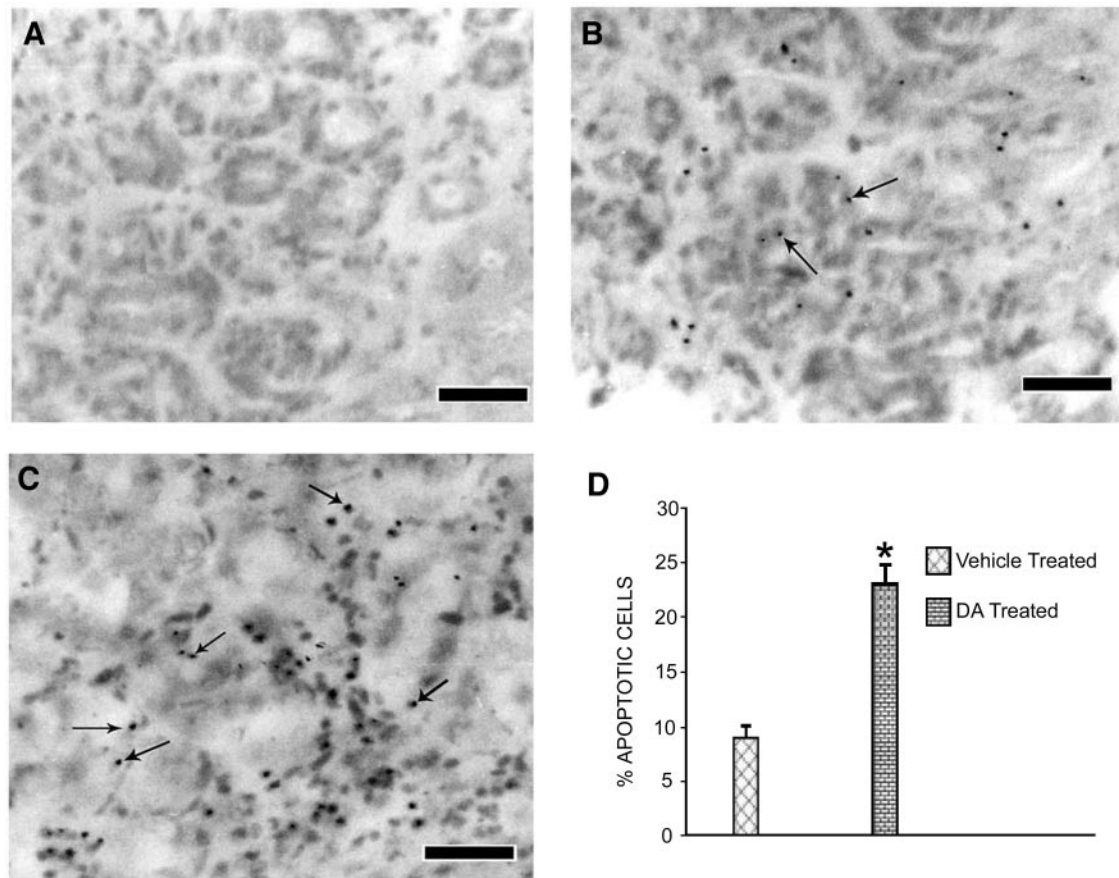


Fig. 4 Effect of dopamine (DA) on tumor cell apoptosis in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced gastric cancer in rats as determined by terminal deoxynucleotidyl transferase-mediated nick end labeling assay. A, negative control (without terminal deoxynucleotidyl transferase enzyme). Vehicle treatment (B) and DA treatment (C) show increased apoptosis of tumor cells. Apoptotic index was determined by the ratio of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells and total number of cells (\*,  $P < 0.05$ ). A–C, scale bars: 50  $\mu$ m.

D<sub>2</sub> DA receptors in tumor endothelial cells isolated from these tumor-bearing mice (Fig. 6A). The D<sub>2</sub> DA receptors were absent in Hs746T gastric cancer cells (Fig. 6A). Furthermore, VEGF is thought to induce angiogenesis by phosphorylating VEGFR-2, and our recent article had indicated that DA inhibits VEGFR-2 phosphorylation *in vitro* (13). In the present investigation, we found that DA treatment strongly inhibited VEGF-induced

phosphorylation of VEGFR-2 in tumor endothelial cells isolated from Hs746T-malignant gastric tumors (Fig. 6B) and pretreatment with domperidone, a specific peripheral D<sub>2</sub> DA receptor antagonist, significantly abrogated this effect (Fig. 6B), indicating the specificity of DA action. The other two catecholamines, epinephrine and norepinephrine, did not inhibit either angiogenesis or tumor growth (data not shown).

Table 5 Effect of DA administration on the growth of Hs746T human gastric tumor-bearing nude mice<sup>a</sup>

Results are mean  $\pm$  SE.

	No. of animals	Tumor volume (mm <sup>3</sup> )
Vehicle treated	12	311.5 $\pm$ 11.9
DA treated	12	106.0 $\pm$ 7.4 <sup>b</sup>
Domperidone + DA	12	302.7 $\pm$ 12.8

<sup>a</sup> DA, dopamine.

<sup>b</sup>  $P < 0.05$  when compared with the vehicle-treated control group. DA treatment started when tumor volume was 100 mm<sup>3</sup> and continued for 10 consecutive days at a dose of 50 mg/kg/day i.p. Domperidone (10 mg/kg/day i.p.) was administered before DA treatment everyday. Tumor diameter was measured 24 h after the completion of treatment.

## DISCUSSION

Endogenous DA produced in substantial amounts in normal stomach tissues regulates several physiological functions of the stomach, including reduction of gastric acid secretion, stimulation of bicarbonate, and mucus secretion (2–4). Here, we show for the first time that DA is depleted in both human gastric cancer and MNNG-induced adenocarcinoma of the stomach in rats. Furthermore, this finding is also significant because our results indicate that there is a significant retardation in the growth of gastric cancer after treatment with exogenous DA at a low nontoxic dose.

VEGF is thought to be the single most important cytokine essential for tumor angiogenesis (6–10), and we had recently demonstrated that DA specifically and significantly inhibits



VEGF-mediated angiogenesis (13). DA acted through D<sub>2</sub> DA receptors to induce endocytosis of VEGFR-2, which is critical for promoting angiogenesis, thereby preventing VEGF binding, receptor phosphorylation, and subsequent signaling steps (13). Malignant human gastric tumors secrete VEGF and therefore are highly angiogenic tumors (8). In the present experiment, MNNG-induced malignant gastric tumor-bearing rats showed significant elevation of plasma VEGF, and this correlated well with their angiogenic nature. There is also a recent article that indicates that the human gastric cancer cell line Hs746T selected for our experiment also secretes considerable amount of VEGF (15). Therefore, one of the important mechanisms by which DA may retard gastric cancer growth is through inhibition of VEGF-mediated angiogenesis. Our present results confirm that DA retards the growth of gastric cancer by inhibiting VEGF-induced angiogenesis. The underlying mechanism of this action has been shown to be mediated by inhibition of VEGFR-2 phosphorylation in tumor endothelial cells involving DA D<sub>2</sub> receptors present on these cells as treatment with domperidone, a specific peripheral DA D<sub>2</sub> receptor antagonist, showed significant abrogation of this inhibitory effect of DA. However, there may be other mechanisms also by which DA may inhibit tumor growth (29). It would also be noteworthy to mention here that there are few contradictory studies that have shown that administration of a DA agonist stimulated MNNG-induced carcinogenesis and a DA antagonist inhibited MNNG-induced carcinogenesis (30–32). However, in those articles, the authors had only shown the effects of DA agonist and antagonist on the process of MNNG-induced carcinogenesis (incidence of malignant gastric tumors), and nothing was mentioned regarding the effects of those agonist and antagonist on the growth and progression of a developed gastric cancer. In addition, there is also an article that indicates that DA inhibits the process of MNNG-induced glandular stomach carcinogenesis in rats (33). However, that study was also limited to the incidence of gastric cancer in the rats, and no effect of DA on the growth and progression of malignant gastric tumor was mentioned (33). Therefore, in brief, the present results are of both physiological and clinical significance because it has been shown for the first time that there is an inverse correlation between endogenous stomach DA and gas-

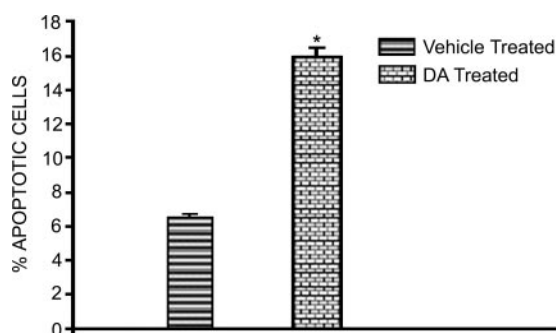


Fig. 5 Effect of dopamine (DA) on tumor cell apoptosis as determined by terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Increased apoptosis of tumor cells in Hs746T gastric cancer-bearing nude mice after DA treatment. Apoptotic index was determined by the ratio of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells and total number of cells (\*,  $P < 0.05$ ).

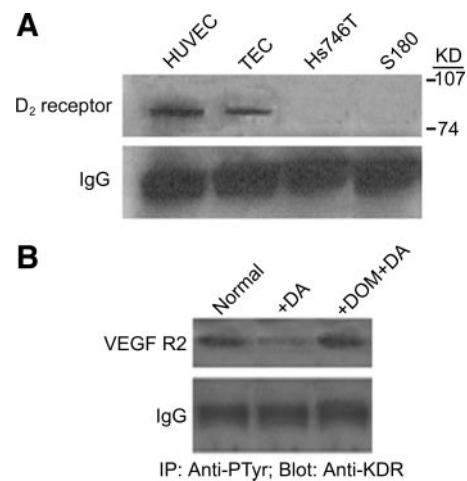


Fig. 6 A, immunoblot shows Mr ~85,000 D<sub>2</sub> dopamine (DA) receptor in extracts of tumor endothelial cells (TECs) of Hs746T, grown in nude mice and in positive control human umbilical vascular endothelial cells (HUVECs) but not in Hs746T gastric cancer cells and negative control sarcoma 180 tumor cells. The figure is representatives of six separate experiments with similar results. B, DA treatment inhibits vascular endothelial growth factor (VEGF)-induced phosphorylation of VEGF receptor-2 (VEGFR-2) but prior treatment with D<sub>2</sub> DA receptor antagonist domperidone has no effect.

tric cancer. These results also indicate a direct gastric cancer growth inhibitory action of nontoxic dose of DA *in vivo*.

Finally, gastric cancer is one of the leading causes of cancer death worldwide (34). Radiation therapy or chemotherapy does not affect the length and quality of life of patients with advanced gastric cancer (8). Thus, novel therapies are needed to target the progression of these tumors. DA is already in clinical use with an established safety record (35). Therefore, DA can be tested without the usual delays that would be required to assess the safety of a new drug in patients with gastric cancer in which VEGF-mediated angiogenesis has an important pathogenic role (8). In addition, information concerning the potent antitumor angiogenic activity of DA in gastric cancer needs to be disseminated widely and rapidly so that clinical trials can be started as soon as possible to combat this very common cancer, which, as of yet, has no significant treatment.

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