Dopamine Increases the Efficacy of Anticancer Drugs in Breast and Colon Cancer Preclinical Models

Chandrani Sarkar,[1,3] Debanjan Chakroborty,[1,3] Uttio Roy Chowdhury,[1]
Partha Sarathi Dasgupta,[1] and Sujit Basu[2,3,4]

Abstract

Purpose: Because neurotransmitter dopamine inhibits vascular permeability factor/vascular endothelial growth factor (VEGF) – induced angiogenesis and as anti-VEGF agents act synergistically with anticancer drugs, we therefore investigated whether dopamine can increase the efficacies of these drugs.

Experimental Design: The effect of dopamine was investigated in human breast cancer – (MCF-7) and colon (HT29) cancer – bearing mice. Experimental groups received either dopamine or doxorubicin or dopamine plus doxorubicin in MCF-7 tumor-bearing mice, and either dopamine or 5-fluorouracil or dopamine plus 5-fluorouracil in HT29-bearing mice. Thereafter, tumor growth, angiogenesis, tumor cell apoptosis, life span, and the effect of dopamine on the growth and survival of tumor cells *in vitro* were determined. Finally, the effects of dopamine on tumor vascular permeability; on VEGF receptor-2, mitogen-activated protein kinase, and focal adhesion kinase phosphorylation; and also on the proliferation and migration of tumor endothelial cells were investigated.

Results: Dopamine, in combination with anticancer drugs, significantly inhibited tumor growth and increased the life span when compared with treatment with dopamine or anticancer drugs alone. Dopamine had no direct effects on the growth and survival of tumor cells. The antiangiogenic action of dopamine was mediated by inhibiting proliferation and migration of tumor endothelial cells through suppression of VEGF receptor-2, mitogen-activated protein kinase, and focal adhesion kinase phosphorylation.

Conclusion: Our study shows that dopamine significantly enhances the efficacies of commonly used anticancer drugs and also indicates that an inexpensive drug like dopamine, which is being extensively used in the clinics, might have a role as an antiangiogenic agent for the treatment of breast and colon cancer.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

The importance of angiogenesis as an integral component of tumor growth has fueled considerable efforts to develop efficient treatment regimens consisting of antiangiogenic agents that may inhibit the formation of new blood vessels in tumors and thereby the growth of these tumors (1 – 3). Angiogenesis inhibitors are considered to be relatively noncytotoxic and also these antiangiogenic agents exhibit different mechanisms of tumor growth inhibition than conventional anticancer agents (2 – 4). A major issue in oncology at present is the development of effective adjuvant therapy following resection of primary tumors to inhibit the growth of cancer cells remaining either locally or at remote sites and having the potential to grow into a recurrent tumor mass. Antiangiogenic therapy could restrain such lesion or even drive those cancer cells to apoptosis (1 – 6). Therefore, administration of antiangiogenic drugs in combination with anticancer drugs may be a better therapeutic strategy due to significant tumor growth – retarding property of these antiangiogenic agents (5, 6).

Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is the prime cytokine reported to induce tumor angiogenesis by stimulating proliferation and migration of endothelial cells (1, 2, 7). VPF/VEGF is also reported to be expressed in most of the malignant tumors, and treatments that interfere with VPF/VEGF functions have shown promising results (1, 2, 5 – 7). Also, angiogenesis inhibitors that interfere with the expression or signaling of VPF/VEGF on tumor endothelial cells are reported to enhance the survival of cancer patients when used in combination with conventional cytotoxic

Authors' Affiliations: [1]Signal Transduction and Biogenic Amines Laboratory and [2]Department of Medical Oncology, Chittaranjan National Cancer Institute, Kolkata, India; and [3]Department of Biochemistry and Molecular Biology and [4]Mayo Clinic Cancer Center, Mayo Clinic, Rochester, Minnesota

Received 7/19/07; revised 12/10/07; accepted 1/10/08.

Grant support: Council of Scientific and Industrial Research Government of India grant [27 (0120)/03/EMR-II; P.S. Dasgupta and S. Basu] and fellowship [27 (F.) No. 9/30 (23)/2001-EMR-1; C. Sarkar] and NIH grants CA118265 and CA 124763 (S. Basu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: C. Sarkar and D. Chakroborty contributed equally to this work.

Requests for reprints: Partha Sarathi Dasgupta, Signal Transduction and Biogenic Amines Laboratory, Chittaranjan National Cancer Institute, 37 S.P. Mukherjee Road, Kolkata 700026, India. Phone: 91-33-24765101, ext. 324; E-mail: partha42002@yahoo.com or Sujit Basu, Department of Biochemistry and Molecular Biology and Cancer Center, Mayo Clinic, Gugg 1793, 200 First Street Southwest, Rochester, MN 55905. Phone: 507-284-1344; E-mail: basu.sujit@mayo.edu.

©2008 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-07-1778

anticancer agents (5–7). However, due to emergence of resistance to the currently used antiangiogenic drugs, it is essential to identify newer and effective antiangiogenic agents for the treatment of cancer (5, 7).

Dopamine is a catecholamine neurotransmitter that we recently showed to inhibit VPF/VEGF–induced angiogenesis (8). We have also shown that endogenous dopamine could be a potent regulator of tumor angiogenesis and its subsequent growth (9). Furthermore, as there are now several reports which indicate that antiangiogenic agents act synergistically with conventional anticancer drugs and because this combination is more effective than antiangiogenic agents used alone (5–7), it is prudent to investigate the potential of dopamine as an antiangiogenic drug in combination with other anticancer drugs. Accordingly, in the present study, we determined the efficacy of dopamine either alone or in combination with conventional anticancer drugs like doxorubicin or 5-fluorouracil (5-FU) in xenotransplanted human breast tumor (MCF-7) and orthotopically implanted human colon cancer (HT29), respectively. Our results for the first time showed that dopamine alone was effective in retarding the growth of these tumors by inhibiting tumor angiogenesis and most importantly dopamine showed significant synergism with conventional anticancer drugs. Also, this combination schedule significantly increased the survival of the tumor-bearing animals when compared with treatment with a single drug.

Materials and Methods

Materials

Dopamine was obtained from Abbott Laboratories; doxorubicin was from Pharmacia; and 5-FU was from Cadila Pharmaceuticals. CD31 rat anti-mouse monoclonal antibody and biotinylated anti-rat secondary antibody were from BD PharMingen. Vectastain Elite ABC kit was from Vector Laboratories. Tumor TACS *in situ* apoptosis detection kit was from R&D Systems. Matrigel was from BD Biosciences. Collagenase and DNase were from Roche Diagnostics Corporation. VEGF receptor-2 (VEGFR-2), CD31, and CD34 monoclonal antibodies for flow cytometry analysis were from BD Biosciences PharMingen. VEGFR-2, phospho–mitogen-activated protein kinase (MAPK), and phospho–focal adhesion kinase (FAK) antibodies were from Santa Cruz Biotechnology. Phosphotyrosine antibody was purchased from Upstate Biotechnology. 5-Bromo-2′-deoxyuridine (BrdUrd) labeling and detection kit was from Roche Applied Science. [^{3}H]thymidine was from BARC, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was from Roche Applied Science.

Animals

Four- to 6-wk-old nude mice were housed under pathogen-free conditions with a 12 h light/12 h dark schedule and fed autoclaved standard pellets and water *ad libitum* throughout the experiment. All procedures were approved by the institutional care and use committee of Chittaranjan National Cancer Institute, Kolkata, India, and Mayo Clinic, Rochester, MN.

Table 1. Treatment groups for human breast cancer (MCF-7)–bearing athymic nude mice

Follow-up group	Vehicle	DA	DXR	DA + DXR
A. Sacrifice on day 8	8	8	8	8
B. Follow to death	14	14	14	14

Abbreviations: DA, dopamine; DXR, doxorubicin.

Table 2. Treatment groups for nude mice bearing orthotopically implanted human colon cancer (HT29)

Follow-up group	Vehicle	DA	5-FU	DA + 5-FU
A. Sacrifice on day 8	8	8	8	8
B. Follow to death	14	14	14	14

Cell lines

MCF-7 human breast cancer and HT29 human colon cancer cell lines were purchased from the American Type Culture Collection. MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and HT29 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum. Adherent monolayer cultures were maintained on plastic dishes and incubated at 37°C in a mixture of 5% CO_2 and 95% air.

Tumor transplantation and dopamine treatment

MCF-7. The human breast cancer cell line MCF-7 (2×10^6 cells) was established by s.c. injection into the right flank of female athymic nude mice. Briefly, tumor cells were detached by trypsinization, harvested by centrifugation, resuspended in serum free medium (200 μL), and mixed with equal volume of cold Matrigel (10 mg/mL). Cells were injected s.c. into 4- to 6-wk-old nude mice preimplanted with 60-d release estradiol pellets (10). Tumor volume was measured twice weekly and animals with tumor volume ~100 mm^3 were randomly divided into two main groups, A and B (Table 1). The animals within these two groups were further randomized to one of the four treatment groups, including vehicle, dopamine (50 mg/kg/d i.p. × 7 continuous days), doxorubicin (5 mg/kg/i.p./single dose), and dopamine (50 mg/kg/d i.p. × 7 continuous days) + doxorubicin (5 mg/kg/i.p./single dose; Table 1). Group A animals were sacrificed on day 8, and group B animals were followed to death. All experiments, except the survival study (14 animals per group was used for the survival study), were done in triplicate.

HT29. To produce cecal tumors, HT29 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Thereafter, trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for injection of 1×10^6 cells in 50 μL of HBSS into the cecal wall of 4- to 6-wk-old nude mice (11). Briefly, athymic nude mice were anesthetized with ketamine/xylazine, and the cecum was exteriorized through a small abdominal incision. Viable (>90%) HT29 cells (1×10^6 in 50 μL HBSS) were injected into the cecal wall from the serosal side with a 30-gauge needle. The formation of a visible bulla between the submucosal and subserosal tissues and the lack of extracecal leakage of fluid were the criteria for a successful injection. The cecum was returned to the abdominal cavity and the wound was closed in one layer (12). Four weeks after tumor implantation, the abdomen of the animals were surgically reopened and thereafter closed to detect tumors in the cecum. Animals with visible HT29 tumors in the cecum were randomly divided into two main groups, A and B (Table 2). The animals within these two groups were further randomized to one of the four treatment groups, including vehicle, dopamine (50 mg/kg/d i.p. × 7 continuous days), 5-FU (20 mg/kg i.p. for 5 continuous days), and dopamine (50 mg/kg/d i.p. × 7 continuous days) + 5-FU (20 mg/kg i.p. for 5 continuous days; Table 2). Group A animals were sacrificed on day 8 and group B animals were followed to death. All experiments, except the survival

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

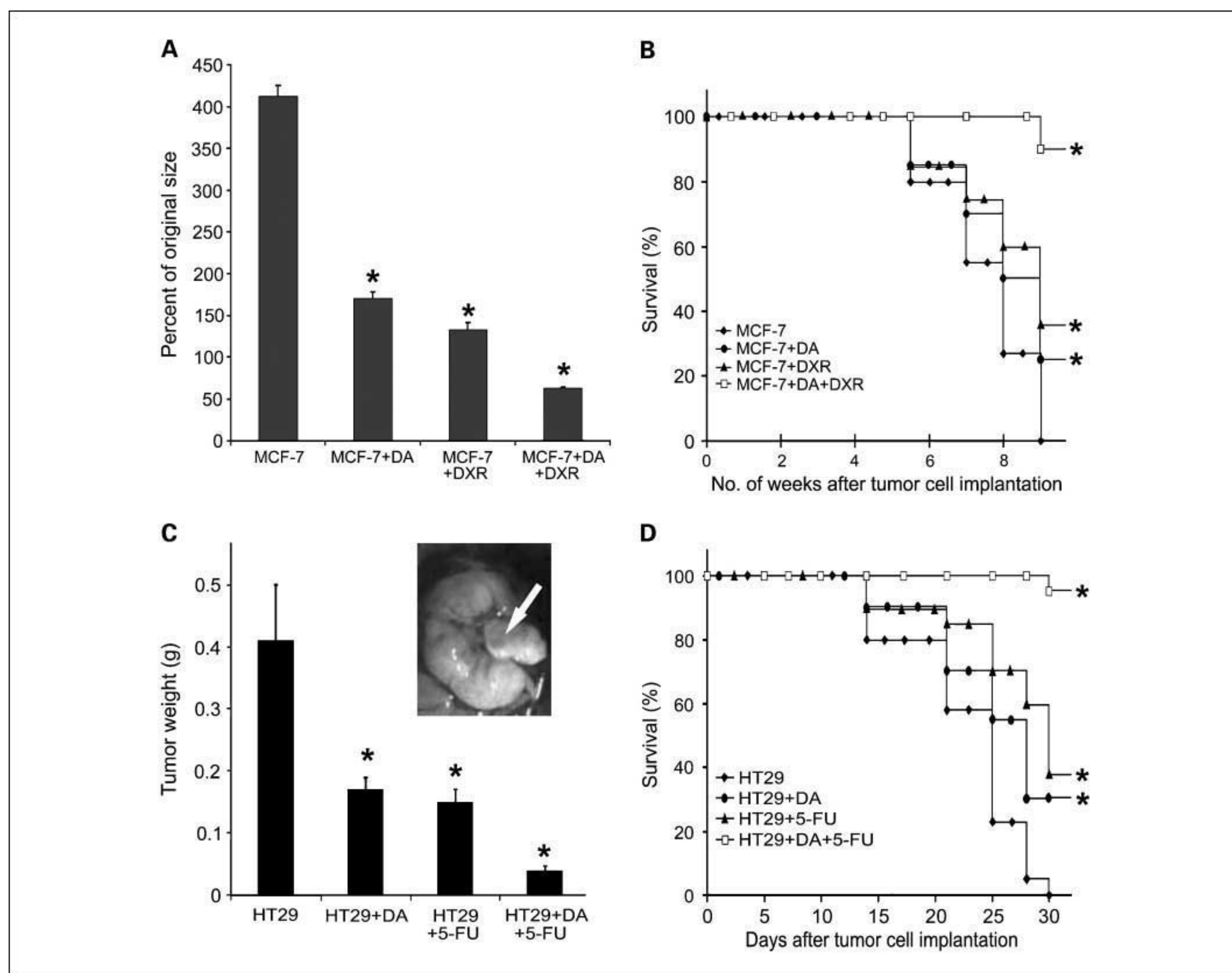

Fig. 1. Effect of dopamine and anticancer drugs (doxorubicin and 5-FU), alone or in combination, on growth of human breast (*A*) and colon tumor (*C*) in nude mice and survival of respective tumor-bearing mice (*B* and *D*). Dopamine (50 mg/kg/d × 7 d) was administered i.p. when the tumor reached a volume of 100 mm^3 (mentioned as original tumor volume in the figure). Results are expressed as percent of original tumor volume (100 mm^3) after completion of treatment. *A,* marked tumor growth inhibition from the initial tumor volume of 100 mm^3 was shown only when MCF-7 – bearing nude mice were treated with a combination of dopamine plus doxorubicin. *B,* tumor-bearing (MCF-7) mice receiving combination therapy showed the most significant increase in survival. *C,* similarly, combination treatment with dopamine and 5-FU also caused significant inhibition of tumor growth in mice bearing orthotopic colon cancer and was more effective than treatment with 5-FU alone. *Inset,* HT29 cell grown orthotopically in cecal region of nude mice. *D,* maximum increase in survival was observed in HT29 colon cancer – bearing mice receiving combination treatment than treatment with anticancer drug alone. Results are mean of three separate experiments; bars, SE (n = 8 for each experimental group. Statistical analysis of survival was evaluated by the Kaplan-Meier method (*, $P < 0.05$).

study (14 animals per group was used for the survival study), were done in triplicate.

Tumor growth measurement

For s.c. MCF-7 breast cancer, tumor growth was determined by caliper measurement of the largest diameter and its perpendicular using the formula, tumor volume (mm^3) = $0.5 \times a \times b^2$, where a is the largest diameter and b is its perpendicular (13). At the completion of treatment, group A animals were sacrificed and their tumor tissues were collected by dissection. Thereafter, a portion of these tumor tissues was formalin fixed and embedded in paraffin, and another portion was snap frozen in liquid nitrogen and stored at -80°C for immunohistochemistry (11).

Similarly, for orthotopic HT29 colon cancer, group A animals were sacrificed after completion of the treatment. Thereafter, their tumor tissues were collected by dissection and tumor growth was determined by weighing the tumor mass. Finally, a portion of these tumor tissues was formalin fixed and embedded in paraffin, and another portion was snap frozen in liquid nitrogen and stored at -80°C for immunohistochemistry (11).

Immunohistochemistry

Tumor tissue samples were fixed in 4% paraformaldehyde, rinsed in PBS, transferred to 30% sucrose in PBS at 4°C, and frozen in optimum cutting temperature compound. Immunohistochemistry was done on frozen tissue sections using rat anti-mouse monoclonal antibody against CD31 using the Vectastain Elite ABC kit following the manufacturer's protocol. Sections were counterstained with hematoxylin. Microvessel density was quantitated by analyzing 10 random fields per section (9).

In vivo tumor cell proliferation

The cellular kinetics of the tumor specimens were determined by immunohistochemical staining of BrdUrd. One hour before sacrificing the animals, BrdUrd (20 mg/kg of body weight) was injected i.p. into each animal. Subsequently, the animals were sacrificed and tumors

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

were excised and fixed in buffered formalin and embedded in paraffin. Cross-sections of the tissues were cut at 4-μm thickness. Tissue sections were then immunostained by using BrdUrd labeling and detection kit. The percentage of stained cells was counted and expressed as labeling index (14).

In vivo tumor cell apoptosis

Tumor cell apoptosis was assessed by measuring DNA fragmentation in a standard terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay according to the instruction of the manufacturer (R&D Systems). The number of apoptotic cells was expressed as apoptotic index (15).

Separation of tumor endothelial cells

A suspension of tumor cells (MCF-7 and HT29) was made by passage of viable tissue through sieve and treatment with collagenase and DNase. The cells were washed and the RBC was lysed with PharMLyse (BD PharMingen). The cell pellets were then resuspended in fluorescence-activated cell sorting (FACS) buffer (1× PBS + 1% bovine serum albumin), preblocked with an F_c block (CD16/32), and then incubated with phycoerythrin-conjugated anti–VEGFR-2 (1:100), CD31 (1:100), and CD34 (1:100). Tumor endothelial cells were collected by FACS (9).

Immunoprecipitation and immunoblotting

Cell lysates were immunoprecipitated with different antibodies (1:100 dilution, except phospho-MAPK antibody was used at 1:1,000 dilution), and immunocomplexes were captured on protein A–agarose beads. Samples were subjected to SDS-PAGE and then transferred to polyvinyl difluoride membranes and immunoblotted. Antibody-reactive bands were detected by enzyme-linked chemiluminescence (Pierce Biotechnology, Inc.) and quantified by laser densitometry (16).

Vascular permeability assay

After completion of treatment with dopamine, Evans blue dye (100 μL of a 1% solution in 0.9% NaCl) was injected i.v. into mice. After 30 min, the animals were euthanized, and the whole tumor was removed. Evans blue dye was extracted from the tumor by incubation with formamide for 5 d at room temperature, and the absorbance of extracted dye was measured at 620 nm (17).

Cell proliferation and viability assay *in vitro*

Tumor cells (2×10^3) or tumor endothelial cells were seeded in 24-well plates, cultured for 2 d, serum starved (0.1% serum) for 24 h, and then treated with either dopamine (1.2 nmol/ml) and/or various dopamine receptor antagonists, followed 5 min later by addition of 10 ng/mL VPF/VEGF or basic fibroblast growth factor. After culturing for 20 h, 1 μCi [^{3}H]thymidine was added to each well and 4 h later, cells were collected for measurement of trichloroacetic acid–precipitable radioactivity. Cell viability was determined using trypan blue dye exclusion or the MTT assay (8).

Cell migration assay *in vitro*

Serum-starved tumor endothelial cells were washed twice with PBS and incubated with 4 mL solution (0.2 mg/mL collagenase, 0.2 mg/mL soybean trypsin inhibitor, 1 mg/mL BSA, 2 mmol/L EDTA in PBS) for 20 min at 37°C. Cells (1×10^5 per well) were seeded into Vitrogen (30 μg/mL)–coated transwell chambers that were inserted into 24-well plates containing 1 mL of the same medium. After incubation at 37°C for 1 h, dopamine or dopamine D_2 receptor antagonists were added. VPF/VEGF was then added to a final concentration of 10 ng/mL. Following incubation for 2 h, the transwell membrane was stained with Cyquant DNA stain and migrating cells were counted in a spectrofluorometer (8).

Statistics

The Kaplan-Meier method was used to determine the estimated survival curves and comparisons between the curves were done by log-rank test. Mean and SE were calculated. Differences among groups were evaluated by ANOVA and the unpaired Student's test or Dunn's multiple comparison tests. $P < 0.05$ was considered significant (8, 18).

Results

Effect of dopamine or doxorubicin or dopamine + doxorubicin on growth and survival of human breast cancer (MCF-7) in nude mice. The treatment started when the tumor volume in the MCF-7–bearing mice was ~100 mm^3. At the end of the treatment, the tumor volumes in the vehicle, dopamine, doxorubicin, and dopamine + doxorubicin–treated animals were 413, 171, 133, and 63 mm^3, respectively. Therefore, the tumor volumes in the vehicle-, dopamine-, and doxorubicin-treated animals were 413%, 171%, and 133%, respectively, of the original size (~100 mm^3). In contrast, the tumor volume in the dopamine + doxorubicin–treated animals was only 63% of the original volume (100 mm^3; Fig. 1A). Similarly, there was 24% increase in the life span of the animals treated with dopamine and 38% increase in the life span of the animals treated with doxorubicin when compared with vehicle-treated controls. However, striking increase in the life span (90%) was observed in animals treated with dopamine + doxorubicin

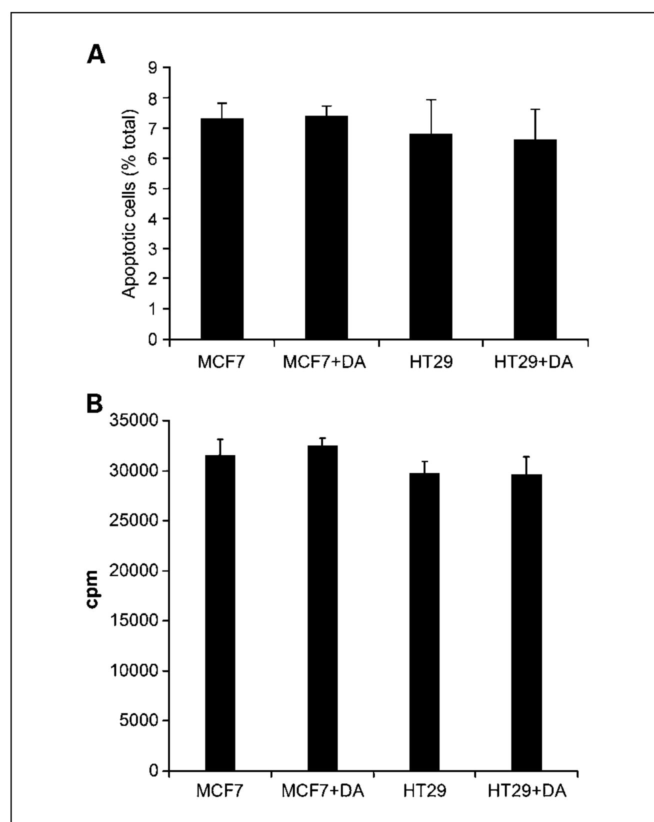

Fig. 2. Treatment with 1.2 nmol/ml dopamine had no effect on the survival and proliferation of MCF-7 and HT29 cells *in vitro*. *In vivo* dopamine (*DA*; 50 mg/kg/7 d) injection results in 1.2 nmol/ml plasma dopamine concentration. No significant direct effect of this concentration of dopamine on tumor cell survival (*A*) and proliferation (*B*) was evident *in vitro* on both MCF-7 and HT-29, respectively. The proliferation was assayed by [^{3}H]thymidine incorporation and cell survival by MTT assay.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

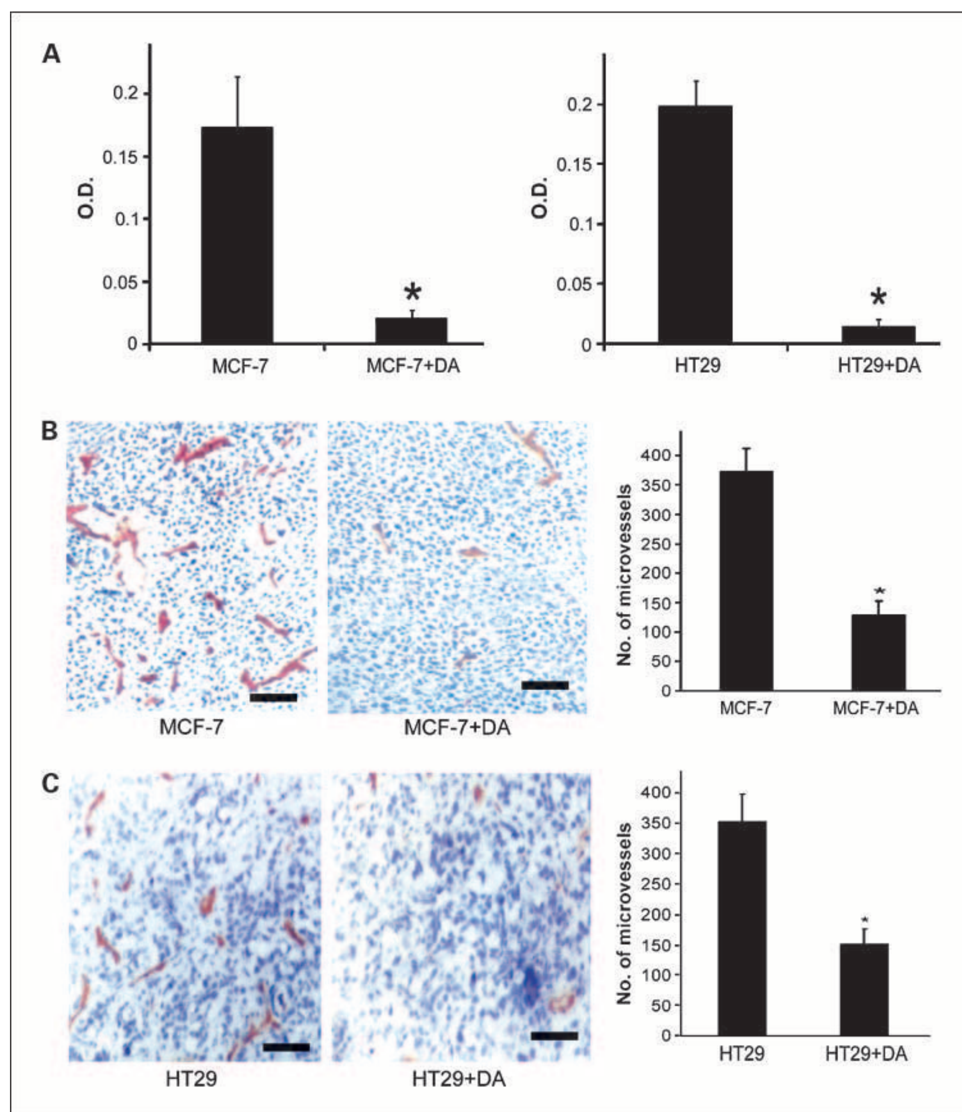

Fig. 3. Dopamine treatment significantly decreased vascular permeability and microvessel density in tumor tissue of MCF-7 and HT29. *A,* dopamine treatment (50 mg/kg/d × 7 d) caused significant inhibition of vascular permeability in tumor tissues of human breast and colon tumor. The vascular permeability was colorimetrically assessed from the degree of extravasation of Evans blue dye from tumor vessels. *B,* dopamine treatment caused significant reduction in the number of microvessels in tumor sections of dopamine-treated MCF-7 tumor-bearing mice. Microvessel density was evaluated by immunohistochemical analysis of CD31, an endothelial cell surface – specific marker. *C,* significant inhibition of CD31 expression following dopamine treatment was also observed in the tumor tissue of HT29-bearing nude mice. Columns, mean of three separate experiments; bars, SE (n = 8 for each experimental group; *, $P < 0.05$).

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

(Fig. 1B) compared with the vehicle-treated controls. Also, 2 of 14 animals receiving combination of dopamine + doxorubicin showed tumor-free survival for the period of our experiments (until all the animals in the control groups died). It is to be noted here that treatment of the tumor-bearing animals with specific dopamine D_2 receptor antagonist domperidone before dopamine treatment abrogated the effect of dopamine, thereby indicating that the action of dopamine was through dopamine D_2 receptors (data not shown).

Effect of dopamine or 5-FU or dopamine + 5-FU on growth and 5-FU or dopamine + 5-FU on survival of human colon cancer (HT29) in nude mice. At the end of the treatment, dopamine treatment showed 58% decrease in tumor growth (Fig. 1C) and 30% increase in the life span when compared with vehicle-treated controls (Fig. 1D). Furthermore, 5-FU treatment alone caused 63% reduction in tumor growth (Fig. 1C) and there was also 38% increase in survival of the hosts in these 5-FU – treated animals compared with vehicle-treated controls (Fig. 1D). However, the combination treatment (dopamine + 5-FU) showed maximum inhibition of tumor growth (95%; Fig. 1C) and a marked increase in survival (97%) of these animals was noted compared with vehicle-treated controls (Fig. 1D). Also, 3 of the 14 animals receiving combination treatment showed disease-free survival throughout the period of experiments (until all the animals in the control groups died). It is to be noted here that treatment of the tumor-bearing animals with specific dopamine D_2 receptor antagonist domperidone before dopamine treatment abrogated the effect of dopamine, thereby indicating that the action of dopamine was through dopamine D_2 receptors (data not shown).

***Effect of dopamine on the proliferation and survival of tumor cells* in vitro.** Thereafter, to investigate whether dopamine had any direct effect on MCF-7 breast cancer or HT29 colon cancer cells, these cells were treated *in vitro* with dopamine at a concentration of 1.2 nmol/ml. This concentration of dopamine was particularly selected because the antiangiogenic dose of dopamine (50 mg/kg i.p.), which we had used in our *in vivo* experiments, raised the plasma dopamine level of mice to this level at 1 minute after dopamine administration and, most importantly, this increase in plasma dopamine level in mice is also comparable with the plasma level reached in human

subjects treated with an i.v. infusion of dopaminergic dose of dopamine (15). It was observed that dopamine had no direct effects on the proliferation and survival of these cancer cells as determined by thymidine incorporation and MTT assay (Fig. 2).

***Effect of dopamine on tumor vascular permeability and microvessel density* in vivo.** Recent reports indicate that anti–VPF/VEGF agents can increase the concentrations of conventional anticancer drugs in the tumor tissues by decreasing vascular permeability, which in turn increases the efficacies of these drugs (2, 19, 20). Therefore, the effects of dopamine on vascular permeability were determined to elucidate the mechanism of synergism of dopamine with conventional antineoplastic agents. The results indicated that dopamine significantly inhibited tumor vascular permeability ($P < 0.05$) in mice bearing breast or colon tumors (Fig. 3A). Furthermore, this decrease in tumor vascular permeability was also accompanied by significant inhibition of tumor angiogenesis as evident from marked reduction of microvessel density ($P < 0.05$) in the tumor tissues (Fig. 3B and C). The other two members of the catecholamine family, namely epinephrine and norepinephrine, had no synergistic effects with antineoplastic drugs (data not shown).

***Role of dopamine on apoptosis and effect of dopamine or doxorubicin or 5-FU and dopamine + doxorubicin or dopamine + 5-FU on proliferation of tumor cells* in vivo.** In addition to inhibition of tumor angiogenesis, dopamine treatment showed considerable increase in number of tumor apoptotic cells in both MCF-7 and HT29 tumors (Fig. 4A and B). Dopamine also caused significant inhibition in proliferation of the tumor cells (Fig. 4C and D). Similar inhibition in tumor cell proliferation was also observed after treatment with doxorubicin or 5-FU alone (Fig. 4C and D). However, maximum inhibition in tumor cell proliferation was observed in the groups receiving dopamine + doxorubicin or dopamine +5-FU (Fig. 4C and D).

***Dopamine inhibits vascular permeability and microvessel density by down-regulating VEGFR-2, FAK, and MAPK phosphorylation in tumor endothelial cells* in vivo.** VPF/VEGF is the prime cytokine expressed by the majority of human tumors (1, 2, 7). It plays a critical role in inducing vascular permeability, a critical step in the process of tumor angiogenesis (1, 2). The molecular mechanism of this action of VPF/VEGF is mediated through phosphorylation of VEGFR-2 and subsequent phosphorylation of FAK in tumor endothelial cells (21, 22). VPF/VEGF–induced proliferation of endothelial cells is also an important function in the angiogenic cascade and is mainly mediated through the MAPK pathway (23–25). Accordingly, dopamine-mediated inhibition of VEGFR-2, FAK, and MAPK phosphorylation was investigated in tumor

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

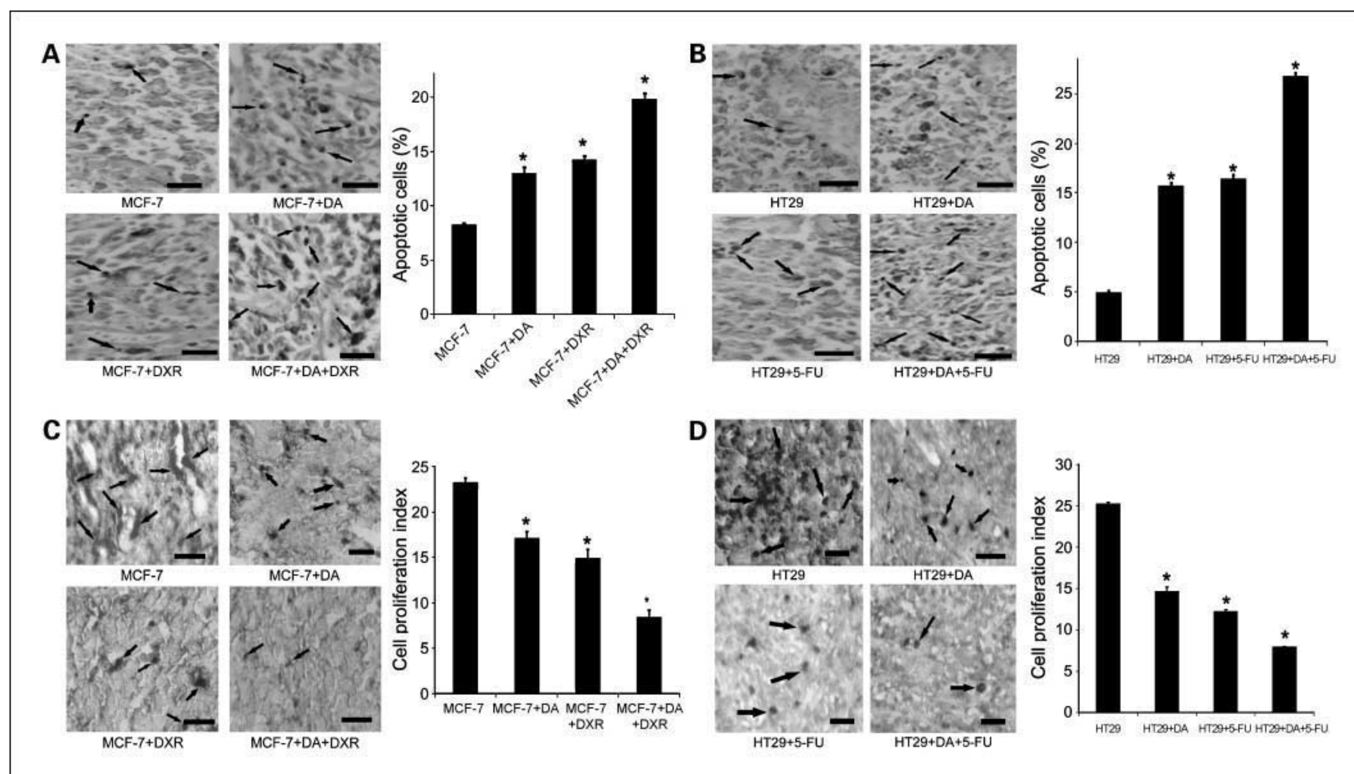

Fig. 4. Effect of dopamine alone or in combination (doxorubicin or 5-FU) on tumor cell apoptosis and on tumor cell proliferation in human breast – and colon cancer – bearing nude mice. *A* and *B,* treatment with dopamine caused significant increase in tumor cell apoptosis in MCF-7 and HT29-bearing mice, when compared with the vehicle-treated control. The highest numbers of apoptotic tumor cells were evident following dopamine + doxorubicin (*DXR*) and dopamine + 5-FU treatment and dopamine + 5-FU. Graphical representation shows the number of apoptotic cells in each experimental group. Apoptosis was determined by tumorTACS apoptosis detection kit from R&D Systems, using the TUNEL assay. *C* and *D,* dopamine, doxorubicin, and 5-FU, when used alone, caused significant reduction in tumor cell proliferation. However, the combination treatment with dopamine and anticancer drug was most effective in inhibiting tumor cell proliferation *in situ* in MCF-7– and HT29-bearing nude mice. Cell proliferation was assessed by immunohistochemical detection of BrdUrd incorporation in tumor cells. Graphs represent cell proliferation index of various experimental groups. Columns, mean of three separate experiments; bars, SE (n = 8 for each experimental group; *, $P < 0.05$).

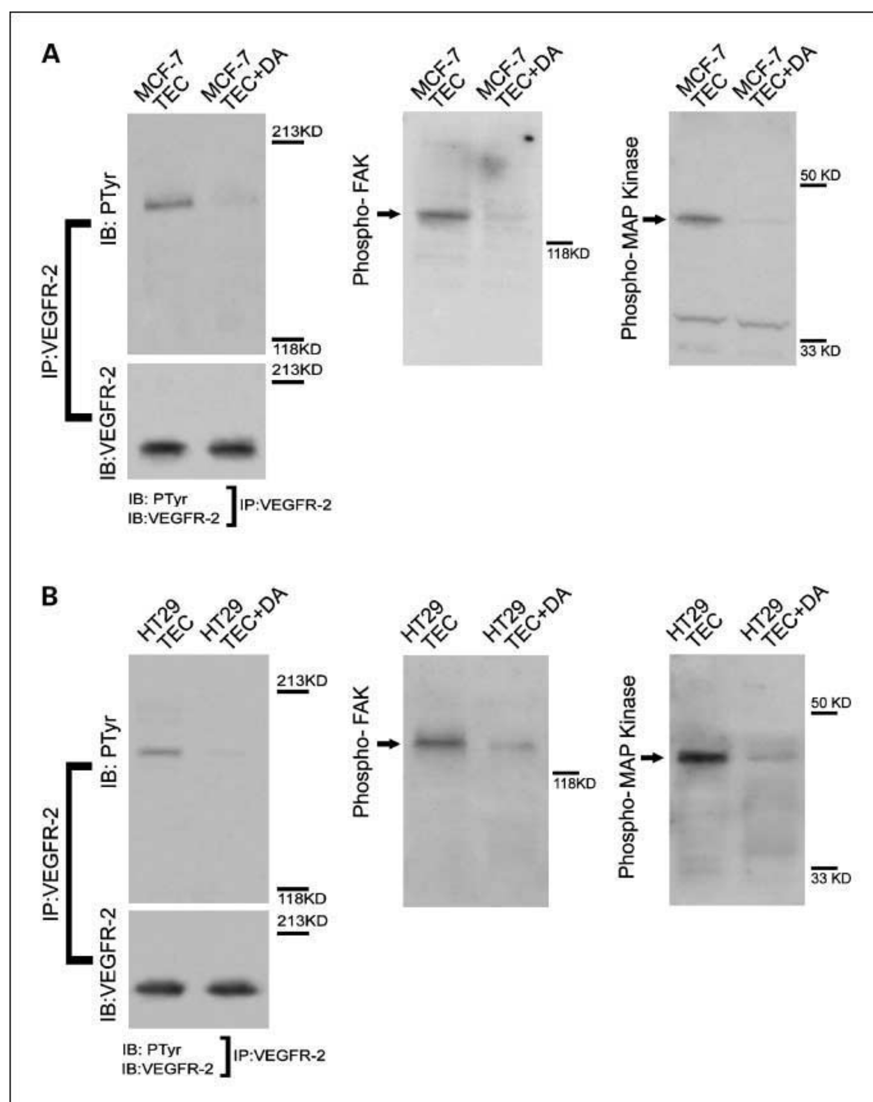

Fig. 5. Dopamine inhibits phosphorylation of VEGFR-2, FAK, and MAPK in tumor endothelial cells isolated from xenotransplanted human breast (MCF-7) and orthotopically grown human colon (HT29) tumor in nude mice. *A* and *B*, first lane of each blot shows considerable expression of phosphorylated VEGFR-2, FAK, and MAPK in tumor endothelial cells (*TEC*) of vehicle-treated controls. The second lane shows significant inhibition of phosphorylation of VEGFR-2, FAK, and MAPK in tumor endothelial cells from dopamine-treated tumor-bearing mice. IB, immunoblotting; IP, immunoprecipitation.

endothelial cells. Although angiogenesis was associated with phosphorylation of VEGFR-2, FAK, and MAPK (Fig. 5A and B), dopamine-mediated significant inhibition of tumor angiogenesis, on the contrary, was accompanied by significant suppression of VEGFR-2, FAK, and MAPK phosphorylation in tumor endothelial cells (Fig. 5A and B).

***Dopamine specifically inhibits VPF/VEGF–mediated tumor endothelial cell proliferation and migration* in vitro.** The specific direct anti–VPF/VEGF or antiangiogenic action of dopamine was next confirmed because although 1.2 nmol/ml of dopamine significantly inhibited VPF/VEGF–mediated proliferation and migration of tumor endothelial cells *in vitro*, it failed to inhibit tumor endothelial cell proliferation induced by basic fibroblast growth factor (Fig. 6A and B). In addition, dopamine also suppressed VPF/VEGF–induced phosphorylation of VEGFR-2 in these cells (Fig. 6C). Dopamine acted specifically through its D_2 receptors present on the tumor endothelial cells because treatment of these cells with specific dopamine D_2 receptor antagonist, eticlopride, before the treatment with dopamine abrogated the action of dopamine (Fig. 6A-C).

Discussion

Taken together, these results for the first time show that low nontoxic dose of dopamine, which is comparable with the low dopaminergic dose used in humans (15, 26), inhibits the growth of human breast and colon cancer in preclinical models. In addition, there is a synergistic effect when dopamine is used in combination with common anticancer drugs and this combination therapy increases the life span of tumor-bearing animals.

Because both the human tumors used in our experiments induce VPF/VEGF–mediated tumor angiogenesis and growth (27, 28) and because dopamine has been reported to inhibit the actions of VPF/VEGF (8), it can be concluded that the antiangiogenic effect of dopamine is due to inhibition of the functions of VPF/VEGF. In addition, VPF/VEGF–induced microvascular hyperpermeability is a critical step in the process of tumor angiogenesis (1, 2, 29). It has been further reported that VPF/VEGF primarily mediates its action by acting mainly through VEGFR-2 receptors present on the endothelial cells (1, 2). Recent reports suggest that FAK is an important molecule in VPF/VEGF–mediated signaling pathway (21, 30–32) and its

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

phosphorylation leads to increased vascular hyperpermeability (30, 31). In the present study, dopamine showed a unique property in inhibiting hyperpermeability of tumor microvessels and thereby angiogenesis in human breast and colon malignant tumors by suppressing phosphorylation of VEGFR-2 and FAK in tumor endothelial cells, the two critical components of VPF/VEGF signaling cascade. This, in turn, led to inhibition of tumor growth. It is to be noted here that there are now reports that indicate that targeting FAK could be an option for developing novel anticancer therapy (33 – 35).

Furthermore, proliferation and migration are other two important functional aspects of endothelial cells for the process of angiogenesis and VPF/VEGF – induced activation of MAPK is critical for these processes (23 – 25). In untreated tumor-bearing mice, MAPK phosphorylation was significantly increased in tumor endothelial cells, which correlated well with angiogenesis in tumor tissues. In contrast, treatment of tumor-bearing mice with dopamine significantly inhibited phosphorylation of MAPK in tumor endothelial cells and this was accompanied with significant inhibition of tumor angiogenesis. Although we had previously reported that endogenous dopamine regulates VPF/VEGF – induced phosphorylation of VEGFR-2, FAK, and MAPK in normal mesenteric endothelial cells (16), our present results indicating dopamine-mediated inhibition of VEGFR-2, FAK, and MAPK phosphorylation in tumor endothelial cells is significant because critical functional and genetic differences have been now been discovered between tumor and normal endothelial cells (36 – 41). Therefore, our study enables to dissect the detailed mechanism of antiangiogenic effect of dopamine on tumor endothelial cells.

Finally, in contrast to the presently available VPF/VEGF inhibitors in the clinics, the price of dopamine (a small molecule) or its specific D_2 agonists are much cheaper. This, in turn, may be very important in perspective of health economics for using the limited funds allocated for the treatment of cancer (42, 43). In addition, although new serious toxicities are being reported with the use of presently available anti – VPF/VEGF agents (5, 7), dopamine is a well-established drug with known and manageable toxicity (44). Furthermore, recent clinical reports have indicated the therapeutic efficacy of dopamine or its D_2-specific agonists in the treatment of ovarian hyperstimulation syndrome, a serious disorder that is many times fatal (45, 46). The pathogenesis of this disorder is due to VEGFR-2 – mediated increase in vascular permeability. Dopamine or its D_2 agonists have shown to cure ovarian hyperstimulation syndrome patients by inhibiting the function of VEGFR-2 (45, 46). Thus, our present preclinical data need to be translated rapidly to the clinics for the treatment of breast and colon cancer.

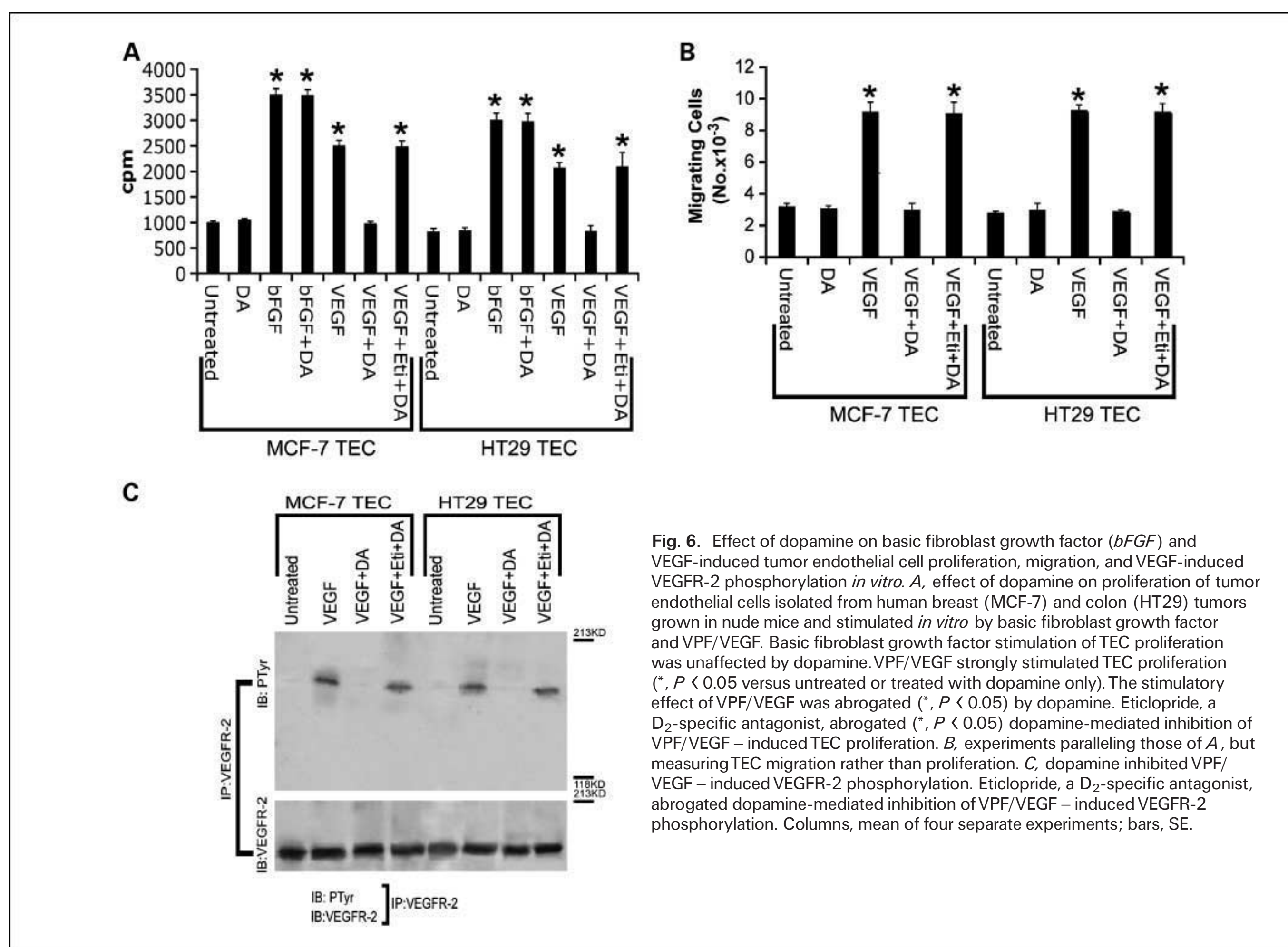

Fig. 6. Effect of dopamine on basic fibroblast growth factor (*bFGF*) and VEGF-induced tumor endothelial cell proliferation, migration, and VEGF-induced VEGFR-2 phosphorylation *in vitro*. *A,* effect of dopamine on proliferation of tumor endothelial cells isolated from human breast (MCF-7) and colon (HT29) tumors grown in nude mice and stimulated *in vitro* by basic fibroblast growth factor and VPF/VEGF. Basic fibroblast growth factor stimulation of TEC proliferation was unaffected by dopamine. VPF/VEGF strongly stimulated TEC proliferation (*, $P < 0.05$ versus untreated or treated with dopamine only). The stimulatory effect of VPF/VEGF was abrogated (*, $P < 0.05$) by dopamine. Eticlopride, a D_2-specific antagonist, abrogated (*, $P < 0.05$) dopamine-mediated inhibition of VPF/VEGF – induced TEC proliferation. *B,* experiments paralleling those of *A*, but measuring TEC migration rather than proliferation. *C,* dopamine inhibited VPF/VEGF – induced VEGFR-2 phosphorylation. Eticlopride, a D_2-specific antagonist, abrogated dopamine-mediated inhibition of VPF/VEGF – induced VEGFR-2 phosphorylation. Columns, mean of four separate experiments; bars, SE.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025

References

1. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol 2002;20:4368–80.
2. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005;23:1011–27.
3. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007;6:273–86.
4. Reimer CL, Agata N, Tammam JG, et al. Antineoplastic effects of chemotherapeutic agents are potentiated by NM-3, an inhibitor of angiogenesis. Cancer Res 2002;62:789–95.
5. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 2006;3:24–40.
6. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004; 350:2335–42.
7. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature 2005;438:967–74.
8. Basu S, Nagy JA, Pal S, et al. The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor. Nat Med 2001;7:569–74.
9. Basu S, Sarkar C, Chakroborty D, et al. Ablation of peripheral dopaminergic nerves stimulates malignant tumor growth by inducing vascular permeability factor/vascular endothelial growth factor-mediated angiogenesis. Cancer Res 2004;64:5551–5.
10. Noël A, Hajitou A, L'Hoir C, et al. Inhibition of stromal matrix metalloproteases; effects on breast- tumor promotion by fibroblasts. Int J Cancer 1998;76:267–73.
11. Yokoi K, Thaker PH, Yazici S, et al. Dual inhibition of epidermal growth factor receptor and vascular endothelial growth factor receptor phosphorylation by AEE788 reduces growth and metastasis of human colon carcinoma in an orthotopic nude mouse model. Cancer Res 2005;65:3716–25.
12. Morikawa K, Walker SM, Jessup JM, Fidler IJ. *In vivo* selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. Cancer Res 1988;48:1943–8.
13. Schirner M, Hoffmann J, Menrad A, Schneider MR. Antiangiogenic chemotherapeutic agents: characterization in comparison to their tumor growth inhibition in human renal cell carcinoma models. Clin Cancer Res 1998;4:1331–6.
14. Yamane T, Takahashi T, Kuwata K, et al. Inhibition of N-methyl-N′-nitro-N-nitrosoguanidine induced carcinogenesis by (-)-epigallocatechin gallate in the rat glandular stomach. Cancer Res 1995;55:2081–4.
15. Chakroborty D, Sarkar C, Mitra RB, Banerjee S, Dasgupta PS, Basu S. Depleted dopamine in gastric cancer tissues: dopamine treatment retards growth of gastric cancer by inhibiting angiogenesis. Clin Cancer Res 2004;10:4349–56.
16. Sarkar C, Chakroborty D, Mitra RB, Banerjee S, Dasgupta PS, Basu S. Dopamine *in vivo* inhibits VEGF-induced phosphorylation of VEGFR-2, MAPK, and focal adhesion kinase in endothelial cells. Am J Physiol Heart Circ Physiol 2004;287:H1554–60.
17. Satchi-Fainaro R, Mamluk R, Wang L, et al. Inhibition of vessel permeability by TNP-470 and its polymer conjugate, caplostatin. Cancer Cell 2005;7:251–61.
18. Yao M, Kargman S, Lam EC, et al. Inhibition of cyclooxygenase-2 by rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice. Cancer Res 2003;63:586–92.
19. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 2005;307:58–62.
20. Daldrup-Link HE, Okuhata Y, Wolfe A, et al. Decrease in tumor apparent permeability-surface area product to a MRI macromolecular contrast medium following angiogenesis inhibition with correlations to cytotoxic drug accumulation. Microcirculation 2004;11:387–96.
21. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. J Biol Chem 1997;272: 15442–51.
22. Bates DO, Harper SJ. Regulation of vascular permeability by vascular endothelial growth factors. Vascul Pharmacol 2002;39:225–37.
23. Gille H, Kowalski J, Li B, et al. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. J Biol Chem 2001;276:3222–30.
24. Meadows KN, Bryant P, Pumiglia K. Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. J Biol Chem 2001; 276:49289–98.
25. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovasc Res 2001;49:568–81.
26. Zaroslinski JF, Possley LH, Schwartz RA, Morris RN, Carone FA, Browne RK. The pharmacology and subacute toxicology of dopamine. Proc R Soc Med 1977;70 Suppl 2:2–6.
27. Bogin L, Degani H. Hormonal regulation of VEGF in orthotopic MCF-7 human breast cancer. Cancer Res 2002;62:1948–51.
28. Nguyen QD, Rodrigues S, Rodrigue CM, et al. Inhibition of vascular endothelial growth factor (VEGF)-165 and semaphorin 3A-mediated cellular invasion and tumor growth by the VEGF signaling inhibitor ZD4190 in human colon cancer cells and xenografts. Mol Cancer Ther 2006;5:2070–7.
29. Dvorak HF. Angiogenesis: update 2005. J Thromb Haemost 2005;3:1835–42.
30. Takahashi N, Seko Y, Noiri E, et al. Vascular endothelial growth factor induces activation and subcellular translocation of focal adhesion kinase (p125FAK) in cultured rat cardiac myocytes. Circ Res 1999;84: 1194–202.
31. Eliceiri BP, Puente XS, Hood JD, et al. Src-mediated coupling of focal adhesion kinase to integrin $\alpha(v)\beta5$ in vascular endothelial growth factor signaling. J Cell Biol 2002;157:149–60.
32. Abu-Ghazaleh R, Kabir J, Jia H, Lobo M, Zachary I. Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells. Biochem J 2001;360: 255–64.
33. McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer—a new therapeutic opportunity. Nat Rev Cancer 2005;5:505–15.
34. van Nimwegen MJ, van de Water B. Focal adhesion kinase: a potential target in cancer therapy. Biochem Pharmacol 2007;73:597–609.
35. Halder J, Kamat AA, Landen CN, Jr., et al. Focal adhesion kinase targeting using *in vivo* short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. Clin Cancer Res 2006;12:4916–24.
36. Bussolati B, Deambrosis I, Russo S, Deregibus MC, Camussi G. Altered angiogenesis and survival in human tumor-derived endothelial cells. FASEB J 2003;17:1159–61.
37. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. Science 2000;289:1197–202.
38. Amin DN, Hida K, Bielenberg DR, Klagsbrun M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. Cancer Res 2006;66:2173–80.
39. Walter-Yohrling J, Morgenbesser S, Rouleau C, et al. Murine endothelial cell lines as models of tumor endothelial cells. Clin Cancer Res 2004;10:2179–89.
40. Hellebrekers DM, Melotte V, Vire E, et al. Identification of epigenetically silenced genes in tumor endothelial cells. Cancer Res 2007;67:4138–48.
41. Chung I, Wong MK, Flynn G, Yu WD, Johnson CS, Trump DL. Differential antiproliferative effects of calcitriol on tumor-derived and matrigel-derived endothelial cells. Cancer Res 2006;66:8565–73.
42. Nadler E, Eckert B, Neumann PJ. Do oncologists believe new cancer drugs offer good value? Oncologist 2006;11:90–5.
43. Viale PH, Fung A, Zitella L. Advanced colorectal cancer: current treatment and nursing management with economic considerations. Clin J Oncol Nurs 2005;9:541–52.
44. Katzung BG, editor. Basic and clinical pharmacology. Stamford (CT): Appleton and Lange; 2004.
45. Manno M, Tomei F, Marchesan E, Adamo V. Cabergoline: a safe, easy, cheap, and effective drug for prevention/treatment of ovarian hyperstimulation syndrome? Eur J Obstet Gynecol Reprod Biol 2005;122:127–8.
46. Alvarez C, Marti-Bonmati L, Novella-Maestre E, et al. Dopamine agonist cabergoline reduces hemoconcentration and ascites in hyperstimulated women undergoing assisted reproduction. J Clin Endocrinol Metab 2007;92:2931–7.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/14/8/2502/1980508/2502.pdf by guest on 27 March 2025