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Advances in Brief

Ablation of Peripheral Dopaminergic Nerves Stimulates Malignant Tumor Growth by Inducing Vascular Permeability Factor/Vascular Endothelial Growth Factor-Mediated Angiogenesis

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Abstract

Many important physiological and pathological processes are modulated by angiogenesis. It has been shown that initiation of this angiogenic process is an essential early step in the progression of malignant tumors. We report here that ablation of peripheral dopaminergic nerves markedly increased angiogenesis, microvessel density, microvascular permeability, and growth of malignant tumors in mice. Endogenous peripheral dopamine acted through D_2 receptors as significantly more angiogenesis and tumor growth was observed in D_2 dopamine receptor knockout mice in comparison with controls. The vascular endothelial growth factor receptor 2 phosphorylation, which is critical for promoting angiogenesis, was also significantly more in tumor endothelial cells collected from the dopamine-depleted and D_2 dopamine receptor knockout animals. These results reveal that peripheral endogenous neurotransmitter dopamine might be an important physiological regulator of vascular endothelial growth factor-mediated tumor angiogenesis and growth and suggest a novel link between endogenous dopamine, angiogenesis, and tumor growth.

Introduction

Angiogenesis, the formation of new blood vessels is essential for the growth and progression of malignant tumors (1). Although the process of angiogenesis is tightly regulated in normal physiological conditions as a result of an intricate balance between stimulators and inhibitors of angiogenesis (1), it becomes aberrant in cancer because tumor cells produce excessive amounts of factors that stimulate angiogenesis (1). Impeded diffusion of nutrients from blood vessels to tumor cells due to high interstitial pressure within the tumor mass, accompanied by excessive tumor cell proliferation, necessitates more new blood vessel formation for the survival of these cells (1). Among the many endogenous factors stimulating angiogenesis, vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is thought to be the single most critical cytokine that promotes tumor angiogenesis, and recent reports indicate that most of the human and animal malignant tumors overexpress VPF/VEGF (1). There are at least three VPF/VEGF receptors, the most important of which is vascular endothelial growth factor receptor 2 (VEGFR-2) for inducing VPF/VEGF-mediated angiogenesis. Inhibition of VEGFR-2 activity has been reported to suppress both primary tumor growth and metastasis (1). Thus, understanding the endogenous regulators of VPF/VEGF-mediated tumor angiogenesis *in vivo* has become an emerging area of research (1). Because pharmacologically administered dopamine cannot cross the blood–brain barrier (2) and our recent results indicate that nontoxic pharmacological dose of dopamine can significantly and specifically inhibit the VPF/VEGF-induced angiogenesis by acting on D_2 dopamine receptors present on endothelial cells (3), we reasoned that peripheral endogenous neurotransmitter dopamine might regulate malignant tumor growth by modulating angiogenesis. Because blood vessels are supplied by peripheral dopaminergic nerves (2, 4), we investigated whether dopamine present in these nerves has any role in controlling VPF/VEGF-mediated tumor angiogenesis and growth. Our results suggest that peripheral endogenous neurotransmitter dopamine might be an important physiological regulator of VEGF-mediated tumor angiogenesis and growth and suggest a novel link between endogenous dopamine, angiogenesis, and tumor growth.

Materials and Methods

Reagents. 6-Hydroxydopamine and ascorbate were obtained from Sigma (St. Louis, MO). 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 were from BD Biosciences PharMingen. Dopamine D_2 receptor and VEGFR-2 antibodies for immunoblot were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phosphotyrosine antibody from Upstate Biotechnology (Lake Placid, NY). The ABC kit was from Vector Labs (Burlingame, CA). Reverse transciption-PCR kit was from Ambion Inc (Austin, TX). Colloidal carbon as Higgins non-waterproof drawing ink was from Sanford (Bellwood, IL). OCT compound was from Miles Diagnostics (Elkhart, IN).

Animals. Four-to-six-week-old male nude mice were purchased from the National Cancer Institute, NIH, Bethesda, MD; 4-to-6-week-old male wild-type C57 BL/6 mice and 4-to-6-week-old male C57 BL/6 dopamine D_2 receptor knockout mice were purchased from The Jackson Laboratory (Bar Harbor, Maine).

Cells. B16 melanoma cell line was obtained from American Type Culture Collection (Manassas, VA)

Mice, Tumor, and Histology. Viable B16 melanoma cells (1×10^5) were injected subcutaneously into syngeneic C57 BL/6 mice. The tumors were measured with microcalipers. B16 melanoma-bearing wild-type C57 BL/6 mice (given injections of either 6-hydroxydopamine dissolved in sterile saline containing 0.01% of the antioxidant ascorbate or of vehicle (5) and dopamine D_2 receptor knockout mice were given intravenous injections of colloidal carbon for permeability assay. Immunohistochemistry was also performed on frozen-tissue sections using Rat anti-CD31 monoclonal antibodies and the ABC kit from Vector Labs. Microvessel density was quantitated by analyzing 10 random fields per section (6).

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Flow Cytometry Analysis. A suspension of B16 melanoma was made by passage of viable tissue through sieve and treatment with collagenase and DNase. The cells were washed and the red blood cells were lysed with PharM Lyse (BD PharMingen, San Diego, CA). The cell pellets were then resuspended in fluorescence-activated cell sorting buffer (1× PBS plus 1% BSA), preblocked with an Fc block (CD16/CD32), and then incubated with primary antibody on ice: phycoerythrin-conjugated anti-VEGF receptor -2 (1:100), CD 31-phycoerythrin (1:100) and CD 34-phycoerythrin (1:100). One million positive tumor endothelial cells were collected by fluorescence-activated cell sorting (7, 8).

Semiquantitative Reverse Transcription-PCR. From isolated endothelial cells, total RNA was isolated by RNA isolation kit (Ambion Inc.). PCR was carried out in a DNA thermocycler (Gene Amp-9700; Applied Biosystem, Forest City, CA) after first denaturation at 94°C for 3 min, and each cycle consisted of denaturation at 94°C for 40 s, annealing at 59°C for 40 s, and extension at 72°C for 80 s. The number of total cycle was 37. In the present experiment, S15rRNA was served as control (9). The sequence of PCR primers for S15 (control) and dopamine D2 receptor were as follows: S15 primers (internal control), 5′-TTCCGCAAGTTCACCTACC-3′ (9) and 5′-CGGGCCGGCCATGCTTTACG-3′ (9); and dopamine D_2 receptor primers, 5′-GCAGCCGAGCTTTCAGGGCC-3′ and 5′-GGGATGTTGCAGTCACAGTG-3′ (Gene Bank accession no. S69899).

Immunoprecipitation and Immunoblotting. Immunoprecipitation and Western blot analysis, using antibodies against VEGFR-2 (1:100) and phosphotyrosine (1:100), was performed on tumor endothelial cells isolated from B16 melanoma.

Statistics. Differences among groups were evaluated by ANOVA and the unpaired Student's *t* test or Dunn's multiple comparison test.

Results

Effect of Ablation of Peripheral Dopaminergic Nerves on Tumor Angiogenesis and Growth. We performed initial experiments to determine whether dopamine present in the peripheral dopaminergic nerve terminals affected the vascular permeabilization and angiogenic response induced by B16 melanoma (B16) a well-characterized VPF/VEGF-secreting mouse tumor (10). The peripheral dopaminergic nerve terminals of syngeneic mice were first ablated by intraperitoneal injection of neurotoxin, 6-hydroxydopamine at a dose of 250 mg/kg (5). This neurotoxin selectively ablates peripheral dopaminergic nerves (5, 11) because 6-hydroxydopamine does not cross the blood–brain barrier in adult animals (5, 11). Furthermore, this neurotoxin does not have any tumor-promoting effect (12), and it also does not induce angiogenesis when injected into normal mice (data not shown).

After being injected subcutaneously into syngeneic peripheral dopaminergic nerve-ablated mice, beginning at 48 h after the last dose of 6-hydroxydopamine injection, B16 melanoma cells induced significantly increased angiogenesis, microvessel density, and microvascular permeability of enlarged, thin-walled, pericyte-poor "mother vessels" (Fig. 1, *A–C,* and *J*) in these animals when compared with their peripheral dopaminergic nerve-intact controls (Fig. 1, *D–F*, and *J*). Tumor volumes were also strikingly larger in these peripheral dopaminergic nerve-ablated mice when compared with their controls (Fig. 1, *A*, *D*, and *K*). Similar results were also observed with Sarcoma 180, another VPF/VEGF-secreting murine tumor (data not shown).

The Role of D_2 Dopamine Receptors Present on Tumor Endothelial Cells. We have recently demonstrated that at nontoxic pharmacological dose, dopamine by acting through the D_2 dopamine receptors strikingly inhibited the vascular permeabilizing and angiogenic activities of VPF/VEGF (3). However, these actions of dopamine were elucidated in cultured normal human umbilical vein endothelial cells (3), in which these cells were not subjected to regulatory mechanisms that may be imposed *in vivo* by neighboring cells (*e.g.,* pericytes), flowing blood, vascular pressure, and so forth (13). Therefore, we investigated the role of endogenous D_2 dopamine receptors present on the tumor endothelial cells in inhibiting angiogenesis and tumor growth. We found that B16 melanoma cells induced significantly increased angiogenesis, microvessel density, microvascular permeability of mother vessels, and tumor growth in D_2 dopamine receptor ($^{-/-}$) C57 BL/6 mice when compared with B16 tumor-bearing wild-type C57 BL/6 controls with intact peripheral nerves (Fig. 1, *D–F*, *G–I*, *J*, and *K*). These results thus indicate that the endogenous peripheral neurotransmitter dopamine modulates tumor angiogenesis and, hence, its growth by acting at the D_2 dopamine receptors present on the tumor endothelial cells. This result was further confirmed, as reverse transciption-PCR, immunoprecipitation, and Western blot analysis demonstrated dopamine D_2 receptors on tumor endothelial cells collected from B16-bearing wild type mice with or without intact peripheral dopaminergic nerves. In contrast, the dopamine D_2 receptors were absent in tumor endothelial cells collected from dopamine D_2 receptor knock out mice (Fig. 2*A* and *B*).

Ablation of Peripheral Dopaminergic Nerves Stimulates VPF/VEGF-induced Angiogenesis. Because it has been recently reported that the action of dopamine is specific for VPF/VEGF and does not affect other mediators of microvascular permeability or endothelial cell proliferation and migration (3), we, therefore, investigated the role of endogenous peripheral dopamine on a stringent model of angiogenesis in which adenoviral vector (Ad-vpf/vegf), which was engineered to express murine VPF/VEGF^{164} under a control of cytomegalovirus promoter (14), was introduced into the ears of athymic mice either with intact or ablated (these mice received 6-hydroxydopamine 250 mg/kg intraperitoneally) peripheral dopaminergic nerves. As the result of alternate splicing, VPF/VEGF is expressed in three different isoforms consisting of 120, 164, and 188 amino acids, respectively, in mice (the human isoforms are one amino acid longer); the 164-amino-acid isoform is most commonly expressed by tumors. The angiogenic response that followed mimicked that found in tumors, proceeding through steps including increased microvascular permeability, tissue edema, fibrin deposition, formation of enlarged, thin-walled, pericyte-poor mother vessels, and subsequent evolution of mother vessels into various types of secondary vessels. This phenomenon of angiogenic response occurred over the course of 1–3 weeks (14) in mice with intact peripheral dopaminergic nerves (Fig. 3). In contrast, significant edema and mother-vessels formation was seen within 2 days in mice with ablated peripheral dopaminergic nerves (Fig. 3), thereby indicating that endogenous peripheral dopamine modulates VPF/VEGF-induced angiogenesis.

Peripheral Dopaminergic Nerve Ablation Increase VEGFR-2 Phosphorylation in Tumor Endothelial Cells. VPF/VEGF is thought to induce angiogenesis by engaging VEGFR-2 (also known as KDR and Flk-1), leading to phosphorylation and a series of downstream signaling events (1, 3, 13). Therefore, we investigated whether endogenous peripheral dopamine inhibited VPF/VEGF-induced phosphorylation in tumor endothelial cells. We found that tumor endothelial cells collected from B16 tumor-bearing mice with ablated peripheral dopaminergic nerves had strikingly more VEGFR-2 phosphorylation when compared with B16 tumor-bearing mice with intact peripheral dopaminergic nerves, thereby indicating that endogenous peripheral dopamine regulates tumor growth by inhibiting VEGFR-2 phosphorylation in tumor endothelial cells. Furthermore we also found markedly more VEGFR-2 phosphorylation in tumor endothelial cells collected from dopamine D_2 receptor knockout mice (Fig. 4). It is to be noted here that we observed a very faint band in normal mice due to the activity of endogenous VPF/VEGF. Also. it is worth mentioning here that we did not find any significant change in plasma VEGF level in B16 tumor-bearing mice with either intact or ablated peripheral dopaminergic nerves and also in B16 tumor bearing dopamine D_2 receptor knockout mice (data not shown), thereby sug-

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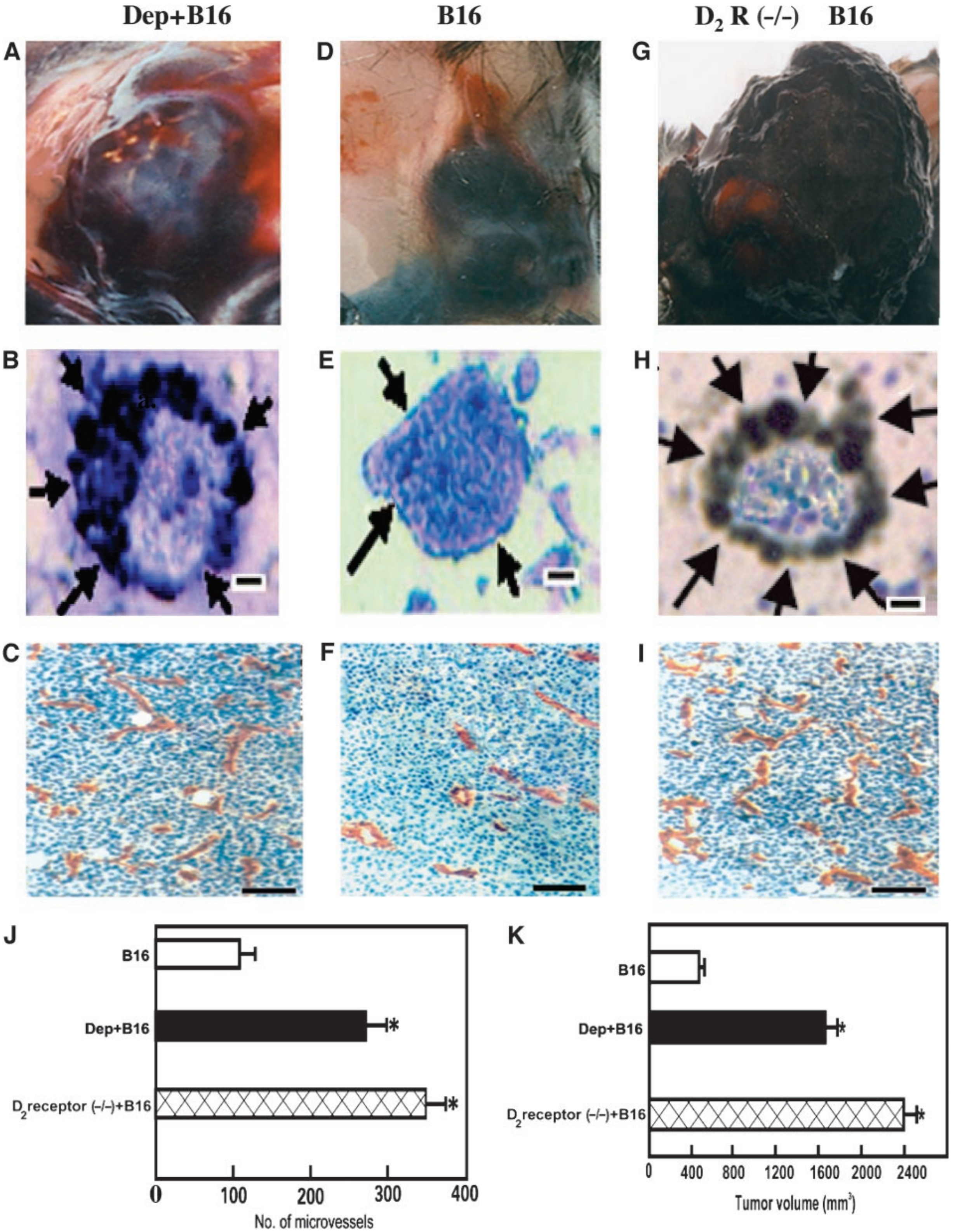

Fig. 1. Effects of ablation of peripheral dopaminergic nerves and D_2 dopamine receptor ($^{-/-}$) on the angiogenesis and growth of B16 melanoma in mice. *A–K,* gross (*A, D, G*) and microscopic (including graphical representation; *B, C, E, F, H, I–K*) appearance of the tumor tissue, 15 days after injection of 1×10^5 B16 tumor cells in 6-hydroxydopamine-induced peripheral dopaminergic nerve-ablated mice, mice with intact peripheral dopaminergic nerve, and D_2 dopamine receptor ($^{-/-}$) mice [*D_2 R (−/−) + B16*]. Mice with ablated peripheral dopaminergic nerve receiving B16 tumor (*Dep+B16 (A)*; *Dep+B16 (J)*] exhibited significantly increased angiogenesis, microvessel density (*A, C, J*), and increased microvascular permeability of many enlarged mother vessels, as determined by their labeling with extravasated colloidal carbon (*black arrows, B*), when compared with angiogenesis, microvessel density (*D, F, J*), and microvascular permeability of enlarged mother vessels as determined by their labeling with extravasated colloidal carbon (*black arrows, E*) observed in B16 tumor-bearing mice with intact peripheral dopaminergic nerve. Tumor volume was also significantly increased in these peripheral dopaminergic nerve-ablated mice when compared with intact peripheral dopaminergic-nerve control mice (*A, D, K*). Also, B16 melanoma-bearing mice with D_2 dopamine receptor ($^{-/-}$) exhibited significantly increased angiogenesis, microvessel density (*G, I, J*), and microvascular permeability of the enlarged mother vessels, as determined by their labeling with extravasated colloidal carbon (*black arrows, H*), and tumor volume in these knockout mice was significantly increased when compared with B 16-bearing mice with intact peripheral dopaminergic nerves (*D, G, K*). *Error bars,* ∗, $P < 0.01$ with regard to intact peripheral dopaminergic nerve-intact mice. *Scale bars: C,* 100 μm; *F,* 100 μm; *I,* 100 μm; *B,* 10 μm; *E,* 10 μm; *H,* 10 μm.

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gesting that endogenous dopamine has no role in the synthesis of endogenous VPF/VEGF.

Discussion

Taken together, our results indicate that the peripheral endogenous neurotransmitter dopamine can significantly modulate microvessel hyperpermeability, angiogenesis, and tumor growth by acting on the D_2 dopamine receptors present on the tumor endothelial cells. Here, we show for the first time that endogenous peripheral dopaminergic system can physiologically regulate VPF/VEGF-induced tumor angiogenesis and growth. This finding is significant because angiogenesis has a central role in many important examples of pathological angiogenesis (neoplasia, rheumatoid arthritis, and wound healing; refs. 1 and 3). Although dopamine has been linked to the pathogenesis of Parkinson disease (15) and schizophrenia (16), our present results for the first time not only demonstrate a unique peripheral physiological role of this important catecholamine neurotransmitter but also suggest that this neurotransmitter may have an important role in VPF/VEGF-mediated tumor angiogenic processes *in vivo*. Dopamine is present in measurable quantities (1 μM at nerve synapses, which are often adjacent to blood vessels) in normal adult tissues (17). Of interest in this regard, immunohistochemical studies have failed to demonstrate tyrosine hydroxylase-positive nerves in malignant tumors (tyrosine hydroxylase is the enzyme required for dopamine synthesis). There is also evidence that tissues immediately surrounding growing tumors have reduced sympathetic innervation (18, 19). In addition, the dopamine concentration in malignant tumors is reported to be significantly reduced, compared with that of normal controls (20). Together these reports indicate that dopamine regulation of the angiogenesis that may be present in normal vascular beds is lost in VPF/VEGF-mediated tumor angiogenesis.

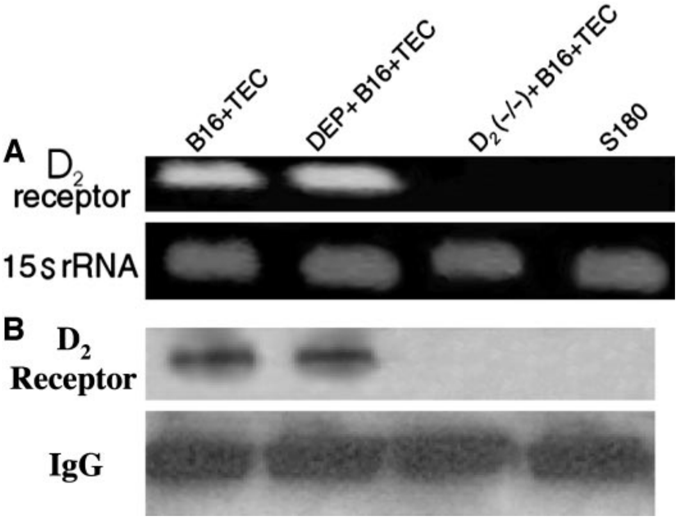

Fig. 2. Expression of D_2 receptor in endothelium. In *A*, semiquantitative reverse transciption-PCR shows D_2 class of dopamine receptor mRNA in tumor endothelial cells (*TEC*) isolated from B16 melanoma. D2 receptor mRNA (*D_2 receptor*) is present in TECs collected from both intact (N+B16) and ablated peripheral dopaminergic nerve (Dep+B16) wild-type control, but D_2 receptor mRNA is absent in TEC isolated from B16 melanoma-bearing D2 receptor knockout mice (D_2 (−/−)+B16) and negative control sarcoma 180 (*S180*) tumor. *15s rRNA*, control. Results are representatives of six separate experiments. In *B*, immunoprecipitation followed by immunoblot shows M_r ~85,000 D_2 dopamine receptor in extracts of TEC isolated from both intact (N+B16) and ablated peripheral dopaminergic nerve (Dep+B16) but absent in TEC isolated from D_2 receptor knock out mice (D_2 (−/−) + B16) and negative control S180 tumor. The figure is representative of six separate experiments.

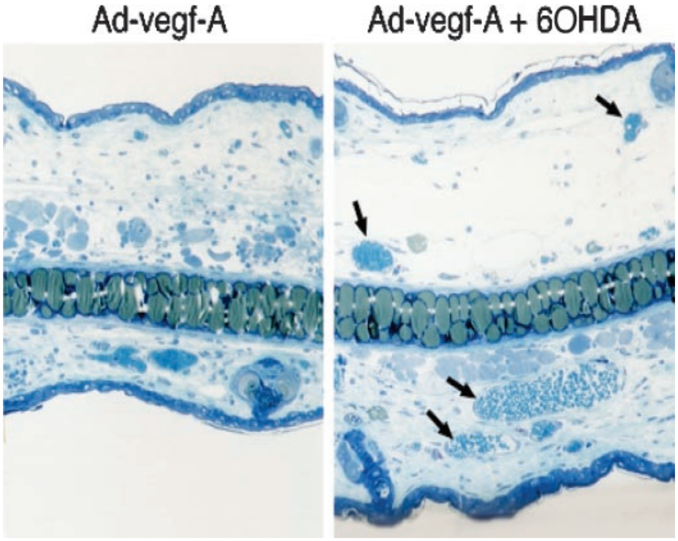

Fig. 3. Microscopic appearance of angiogenesis in the ears of athymic mice 2 days after local injection of 5×10^7 plaque-forming units of Ad-vegf (*left*, *Ad-vegf-A*). 6-hydroxy-dopamine-induced peripheral dopaminergic nerve-ablated mice [treatments with 6-hydroxydopamine (*6OHDA*) for 2 days before virus injection, *right panel*]. Ears of 6OHDA-treated mice show striking angiogenesis as determined by naked eye, or by microscopy. *Arrow*, the mother vessels formation within 2 days. These are 50-μm section.

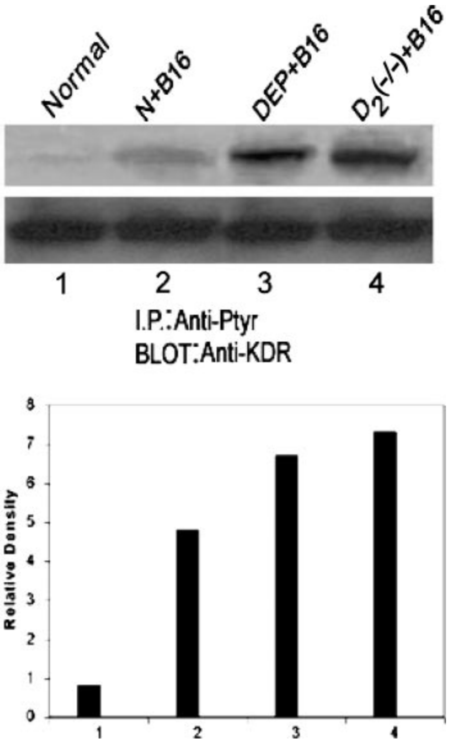

Fig. 4. Increased VPF/VEGF-induced phosphorylation in tumor endothelial cells (TECs) collected from the B16 melanoma-bearing mice with ablated peripheral dopaminergic nerves (*Dep+B16*) or dopamine D_2 receptor knockout mice [*D_2 (−/−)+B16*] when compared with B16-bearing mice with intact peripheral dopaminergic nerves (*N+B16*). Results are representative of six separate experiments. (*I.P.:Anti-Ptyr*, Anti-Phosphotyrosine; BLOT: *Anti-KDR*, anti-VEGFR-2.)

Finally, this study in addition to elucidating a new physiological role of peripheral dopamine in the regulation of tumor angiogenesis, also elucidates the significant physiological role played by dopamine in other pathological conditions in which VPF/VEGF-mediated angiogenesis is an important pathogenic factor. Our results, thus, in addition to revealing a new link between the peripheral dopaminergic system and cancer, also indicate a novel role of the endogenous peripheral neurotransmitter dopamine in regulating several pathological processes by modulating VPF/VEGF-induced angiogenesis.

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