



Dopamine regulates endothelial progenitor cell mobilization from mouse bone marrow in tumor vascularization

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Mobilization of endothelial progenitor cells (EPCs) from the bone marrow and their subsequent participation in neovessel formation are implicated in tumor growth and neovascularization. As the neurotransmitter dopamine (DA) modulates adult endothelial cell function, we hypothesized that DA might have a regulatory role in mobilization of EPCs from the bone marrow niche. We show that there was a significant decrease in bone marrow DA content and an increase in EPC mobilization in tumor-bearing mice associated with tumor neovascularization. DA treatment of tumor-bearing mice inhibited EPC mobilization and tumor growth through its D₂ receptors, as DA treatment failed to inhibit EPC mobilization in tumor-bearing mice treated with a specific DA D₂ receptor antagonist and in tumor-bearing mice lacking the D₂ receptor. In addition, we found that DA, through D₂ receptors, exerted its inhibitory effect on EPC mobilization through suppression of VEGFA-induced ERK1/ERK2 phosphorylation and MMP-9 synthesis. These findings reveal a new link between DA and EPC mobilization and suggest a novel use for DA and D₂ agents in the treatment of cancer and other diseases involving neovessel formation.

Introduction

It is now well established that angiogenesis or neovessel formation is essential for the growth, progression, and metastasis of malignant tumors (1–3). Until recently, endothelial cell proliferation, migration, sprouting, and co-option of preexisting blood vessels were considered to be the principal source of new vessel formation in cancer (1–3), but recent evidence suggests that mobilization of endothelial progenitor cells (EPCs) from the BM is also important for neovascularization in several types of malignant tumors (4–9). Accordingly, mobilization of EPCs from the BM to the peripheral circulation, resulting in circulating EPCs (CEPCs), is the critical step required for these cells to participate in the process of tumor neovascularization (4–7).

In adults, stem cells, including EPCs, reside in the BM, a specific niche that controls their survival, self renewal, differentiation, proliferation, and mobilization (5, 10–12). Therefore, it is important to decipher the molecular environment of the BM with special reference to the identification of factors promoting or inhibiting this process of EPC mobilization from the BM in pathological conditions like cancer, as this mobilization contributes to the process of tumor neovascularization (5, 10, 13, 14). Furthermore, as we have demonstrated that the neurotransmitter dopamine (DA) modulates functions of adult endothelial cells (15, 16), we reasoned that DA might have a regulatory role in controlling mobilization of EPCs from the BM. Because DA is present in the BM (17), we investigated whether BM DA could regulate EPC mobilization in cancer.

Nonstandard abbreviations used: 7AAD, 7-amino-actinomycin D; BMT, BM transplanted; CEPC, circulating EPC; DA, dopamine; Dil-Ac-LDL, Dil-labeled acetylated LDL; EPC, endothelial progenitor cell; rhVEGFA, human recombinant VEGFA; S180, sarcoma 180; TIMP-1, tissue inhibitor of metalloproteinase 1; VPF, vascular permeability factor.

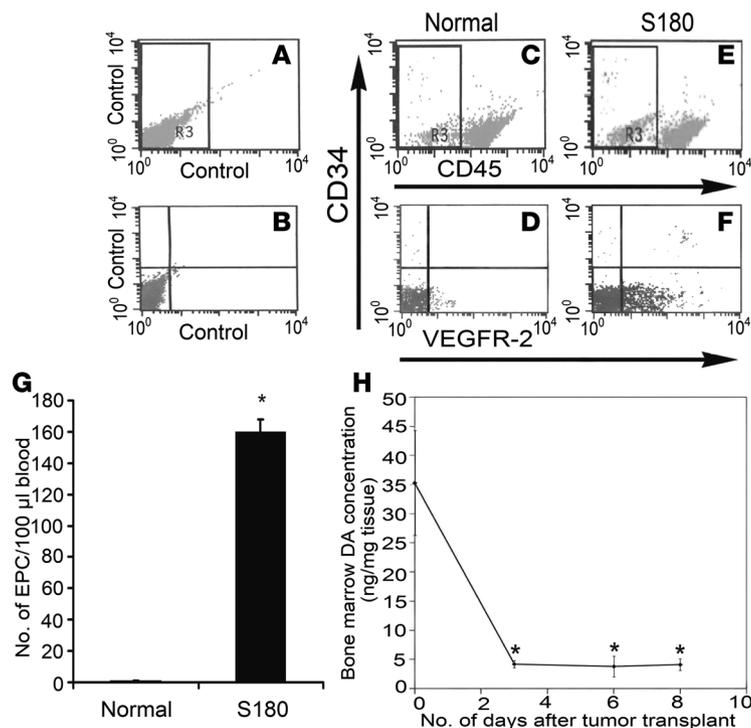
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Results

Increased mobilization of EPCs during tumor growth was associated with decrease in BM DA content. In order to determine the role of BM DA in the regulation of EPC mobilization in cancer, we performed our experiments in sarcoma 180 (S180), a well-characterized mouse tumor (16, 18) that can be transplanted into different strains of mice without concern of rejection, as these tumor cells do not express MHC antigens (19). We initially investigated the correlation between mobilization of EPCs from the BM and the BM DA content in S180-bearing mice. Following i.m. transplantation (1×10^6 cells) into the thighs of Swiss mice, increased numbers of CEPCs (CD45-VEGFR2⁺CD34⁺ cells) (20, 21) were demonstrated in these animals from day 3, which continued until day 6 after tumor transplantation (day of tumor transplantation was considered as day 0 in these experiments) (Figure 1, A–G). Thereafter, to determine the correlation between BM DA content and increased mobilization of EPCs, we examined the status of BM DA in these tumor-bearing animals. Using HPLC, we demonstrated significantly decreased BM DA concentration from day 3 after transplantation in these tumor-bearing animals in comparison with normal controls (Figure 1H). These data therefore indicate an inverse correlation between decreased BM DA content and mobilization of EPCs from the BM into circulation during tumor growth.

DA acted through its D₂ receptors in EPCs to inhibit tumor-induced mobilization of EPCs from BM to circulation and subsequent incorporation of these cells into tumor neovessels. Because DA acts through its specific receptor (22), in order to determine the DA receptor involved in inhibiting mobilization of BM-derived EPCs during malignant tumor growth, we next investigated the effect of DA on EPC mobilization in S180 tumor-bearing mice. DA was administered i.p. to these tumor-bearing animals at a low nontoxic dose (50 mg/kg/day \times 4 days, i.p.) (15, 23) from day 3 after tumor transplantation

**Figure 1**

Transplantation of S180 tumors in normal mice significantly increases CEPC numbers and decreases DA concentration in the BM. (A and B) Corresponding isotype controls. (C–G) Enumeration of CEPC numbers in normal and tumor-bearing mice. Significantly increased numbers of CEPCs were detected in tumor-bearing mice but were undetectable in the blood samples of normal mice. Cells were initially gated to exclude dead cells, debris, and red blood cells. (C and E) Subsequent gate used to select total CD45⁻ cell population. (D and F) Corresponding flow cytometric analysis for detecting CD34⁺VEGFR2⁺ cells in the gated CD45⁻ cell population. The cells represented in the upper-right quadrant are the desired EPC population showing a phenotype of CD45⁻CD34⁺VEGFR2⁺ cells. (G) Absolute number of EPCs (**P* < 0.05). Figures represent EPC frequency on posttransplantation day 6 and are representative of 4 separate experiments for each group. (H) BM DA concentration was found to decrease significantly from day 3 after tumor transplant, showing an inverse correlation with mobilization of EPCs.

and was continued for 6 days after tumor transplantation. After completion of treatment on the sixth day after tumor transplantation, we observed a significantly decreased number of CEPCs in these DA-treated S180-bearing animals when compared with untreated controls (Figure 2, A–F and I). In contrast, pretreatment with specific DA D₂ receptor antagonist eticlopride (10 mg/kg i.p.) completely abrogated this inhibitory effect of DA (Figure 2, A, B, and G–I). In addition, treatment of the tumor-bearing animals with other classes of specific DA receptor antagonists (D₁, D₃, D₄, D₅) prior to DA treatment failed to inhibit the action of DA (data not shown). The 2 other members of the catecholamine family, epinephrine and norepinephrine, which do not interact with DA D₂ receptors, did not reproduce the action of DA (Supplemental Figure 1; available online with this article; doi:10.1172/JCI33125DS1). Since formation of late outgrowth colonies in cultured PBMCs and the abilities of these colonies to uptake Dil-labeled acetylated LDL (Dil–Ac–LDL) are reported to be characteristics of EPCs (24), we examined late outgrowth colony-forming ability of PBMCs collected from both tumor-bearing and tumor-bearing plus DA-treated animals. Although late outgrowth colonies confirmed by Dil–Ac–LDL uptake were demonstrated in cultured PBMCs collected from tumor-bearing animals, late outgrowth colonies were absent in cultured PBMCs collected from DA-treated, tumor-bearing animals (Figure 2, J–M). This result further demonstrated that DA inhibited tumor-induced mobilization of EPCs from the BM to the peripheral circulation and indicated that BM DA, by acting through DA D₂ receptors, might play an important role in regulating mobilization of BM-derived EPCs.

Finally, to confirm the involvement of BM DA and its D₂ receptors in the mobilization of EPCs from the BM to the peripheral circulation, we transplanted S180 tumors i.m. into the thighs of DA D₂^{-/-} receptor C57BL/6 mice, as DA D₂^{-/-} receptor mice are commercially available only in the C57BL/6 background. It is to

be noted here that S180 tumors can also be transplanted into C57BL/6 mice (18, 19) and the growth kinetics of S180 tumors in this strain of mice were similar to that observed in Swiss mice (data not shown). Thereafter, wild-type C57BL/6 mice were also transplanted i.m. with S180 tumor cells. Following tumor transplantation, we observed higher numbers of CEPCs in tumor-bearing DA D₂^{-/-} animals in comparison with tumor-bearing wild-type controls (Figure 2, A, B, N–Q, and T). Also, DA treatment failed to inhibit mobilization of EPCs from the BM to the peripheral circulation in these tumor-bearing DA D₂^{-/-} animals (Figure 2, A, B, and R–T).

We next investigated the effect of DA treatment on the incorporation of EPCs into tumor neovessels by utilizing a BM transplantation model. FVB/N mice underwent BM transplantation from transgenic mice constitutively expressing β-gal encoded by *lacZ* under the transcriptional regulation of an endothelial cell-specific promoter, *Tie2*. At 4 weeks after the BM transplantation, reconstitution of the transplanted BM yielded *Tie2*/LZ/BMT (LZ, *lacZ*; BMT, BM-transplanted) mice in which expression of *lacZ* was restricted to BM-derived cells expressing *Tie2*. Thereafter, these BMT mice were transplanted with S180 tumors because the FVB/N mice were reported to be of Swiss mouse origin (25) and S180 is a transplantable tumor for Swiss mice (16). Furthermore, the growth kinetics of S180 in FVB/N mice were also similar to those of S180 transplanted in Swiss mice (data not shown). We observed incorporation of BM-derived EPCs into the tumor neovessels of untreated S180-bearing BMT mice on day 7 after transplantation. In contrast, tumor neovessels of DA-treated S180-bearing BMT mice did not show any incorporation of BM-derived EPCs (Figure 3, A–C). These results thus confirm that DA inhibited mobilization of EPCs from the BM to the peripheral circulation and thereby inhibited incorporation of these progenitor cells into the tumor neovessels.

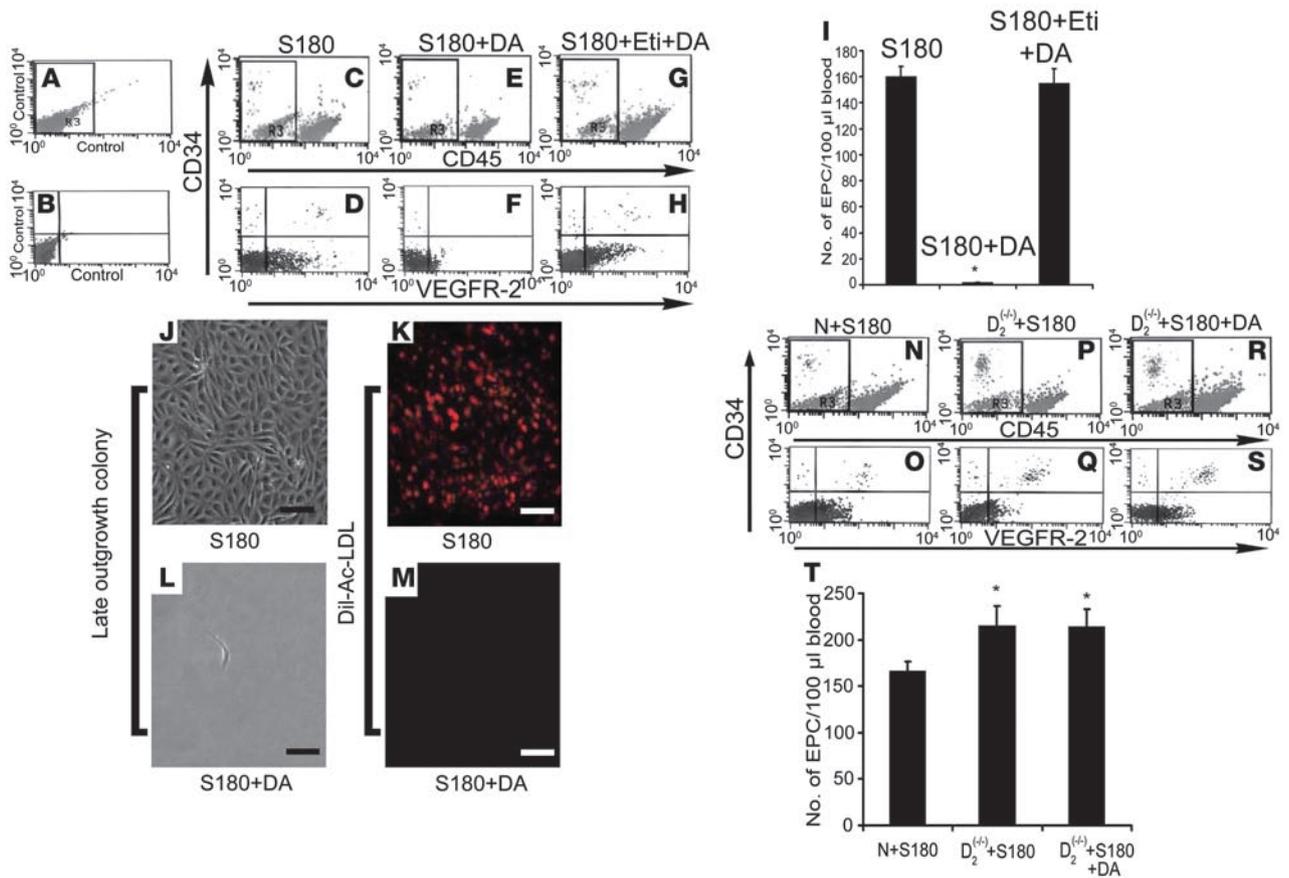


Figure 2

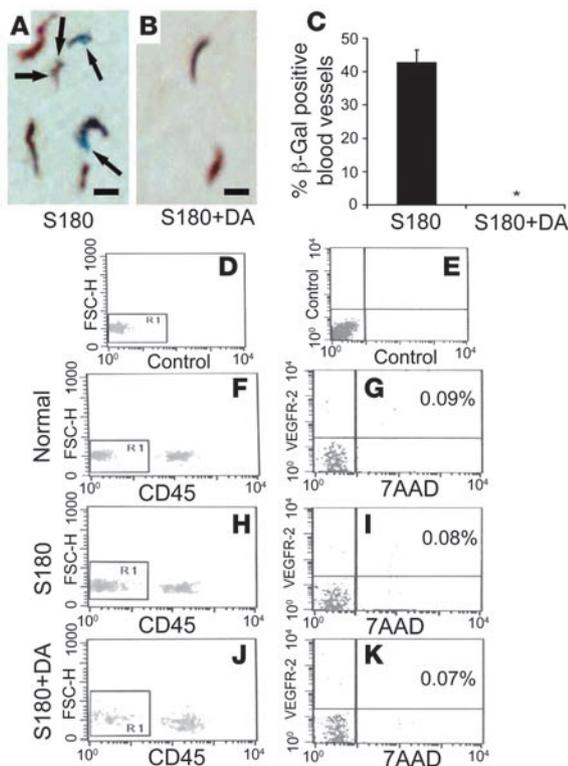
DA, by acting through its D₂ receptors on EPCs, inhibits tumor-induced mobilization of CEPCs and thereby incorporation into the tumor neovasculature. (A and B) Respective isotype controls. (C–I) Flow cytometric analysis of CEPCs in tumor-bearing, tumor-bearing plus DA-treated, and tumor-bearing plus eticlopride (Eti) plus DA-treated mice. CEPCs were detectable in tumor-bearing mice, but were undetectable in the blood of tumor-bearing DA-treated animals. DA had no effect on EPC frequency in tumor-bearing animals treated with the D₂ receptor antagonist eticlopride prior to DA administration, indicating that this inhibition was D₂ receptor mediated. Figures represent EPC frequency on posttransplantation day 6 in both tumor-bearing and DA-treated, tumor-bearing animals after completion of DA treatment schedule. (I) Absolute number of EPCs. (J and K) Late outgrowth colonies from PBMCs of tumor-bearing normal mice. The endothelial nature of the late outgrowth colony-forming cells was confirmed by the uptake of Dil-Ac-LDL. (L and M) Absent late outgrowth colonies from PBMCs of DA-treated, tumor-bearing normal mice. PBMCs isolated from circulation were plated onto fibronectin-coated dishes, and thereafter, late outgrowth colonies were scored. (N–T) Flow cytometric analysis of circulating EPCs in tumor-bearing D₂^(-/-) mice showed significantly higher CEPCs when compared with tumor-bearing wild-type mice (*P* < 0.05). (T) Absolute number of EPCs. DA treatment caused no change in EPC frequency in peripheral blood of tumor-bearing D₂^(-/-) mice. Scale bars: 50 μ m. D₂^(-/-), D₂ receptor–knockout mice. Figures are representative of 4 separate experiments in each group. N, wild type.

Effect of DA on the viability of CD45-VEGFR2⁺ circulating cells. Because decrease in the number of CEPCs following DA treatment might be due to cell death, we investigated the effect of DA treatment on the viability of CD45-VEGFR2⁺ cells (which include CEPCs) in the peripheral blood by 7-amino-actinomycin D (7AAD) staining (26) of both S180 tumor-bearing animals and tumor-bearing animals treated with DA. Accordingly, we enumerated the number of 7AAD⁺ cells in this cell population by flow cytometry. We did not observe any significant alterations in cell viability of CD45-VEGFR2⁺ cells in these 2 groups of animals (Figure 3, D–K). It is to be noted here that since EPCs in the BM are very few in number, we therefore undertook these experiments utilizing circulating CD45-VEGFR2⁺ BM-derived cells (which include EPCs) as previously reported (10).

Effect of DA on growth and angiogenesis of solid S180 tumor in DA D₂^(+/+) and DA D₂^(-/-) mice. Experimental results so far demonstrated that DA inhibits mobilization of EPCs from the BM in tumor-bearing

mice. Recent reports suggest that mobilization of EPCs from the BM to the peripheral circulation and its subsequent incorporation into the tumor vascular bed also contribute to tumor neovascularization (4–9), a critical step for tumor growth because, unless EPCs mobilize from the BM, they cannot participate in tumor neovascularization (5, 10, 13, 14). Therefore, we next investigated whether this DA-induced inhibition of EPC mobilization was associated with inhibition of tumor angiogenesis and its growth.

DA administration (50 mg/kg \times 4 days) significantly inhibited tumor growth (*P* < 0.05) (Figure 4A) by inhibiting angiogenesis in tumor tissue of DA D₂^(+/+) mice (Figures 4, B, C, and F). This result correlated with the inhibition of EPC mobilization by DA. In contrast, microvessel density in tumors of DA D₂^(-/-) mice was significantly higher (*P* < 0.05), and treatment of tumor-bearing DA D₂^(-/-) mice with DA failed to show any effect on tumor growth (Figure 5A) and angiogenesis (Figure 4, D–F) in these animals.

**Figure 3**

DA inhibits incorporation of BM-derived cells into tumor vasculature and has no effect on the viability of CEPCs. (A and B) Tie2/lacZ transgenic BM cells were transplanted into lethally irradiated syngeneic mice, followed by tumor transplantation. Tumor sections were incubated with X-gal and anti-CD31 to enumerate incorporation of lacZ⁺ BM-derived cells into tumor vasculature (arrows). (C) Percentage of β -gal-positive blood vessels. Figures are representative of 3 separate experiments. Scale bars: 50 μ m. (D–K) DA had no effect on viability of CD45-VEGFR2⁺ cells, including EPCs. Viability of cells was determined by flow cytometry based on 7AAD uptake. (D and E) Respective isotype controls. (F–K) After excluding dead cells, debris, and red blood cells, CD45⁻ cells were gated. (F, H, and J) VEGFR2⁺ plus 7AAD⁺ cells were quantitated out of the gated CD45⁻ cell population. (G, I, and K) Upper-right quadrants show the 7AAD⁺ nonviable cells among the CD45-VEGFR2⁺ cells. Figures are representative of 4 separate experiments for each group.

ited VEGFA-induced mobilization of EPCs from the BM to the peripheral circulation.

In vitro VPF/VEGFA-induced mobilization of CD45-VEGFR2⁺ cells was inhibited by DA. To further confirm that DA specifically inhibited VEGFA-induced mobilization of EPCs, *in vitro* studies were undertaken. BM-derived CD45-VEGFR2⁺ cells from normal mice were at first treated with 1 μ M of DA, the physiological concentration of DA found at nerve endings (15), and thereafter these DA-treated cells were seeded into the upper chamber of a modified Boyden chamber. Significant inhibition of VEGFA-induced mobilization of these cells was demonstrated when compared with the mobilization of control cells not treated with DA. However, when these cells were pretreated with eticlopride (10 μ M), a specific DA D₂ receptor antagonist, no such inhibitory effect on mobilization of BM-derived CD45-VEGFR2⁺ cells in the modified Boyden chamber was observed (Figure 6G).

DA-mediated inhibition of EPC mobilization from BM was associated with inhibition of MMP-9 expression and activity in BM of tumor-bearing mice. Recent results indicate the important role of MMPs in the regulation of EPC mobilization from BM (5, 10, 12). In BM, MMPs, especially MMP-9 (10, 12), play a pivotal role in the mobilization of EPCs from the BM to the peripheral circulation. We therefore reasoned that the underlying molecular mechanism of DA-mediated inhibition of EPC mobilization may be due to DA-mediated regulation of BM MMP-9. Thus, we investigated the role of DA on the expression of MMP-9 and its activity in the BM. Immunohistochemical staining of the BM tissues collected from tumor-bearing mice demonstrated increased expression of MMP-9 in comparison with normal controls (Figure 7, A and B), and this correlated with the striking increase in the mobilization of EPCs from the BM to the peripheral circulation in these animals. On the contrary, marked decrease in MMP-9 expression was demonstrated in the BM tissues collected from the tumor-bearing animals treated with DA (Figure 7C). Tumor-bearing D₂^{-/-} mice showed robust expression of MMP-9 in the BM (Figure 7D), and DA treatment had no effect on MMP-9 expression in these S180 tumor-bearing DA D₂^{-/-} mice (Figure 7E). Furthermore, this increase in MMP-9 expression was associated with increase in EPC mobilization from the BM to circulation, thereby demonstrating the importance of endogenous BM DA on MMP-9 expression and therefore EPC mobilization. Next, in order to substantiate the immunohistochemical results showing the regulatory role of DA in MMP-9 expression in the BM, we quantitated the pro-MMP-9 concentration and MMP-9 activity by sandwich ELISA and zymog-

Effect of DA on VPF/VEGFA-mediated mobilization of EPCs from the BM to the peripheral circulation. VEGFA (also known as vascular permeability factor [VPF]) is an important cytokine that induces mobilization of EPCs from BM (4, 5, 8) in tumor-bearing hosts. Therefore, to elucidate whether DA in tumor-bearing hosts exerts an inhibitory effect on VEGFA-induced mobilization of EPCs, we performed further experiments to determine the effect of DA treatment on VEGFA-induced mobilization of EPCs from the BM to the peripheral circulation. Injection of 10 μ g of human recombinant VEGFA (rhVEGFA) *i.p.* into normal mice for 5 continuous days significantly increased the number of CEPCs (CD45-VEGFR2⁺CD34⁺ cells) in these animals, beginning on day 1 of treatment and reaching a peak on days 4–5 of treatment. This result correlated well with the previous reports (27). In contrast, *i.p.* injection of low nontoxic doses of DA (50 mg/kg/day \times 5 days) administered 5 minutes after each rhVEGFA administration (starting from day 1 of rhVEGFA injection) strikingly inhibited VEGFA-induced mobilization of EPCs (Figure 5, A–H and K). It is to be noted here that no significant change in total BM cell count and viability was observed in these animals after DA treatment (data not shown). However, pretreatment (*i.p.*) of these animals with a specific DA D₂ receptor antagonist, eticlopride (10 mg/kg), abrogated this action of DA (Figure 5, A, B, and I–K), thereby indicating that the effect of DA was mediated through its D₂ receptors. The specificity of DA action through its D₂ receptors was further confirmed, as treatment of the animals with other classes of specific DA receptor antagonists (D₁, D₃, D₄, D₅) prior to DA treatment failed to inhibit the action of DA (data not shown). Accordingly, by FACS analysis, DA D₂ receptors were demonstrated in EPCs (CD45-VEGFR2⁺CD34⁺) collected from the BM of these animals (Figure 6, A–F). These results further confirmed that DA inhib-

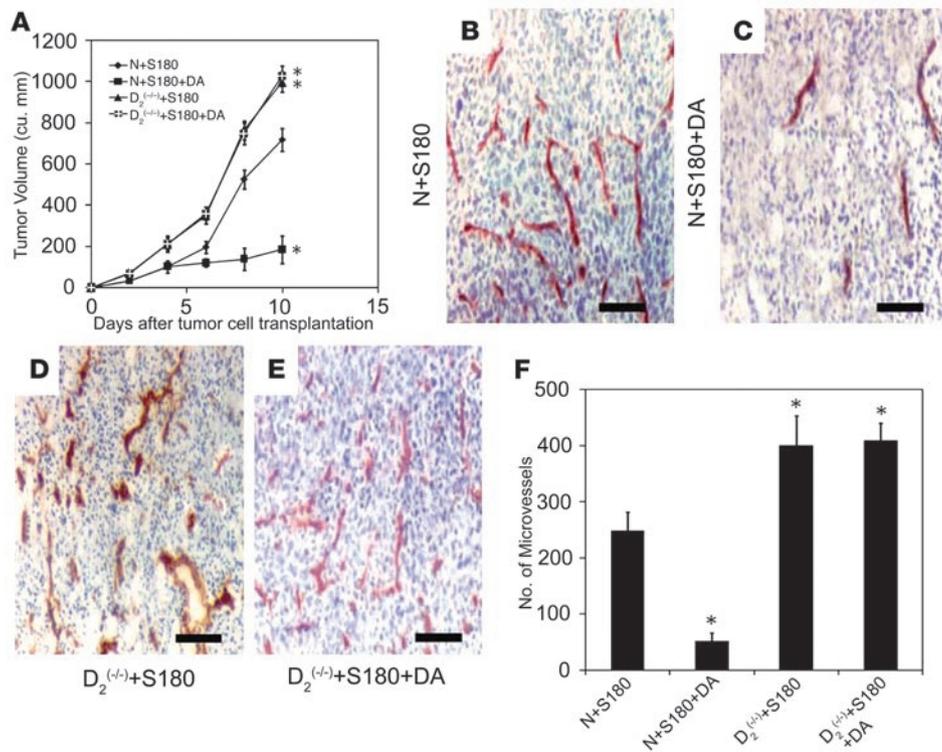


Figure 4

DA inhibits EPC mobilization, which in turn is associated with inhibition of tumor growth and angiogenesis. (A) Graphical representation of the tumor volume of tumor-bearing S180 plus DA and D₂^{-/-} plus S180 plus DA-treated animals. Significant reduction was observed in the tumor volume of DA-treated S180 tumor-bearing animals. In D₂^{-/-} plus S180 animals, tumor growth was higher than in tumor-bearing controls. However, no effect of DA treatment was observed in D₂^{-/-} plus S180 animals. (B, C, and F) Immunohistochemical staining of CD31 (microvessel density), a specific endothelial cell marker, showed significantly reduced microvessel density in tumor tissues following DA treatment in comparison with the vehicle-treated controls. (B, D, and F) Tumor microvessel density was significantly higher in D₂^{-/-} plus S180 animals (*P* < 0.05 compared with respective control). (D–F) DA treatment failed to reduce the microvessel density in tumor-bearing D₂^{-/-} animals. Microvessel density was measured by counting the number of microvessels in 10 randomly chosen high-power microscopic fields within the sections. Figures are representative of 4 separate experiments for each group. **P* < 0.05. Scale bars: 50 μm.

raphy, respectively. These studies were carried out in the supernatants of overnight culture of CD45-VEGFR2⁺ BM cells collected from tumor-bearing and tumor-bearing plus DA-treated animals. Our results indicate significant increases in the pro-MMP-9 concentration and MMP-9 activity in the cell supernatants of untreated, tumor-bearing animals (Figure 7, F and G), which correlated well with the significantly increased mobilization of EPCs observed in tumor-bearing animals. In contrast, DA treatment significantly decreased pro-MMP-9 concentration and MMP-9 activity (*P* < 0.05) (Figure 7, F and G). However, pretreatment with the specific D₂ receptor antagonist eticlopride significantly abrogated the action of DA (Figure 7, F and G). These results thus indicated the involvement of the DA D₂ receptors in DA-mediated regulation of MMP-9 synthesis in these progenitor cells. Accordingly, RT-PCR and Western blot analysis demonstrated the presence of DA D₂ receptors in these BM cells (Figure 7, H and I).

To further confirm the role of BM DA in the regulation of BM MMP-9 concentration and activity, we collected BM-derived CD45-VEGFR2⁺ cells from tumor-bearing DA D₂^{-/-} mice. A significant increase in pro-MMP-9 concentration as well as MMP-9 activity was demonstrated in the culture soup of those BM-derived progenitor cells, and DA treatment failed to inhibit the increase (Figure 7, F and G). It is to be noted here that DA had no effect on

tissue inhibitor of metalloproteinase 1 (TIMP-1) (28), an endogenous inhibitor of MMP-9 (Figure 7J).

DA in vitro inhibited VPF/VEGFA-induced MMP-9 synthesis in CD45-VEGFR2⁺ BM cells. Mobilization of BM-derived EPCs into peripheral circulation occurs in response to VEGFA in different pathological conditions, including cancer (8, 10, 27). This process is also MMP-9 dependent (8, 10, 11, 28, 29). Our previous in vivo experiments have shown that DA inhibited MMP-9 expression and activity in BM of tumor-bearing mice (Figure 7, F and G). Therefore, to identify the specific molecular pathway through which DA regulated EPC mobilization from BM, we determined the direct effect of DA on VEGFA-induced MMP-9 synthesis in BM-derived CD45-VEGFR2⁺ cells (which include EPCs) from normal mice in vitro. Thereafter, the CD45-VEGFR2⁺ cells were treated with VEGFA (50 ng/ml), VEGFA (50 ng/ml) plus DA (1 μM), or VEGFA (50 ng/ml) plus eticlopride (10 μM) plus DA (1 μM) and cultured overnight. A considerable amount of pro-MMP-9 in culture supernatant was demonstrated in cells treated with VEGFA (Figure 8A). In contrast, we observed significant inhibition (*P* < 0.05 in comparison with normal control) of pro-MMP-9 in the supernatant of cultures treated with DA plus VEGFA (Figure 8A). Furthermore, pretreatment with eticlopride, a specific DA D₂ receptor antagonist, significantly abrogated the inhibitory effect of DA (Figure 8A).

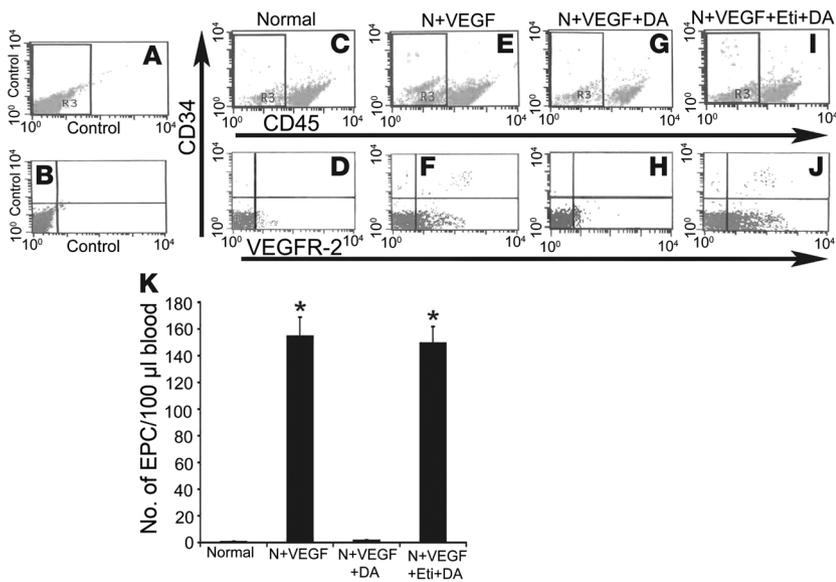


Figure 5 DA inhibited VEGFA-induced mobilization of EPCs from the BM through its D₂ receptors. (A and B) Respective isotype controls. (C–J) Flow cytometric determination of CEPC frequency after completion of DA treatment. rhVEGFA was injected i.p. into normal mice for 5 continuous days. Immediately following VEGFA administration, each mouse received i.p. injection of DA (50 mg/kg body weight) for 5 consecutive days beginning from day 1 of VEGFA administration. CEPC frequency on treatment day 5 after completion of the treatment is represented. (K) Absolute number of EPCs in various groups. Treatment with eticlopride, a DA D₂ receptor antagonist, prior to DA treatment completely abrogated the inhibitory effect of DA. Figures are representative of 4 separate experiments for each group. *P < 0.05.

DA acted through its D₂ receptors to inhibit MMP-9 synthesis by suppressing ERK1/ERK2 phosphorylation in CD45-VEGFR2⁺ cells collected from tumor-bearing animals. Reports from other laboratories have demonstrated that in several cell types, including microvascular endothelial cells, MMP-9 synthesis is under the control of the ERK1/ERK2 pathway (30–32). Accordingly, we investigated to determine whether DA-induced inhibition of MMP-9 synthesis in CD45-VEGFR2⁺ cells of the BM was through the ERK1/ERK2 pathway. In tumor-bearing animals, increased expression of MMP-9 in the BM was associated with increased phosphorylation of ERK1/ERK2 in CD45-VEGFR2⁺ BM-derived progenitor cells (Figure 8B). In contrast, significantly decreased MMP-9 expression in the BM of the DA-treated, tumor-bearing animals (Figure 8B) was associated with significantly decreased phosphorylation of ERK1/ERK2 in BM-derived CD45-VEGFR2⁺ progenitor cells collected within 15 minutes after DA treatment from the tumor-bearing animals. However, pretreatment with specific DA D₂ receptor antagonist eticlopride (10 mg/kg i.p.) abrogated this action of DA in the CD45-VEGFR2⁺ progenitor cells (data not shown).

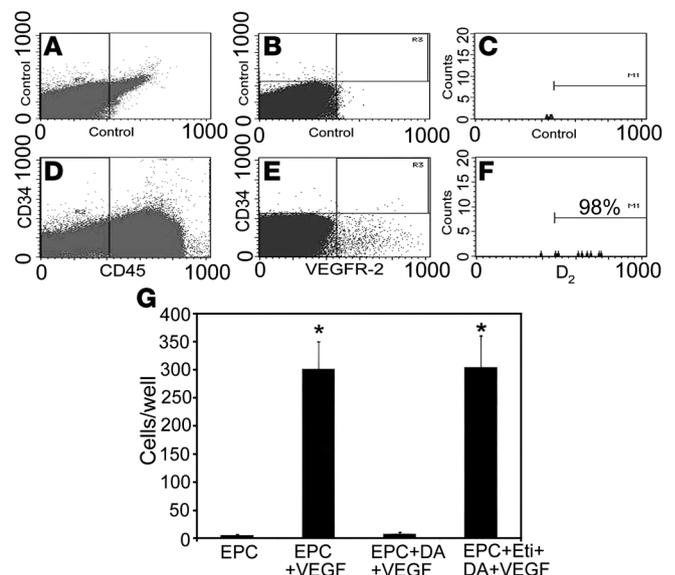
Furthermore, to confirm the role of BM DA and its D₂ receptors in the regulation of MMP-9 synthesis in BM-derived CD45-VEGFR2⁺ progenitor cells, we next investigated phosphorylation

of ERK1/ERK2 in CD45-VEGFR2⁺ BM-derived progenitor cells in tumor-bearing DA D₂^{-/-} mice. Significantly increased synthesis of MMP-9 was associated with significantly increased phosphorylation of ERK1/ERK2 in CD45-VEGFR2⁺ BM-derived cells collected from S180-bearing DA D₂^{-/-} mice (Figure 8B), and DA treatment failed to inhibit this enhanced phosphorylation of ERK1/ERK2 in these D₂ receptor-knockout mice (Figure 8B).

In vitro DA inhibited VEGFA-induced MMP-9 synthesis in CD45-VEGFR2⁺ BM-derived cells by suppressing ERK1/ERK2 phosphorylation. VEGFA activates MMPs, especially MMP-9 (10, 28, 29), which subsequently promote the mobility of EPCs from the BM to the peripheral circulation by release of soluble kit ligand (sKITL) (5, 10, 29), and VEGFA has been reported to be the most critical cytokine that induces mobilization of EPCs in cancer (33, 34). We therefore elucidated the molecular mechanisms of DA-mediated inhibition of MMP-9 synthesis in CD45-VEGFR2⁺ BM cells *in vitro*. We observed that DA (1 μ M)

Figure 6

Four-color flow cytometry showing presence of DA D₂ receptors on EPCs; DA inhibits VEGFA-induced migration of EPCs *in vitro*. (A–C) Respective isotype controls. (D) After excluding dead cells and debris, total CD45⁻ cells were gated (gate R2). (E) CD45⁻ cells were further analyzed for the presence of CD34 and VEGFR2. Upper-right quadrant represents CD45-CD34⁺VEGFR2⁺ EPCs. These cells were further gated (gate R3) to analyze the presence of D₂ receptors. (F) Histogram showing the presence of D₂ receptors in 98% of the CD45-CD34⁺VEGFR2⁺ EPCs. (G) Migration assay of cultured EPCs. DA was found to exert significant inhibition on VEGFA-induced migration of EPCs in a modified Boyden chamber. Treatment with eticlopride, a DA D₂ receptor antagonist, prior to DA treatment, completely abrogated the inhibitory effect of DA. Figures are representative of 4 separate experiments for each group. *P < 0.05.



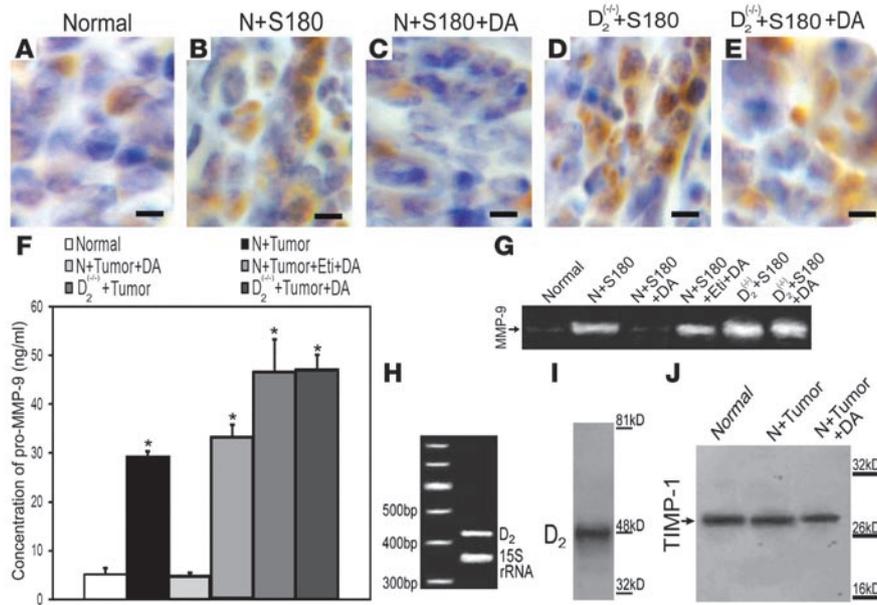


Figure 7

DA, by acting on its D₂ receptors, downregulates MMP-9 expression and activity in BM-derived CD45-VEGFR2⁺ cells without affecting TIMP-1. (A–E) Immunohistochemical analysis of BM sections showed increased expression of MMP-9 in BM cells of S180 tumor-bearing mice whereas normalization of MMP-9 expression was observed in wild-type (N) plus S180 plus DA-treated mice. S180 tumor-bearing D₂^(-/-) animals showed more robust expression of MMP-9 than respective controls. However, DA treatment had no effect on MMP-9 expression in these animals. (F) Concentration of pro-MMP-9 in overnight cultures of CD45-VEGFR2⁺ BM cells. Cells were isolated from BM by MACS cell-sorting system; pro-MMP-9 increased significantly in tumor-bearing animals. *P < 0.05. DA inhibited the increased pro-MMP-9 level to normal. However, prior eticlopride treatment abrogated this inhibitory effect of DA. In tumor-bearing D₂^(-/-) animals, culture showed higher pro-MMP-9 level, and DA treatment failed to show any effect on pro-MMP-9 concentration. (G) Assessment of MMP-9 activity in culture soup of CD45-VEGFR2⁺ cells from BM, by gelatin zymography. The separated cells from different experimental groups were cultured overnight in serum-free media, and the supernatant was assayed by gelatin zymography. Culture of CD45-VEGFR2⁺ cells from tumor-bearing hosts showed significantly increased activity of MMP-9 compared with normal, which was significantly inhibited by DA treatment. Culture of CD45-VEGFR2⁺ cells from tumor-bearing D₂^(-/-) mice showed significantly increased MMP-9 activity than respective control. However, DA treatment had no effect on MMP-9 activity. Figures are representative of 4 separate experiments for each group. Scale bars: 50 μm. (H–J) DA D₂ receptors are present on EPCs, and DA treatment had no effect on TIMP-1, the endogenous inhibitor of MMP-9 synthesis. TIMP-1 was evaluated by Western blot from CD45-VEGFR2⁺ BM cells. Figures are representative of 4 separate experiments for each group.

significantly inhibited VEGFA-induced phosphorylation of ERK1/ERK2 in these cells and thereby downregulated MMP-9 synthesis in these cells (Figure 8C).

Discussion

Neovascularization is required for the growth and progression of malignant tumors (1–3). Both human and animal studies have indicated that BM-derived EPCs play an important role in this process (4–7, 14). There are also recent reports that indicate that BM-derived EPCs form vascular niches, to which tumor cells subsequently migrate to form metastatic foci (35). These studies thus emphasize the importance of mobilization of EPCs from the BM in tumor neovascularization. Furthermore, it is now well established that VPF/VEGFA is the prime cytokine that regulates this dynamic process of EPC mobilization from the BM to the peripheral circulation in cancer (10, 33, 34). In animals, EPCs are mobilized into the circulation and thereafter incorporated into the vascular bed within 48 hours after tumor transplantation (4, 5, 27). In addition to VPF/VEGFA, MMP-9 has also been identified as a specific mediator of EPC mobilization from the BM to the circulation (10–12, 28, 29). Several reports indicate that VPF/VEGFA-mediated MMP-9 activation is

required for EPC mobilization (5, 10–12, 28, 29), and this activity of MMP-9 correlates well with the efficient mobilization of EPCs (10–12, 29).

In the present investigation, we indicate a new molecular pathway that regulates tumor-induced mobilization of EPCs from the BM. Our results demonstrate for what we believe is the first time that there is a significant decrease in the concentration of BM DA during malignant tumor growth, which in turn is inversely correlated with the mobilization of EPCs from the BM to the peripheral circulation. In addition, we have also shown that the increased expression of MMP-9 in the BM of tumor-bearing or VPF/VEGFA-treated animals is associated with increased mobilization of EPCs (5, 10). However, DA treatment significantly decreases tumor-induced or VPF/VEGFA-induced EPC mobilization from the BM, and this decrease in EPC mobilization is associated with a decrease in BM MMP-9 expression. These results thus indicate a regulatory role of DA in MMP-9 synthesis in the BM. In addition, because we have demonstrated that DA inhibits mobilization of EPCs by suppressing VPF/VEGFA-induced MMP-9 activation via the ERK1/ERK2 pathway, this study also provides what we believe is a novel mechanistic insight into the regulatory role of neurotransmitter DA in regulating mobilization of EPCs from the BM in cancer.

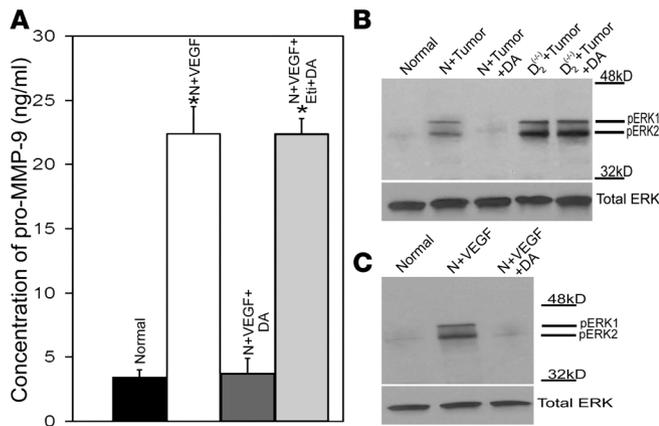


Figure 8 DA inhibits VEGFA-induced pro-MMP-9 synthesis and ERK1/ERK2 phosphorylation in CD45-VEGFR2⁺ BM cells. (A) In vitro, DA inhibits VEGFA-induced pro-MMP-9 synthesis in CD45-VEGFR2⁺ BM cells. Pro-MMP-9 was assayed from supernatants of cultured CD45-VEGFR2⁺ BM cells by sandwich ELISA. CD45-VEGFR2⁺ cells were isolated from BM of normal mice using MACS cell-sorting system and cultured overnight in serum-free medium with VEGFA, VEGFA plus DA, and VEGFA plus eticlopride plus DA. Synthesis of pro-MMP-9 was found to be significantly increased in supernatants from cells treated with VEGFA, which was normalized in cells treated with VEGFA plus DA. **P* < 0.05. However, treatment with eticlopride (DA D₂ receptor antagonist) prior to DA administration abrogated the effect of DA. Results are mean ± SEM for 4 separate experiments. (B) Western blot analysis of CD45-VEGFR2⁺ BM cells showed considerably increased phosphorylation of ERK1/ERK2 in tumor-bearing mice compared with normal controls, whereas DA treatment inhibited phosphorylation. In D₂^{-/-} plus tumor-bearing animals, CD45-VEGFR2⁺ BM cells showed increased phosphorylation, but DA treatment had no effect. (C) When CD45-VEGFR2⁺ EPCs isolated from BM of normal animals were cultured overnight in presence of VEGFA, increased phosphorylation of ERK1/ERK2 was observed in Western blot analysis when compared with the untreated cells. However, the phosphorylation was significantly inhibited within 15 minutes of DA treatment, showing that DA inhibits VEGFA-induced phosphorylation of ERK1/ERK2. An antibody against total ERK was used to determine equal loading. Figures are representative of 4 separate blots.

In addition to our present investigation, there is also a recent paper in which the authors have indicated that DA under normal conditions stimulated GM-CSF-mediated mobilization of human immature CD34⁺ hematopoietic cells in vitro via DA D₃ and D₅ receptors (36). This stimulatory action of DA on immature CD34⁺ hematopoietic cells contradicts our present data demonstrating DA-mediated inhibition of mobilization of BM-derived EPCs because there are major differences between these 2 investigations. In our present experiments, we have shown that in malignant tumor-bearing animals, DA inhibits VEGFA-mediated mobilization of EPCs from the BM in vivo through the DA D₂ receptors. Thus, the dissimilarities between these 2 reports can be attributed to the differences in selection of experimental conditions, cell types, cytokines, and DA receptors between the 2 groups. Furthermore, it will be interesting to mention here that since a subset of macrophages can be EPCs (37) and because a recent report indicates synthesis and release of catecholamines by macrophages (38), future studies may be undertaken to investigate the role of DA on these cells.

Finally, as there are now several reports that indicate that mobilization of EPCs from the BM to the peripheral circulation and its subsequent incorporation into neovessels is important for the pathogenesis of many diseases (4, 5, 7, 8, 11, 14, 27, 39), targeting EPC mobilization may be an important therapeutic intervention for the treatment of these pathological conditions. Moreover, since we had previously reported that DA can inhibit the functions of adult endothelial cells by suppressing phosphorylation of VEGFR2, FAK, and MAPK (15, 16, 40, 41) and because we have now demonstrated that DA, by acting through its D₂ receptors, can inhibit the mobilization of BM-derived EPCs, it is prudent to suggest that inexpensive drugs such as DA or its specific D₂ receptor agonists may be used for the treatment of cancer, in which neovessel formation is essential for the growth and metastasis of the tumors (1–3).

In summary, this study not only reveals a new link between the BM DA and EPC mobilization, but it also indicates that DA or its D₂ receptor agonists and antagonists, which are being extensively used in the clinics (42) at present, may have a therapeutic value in cancer and other diseases in which EPC mobilization plays an important pathogenic role (4, 5, 7, 8, 11, 14, 27, 39).

Methods

Animals, tumors, and VEGFA treatment. The protocols were approved by the Institutional Animal Care and Use Committee of Chittaranjan National Cancer Institute and the Mayo Clinic. Normal Swiss mice (NIH) were treated with 10 µg of rhVEGFA (R&D systems) dissolved in 100 µl of 0.5% BSA (Sigma-Aldrich) (27) and injected i.p. for 5 continuous days. Control mice received equal amounts of BSA. Low nontoxic doses of i.p. DA (50 mg/kg/d) were administered 5 minutes following VEGFA treatment for 5 continuous days. Thereafter, blood and BM cells were collected on day 5, when peak CEPC count was observed. In the S180 tumor model, the increase in CEPCs started from day 3 and continued until day 6 after transplantation. Therefore, in the S180 tumor-bearing animals, DA treatment (50 mg/kg/day i.p.) was started on day 3 after tumor transplantation and was continued for 4 consecutive days. Similar experiments were also undertaken in normal and DA D₂ receptor-knockout C57BL/6 mice (The Jackson Laboratory). Tumor growth was determined by caliper measurement of the largest diameter and its perpendicular. Tumor volume was determined by the following equation: Tumor volume (mm³) = 0.5 × *a* × *b*², where *a* is the largest diameter and *b* is its perpendicular (43).

Flow cytometry. Murine circulating CEPCs were determined by flow cytometry using a panel of fluorochrome-conjugated monoclonal antibodies reacting with CD45 (to exclude hematopoietic cells) and endothelial markers VEGFR2 and CD34 (BD Biosciences – Pharmingen). Following red cell lysis, CEPCs were enumerated by flow cytometry (FACSCalibur; BD Biosciences) using gates to exclude dead cells, debris, and platelets. Percentage of stained cells was finally determined after comparing them with matched isotype controls (26, 27).

Assay of BM DA by HPLC with electrochemical detection. The animals were sacrificed, their long bones were dissected, and the BM was flushed out with 5 ml of ice-cold 0.6 M perchloric acid containing 1.7 mg/ml ethylene glycol tetra acetic acid and 1.1 mg/ml reduced glutathione. Recovered tissue was weighed, samples were centrifuged for 15 minutes at 2500 g at 0°C, and 1.0 ml of the supernatant was adjusted to pH 8.6 with 6 M potassium hydroxide and processed for determination of DA concentration after alumina batch extraction. Thereafter, 25 µl of 3 M potassium chloride was added to 200 µl of the 0.2 M perchloric acid eluate. Finally, after centrifugation, 50 µl of the supernatant was injected into the HPLC system and assayed (41).



Determination of clonogenic potential of EPCs. PBMCs (0.5×10^6 nucleated cells) were seeded onto fibronectin-coated dishes. The cells were cultured in EGM-2 medium (Clonetics) and supplemented with bFGF and VEGFA (5 and 2 ng/ml, respectively) on every other day. Colonies formed after 2 weeks of culture were considered as late outgrowth colony forming units and were costained with Dil-Ac-LDL (Sigma-Aldrich) (24).

BM transplantation model. BM cells were obtained by flushing the femurs of donor Tie2 transgenic mice (FVB/N-TgN [Tie2 lacZ] 182 Sato; The Jackson Laboratory). Low-density BM mononuclear cells were isolated by density centrifugation. Syngenic FVB/N mice (The Jackson Laboratory) were lethally irradiated with 9.0 Gy (780C TeleCobalt; Theratronix) using Cobalt-60 source and received i.v. infusion via tail vein of 2×10^6 donor BM mononuclear cells. Four weeks after BMT, mice were transplanted with S180 tumor. In the BMT model, where S180 tumor was transplanted, DA treatment was started from day 3 after tumor transplant and was continued for 5 days. Tumors were dissected out, and tumor sections were incubated with X-gal to determine bone lacZ⁺ cells (27).

Effect of DA on tumor angiogenesis. Angiogenesis in the tumor tissues was determined by immunohistochemical staining of CD31, a specific marker of endothelial cells (36, 37). Tumor tissue samples collected from the experimental animals were fixed in 4% paraformaldehyde, rinsed in PBS, transferred to 30% sucrose in PBS at 4°C, and frozen in OCT compound. Immunohistochemistry was performed on frozen tissue sections using rat anti-mouse monoclonal antibody against CD31 using the Vectastain Elite ABC kit following the manufacturer's protocol (Vector). Sections were counterstained with hematoxylin. Microvessel density was quantitated by analyzing 10 random fields per section (44).

In vitro migration assay of CD45-VEGFR2⁺ BM cells. Since EPCs in the BM are few in number, we undertook these experiments utilizing CD45-VEGFR2⁺ BM-derived cells (which include EPCs) as previously reported (10). CD45-VEGFR2⁺ BM cell migration assays were performed using a 48-well microchemotaxis chamber. rhVEGFA was diluted to 10 ng/ml concentrations in EBM-2 medium supplemented with 0.5% BSA, and 25 µl of the final dilution was placed in the lower chamber of a modified Boyden chamber. CD45-VEGFR2⁺ BM-derived cells were harvested by magnetic bead separation (Miltenyi Biotec), and 2×10^5 cells suspended into 50 µl of EBM-2 medium supplemented with 0.5% BSA were seeded into the upper compartment. After incubation at 37°C at 5% CO₂ for 5 hours, the filter was removed and the upper side of the filter containing the nonmigrating cells was scrapped. The filters were thereafter fixed with methanol and stained with Giemsa stain. Cells migrating into the lower chamber were counted in high power field ($\times 100$) in triplicate for each group (15).

Immunohistochemistry of MMP-9. Deparaffinization and rehydration of BM sections were followed by treatment of the BM sections with 0.3% H₂O₂. For MMP-9 staining, sections were incubated overnight with MMP-9 mAbs (R&D Systems) and then further incubated with

secondary antibodies using ABC staining kits (Vector) following the manufacturer's protocol (10).

MMP-9 synthesis and activity assay. CD45-VEGFR2⁺ BM cells were separated by MACS microbeads (Miltenyi Biotec) from different experimental groups and were cultured overnight in serum-free X-VIVO20 medium (BioWhittaker). MMP-9 concentration was assayed in the culture supernatant (1×10^6 cells) by sandwich ELISA (R&D Systems), and activity was determined by gelatin zymography of the overnight culture soup (5×10^3 cells) (10).

Western blot analysis. CD45-VEGFR2⁺ BM cells were lysed, and the lysates were immunoblotted with anti-MAPK, anti-phospho-ERK1/ERK2 (Santa Cruz Biotechnology Inc.), and anti-TIMP-1 (R&D Systems) (15, 40, 41).

PCR analysis of D₂DA receptor. From the isolated CD45-VEGFR2⁺ BM cells, the total RNA was extracted following the manufacturer's protocol (Ambion). Two micrograms of total RNA was reverse transcribed into first-strand cDNA by using poly-dT priming following amplification using a PCR kit (Ambion). The sequences of PCR primers were as follows (16): DA D₂ receptor, 5'-GCAGCCGAGCTTTCAGGGCC-3' and 5'-GGGATGTTGCAGTCA-CAGTG-3'; 15S rRNA (internal control), 5'-TTCGCAAGTTCACCTACC-3' and 5'-CGGGCCGGCCATGCTTTACG-3'.

Statistics. All data are expressed as the mean \pm SEM. Statistical comparisons were performed using Student's *t* test. *P* < 0.05 was considered significant (15, 16).

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