17β-Estradiol Mobilizes Bone Marrow–Derived Endothelial Progenitor Cells to Tumors


Abstract

Neovascularization is critical for tumor growth and development. The cellular mediators for this process are yet to be defined. We discovered that bone marrow–derived endothelial progenitor cells (BM-EPCs), having the phenotype (CD133+, CD34+, and VEGFR-2+), initiate neovascularization in response to TG1-1 mammary cells implanted in the inguinal mammary gland of Tie-2 GFP transgenic mice. The fluorescence tag allowed for tracing the migration of green fluorescent protein–tagged endothelial progenitor cells to tumor tissues. We discovered that 17β estradiol supplementation of ovarectomized mice significantly enhanced BM-EPC–induced neovascularization and secretion of angiogenic factors within the tumor microenvironment. Cell-based system analyses showed that estrogen-stimulated BM-EPCs secretes paracrine factors which enhanced TG1-1 cell proliferation and migration. Furthermore, TG1-1 cell medium supplemented with estrogen-induced BM-EPC mediated tubulogenesis, which was an experimental in vivo representation of the neovascular. Our data provide evidence of BM-EPC mammary tumor cell interactions and identify a novel cellular mediator of tumor progression that can be exploited clinically. [Cancer Res 2008;68(15):6038–42]

Introduction

Neovascularization is a critical process needed for tissue repair (1) and is indispensable for tumor growth and metastases (2, 3). This process requires assistance from bone marrow–derived endothelial progenitor cells (BM-EPCs), phenotypically characterized as CD133+, CD34+, and VEGFR-2+ (4–6). Mobilization and homing of EPCs from the bone is influenced by molecules such as vascular endothelial growth factor and proteases such as matrix metalloproteinase-9 (7, 8). Interestingly, estrogen (E2) has been implicated in the proliferation and mobilization of EPCs by stimulating the expression of EPC nitric oxide synthetase (9). Despite the evidence that breast cancers initiate as E2-responsive (1) and is indispensable for tumor growth and metastases (2, 3), no studies have examined the possible involvement of E2 on EPC mobilization and their contribution to neovascularization. Clinically, circulating EPCs were increased in patients with stage III and IV breast cancers, suggesting that they may correlate with disease (10). In this study, we examined the contribution of EPCs in breast cancer–responsive neovascularization using the well characterized Tie2/green fluorescent protein (GFP) murine model, in which GFP expression is under the endothelial cell–specific receptor tyrosine kinase Tek, (formerly Tie2) promoter. To test our hypothesis of whether E2 mobilizes circulating EPCs that home to implanted breast carcinomas and participate in tumor neovascularization, we used ovarectomized Tg (TIE2GFP)287SATO/J female mice supplemented with E2. We also examined EPC and breast cancer cell interactions, as well as the effect of E2 in both in vivo–based and in vitro–based experimental systems.

Materials and Methods

Animal model and orthotopic implantation of tumor cells. Tg (TIE2GFP)287SATO/J mice (Jackson Laboratorries) were grouped as follows: nonovariectomized (Intact), ovarectomized (OVX), and ovarectomized + E2 supplemented (OVX + E2) by implantation of 90-day release estradiol pellets (1.7 mg/pellet; Innovative Research of America) 1 week prior to surgery. Tumor induction was done by implanting 2 × 106 TG1-1 cells in the cleared mammary fat pad.

EPC mobilization experiments. Mononuclear cells, isolated by using a Histopaque-1083 (Sigma) density gradient, were stained with FITC anti-CD133 antibody (PharMingen) with isotype-matched antibodies (negative control). Immunofluorescence-labeled cells were fixed with 2% paraformaldehyde and analyzed using FACStar flow cytometer (Becton Dickinson) and Cell Quest Software counting 10,000 events per sample.

EPC characterization experiments. Mononuclear cells were cultured in EB2M (Clonetics; ref. 8). Four days after culture on four-wall glass slides coated with rat plasma vitronectin (Sigma) in 0.5% gelatin solution, EPCs were assayed by containing with acetylated LDL-DiI (Biomedical Technologies) and FITC conjugated BS lectin-1 (Vector Laboratories), both of which are markers characteristic of endothelial lineage (8).

TG1-1 tumor cell migration and proliferation. Primary cultures of EPCs from mice were cultured with or without estradiol (10–8 mol/L) 7 days in complete EB2M medium. Medium from EB2M cultures, with or without E2 and ERα inhibitor (ICI 182780, 1 μmol/L) or ERβ inhibitor (MJM II-17B, 1 μmol/L), was harvested and added to TG1-1 tumor cells for 48 h. TG1-1 proliferative activity was measured by tritiated thymidine ([3H]TdR) incorporation for 18 h and migration of TG1-1 cells was measured using a modified Boyden chamber (11).

In vitro EPC tubulogenesis. Primary cultures of EPCs were isolated from intact mouse bone marrow and plated on growth factor–reduced Matrigel-coated (300 μL; Becton Dickinson) dishes in EB2M medium. Cells (50,000 cells/slide) were plated on Matrigel chambers and tubulogenesis monitored 24 h after the addition or not of E2-supplemented TG1-1 cell–conditioned medium.

Results and Discussion

BM-EPCs and TG1-1 cells express ERα. Consistent with earlier observations in the literature, we observed the expression of ERα in
Figure 1. BM-EPCs and TG1-1 cells express ERα. OCT embedded tumor-bearing mammary glands were sectioned and immunofluorescently stained with PE-ERα and 4',6-diamidino-2-phenylindole (DAPI). Areas of neovascularization (green; GFP + EPC). The merged view shows that the tumor tissue, as well as the neovasculature, expresses ERα (A). In addition, Western blots of TG1-1 whole-cell lysates show the expression of ERα (B) and HER-2/neu (C) with MCF-7 as positive control.

Figure 2. Estrogen enhances BM-EPC mobilization and homing to tumors. Isolated mononuclear cells were used to quantitate circulating EPCs. OVX + E2 mice (●) displayed a significant increase in circulating double-positive EPCs (GFP+ and CD133+) when compared with OVX mice (■; A). Circulating EPCs were also authenticated by costaining using acetylated LDL-Dil and FITC-conjugated BS lectin-1 and appear in yellow after the merge (B). A qualitative increase in neovascularization by GFP+ EPCs in tumor-bearing mammary glands of OVX + E2 mice was observed compared with Intact and OVX mice (C). Six random tissue fields/section were counted by two blinded investigators and a significant increase in GFP+ capillaries was observed in the tumor-bearing glands (gray columns) between OVX + E2 and OVX (D); *, statistically significant differences.
tumor-bearing mammary gland sections (Fig. 1A), in neovasculature (merged fields, Fig. 1A), and in TG1-1 cells (Fig. 1B) using immunofluorescence and Western blotting. TG1-1 cells being derived from HER-2/neu background also express HER-2/neu (Fig. 1C).

**E2 induces the mobilization of BM-EPCs into the circulation and homing to tumor tissues.** Implanting 2 × 10^6 or 4 × 10^6 TG1-1 cells/mouse resulted in 100% tumor incidence for all groups of mice with the histology of the tumors consistent through groups and characteristic of TG1-1–induced tumors (Supplementary Fig. S1; Supplementary Table S1). Double-positive cells for GFP (hematopoietic stem cell marker) and CD133 (endothelial lineage) were counted as a representative population of circulating EPCs and E2 significantly increased the number of circulating EPCs in the blood, peaking at day 3 and returning to baseline by day 7 (Fig. 2A). The presence of authentic EPCs was validated by co-staining with markers of endothelial lineage acetylated LDL-DiI and FITC-conjugated BS lectin-1 (Fig. 2B). Vascular structures or capillaries were detected through GFP expression of EPCs. The presence of tumors increased the number of capillaries (Fig. 2C) and E2-mediated effect on capillary enhancement (Fig. 2D). Quantitation of the data revealed a 2-fold enhancement suggesting that E2 not only mediates EPC migration but also participates in capillary formation in response to implanted tumors.

**E2-induced homing of EPCs to tumor tissues is accompanied by enhanced expression of angiogenic and matrix metalloproteinase RNA transcripts.** Further evidence supporting the homing of EPCs to tumor sites, thus leading to neovascularization, was confirmed by immunofluorescence staining of tumors with anti-CD133 (Fig. 3A). The number of CD133-positive cells was significantly higher in OVX + E2 mice (156 ± 2.9) compared with OVX − E2 mice (65 ± 3.1). Also, colocalization of CD133-positive endothelial cells with GFP-positive endothelial cells (red arrows) was observed, suggesting that not only do EPCs home to the tumor-bearing gland but physically associate with new blood vessels, which was considerably enhanced in response to E2. Consistent with previous published literature (12–17), during cardiac injury, we observed an E2-induced enhanced expression of secretory angiogenic paracrine cytokines such as angiopoietin-1, angiopoietin-2, thrombospondin-1, vascular endothelial growth factor, matrix metalloproteases 2 and 9, and basic fibroblast growth factor. Expression of these cytokines indicates that E2 participates not only in neovascularization by recruiting EPCs but also by inducing biochemical components which promote a microenvironment conducive to tumor survival and growth (Fig. 3B).

**E2 enhances TG1-1 cells and EPCs to secrete paracrine cytokines which lead to cell tubulogenesis, migration, and proliferation.** We also present evidence that the mobilized EPCs participate in tubulogenesis, which is a hallmark activity of EPCs in vitro (18). EPCs cultured in vitro with TG1-1–conditioned medium supplemented with E2 displayed the formation of well-organized and defined tubes (Fig. 4A). Examination of the proliferative effect of secreted EPC factors on TG1-1 tumor cells indicated that EPC-conditioned medium supplemented with E2 significantly enhanced TG1-1 proliferation as measured by [3H]TdR incorporation (Fig. 4B). Furthermore, TG1-1 cell migration increased towards E2-conditioned EPC medium as opposed to non−E2−conditioned EPC medium (Fig. 4C). This E2-enhanced migration and proliferative activity was abrogated in the presence of both inhibitors of ERα (IC1) and ERβ (MJM II-17B; Fig. 4B and C), showing the ability of E2 to orchestrate a synergistic interplay.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Estrogen homes BM-EPCs to tumor tissues and enhances angiogenic and matrix metalloproteinase RNA transcripts. Immunofluorescent staining of mammary gland sections show an increased presence of EPCs in OVX + E2 tumor-bearing glands as opposed to OVX and intact tumor-bearing glands. Red arrows, colocalization of GFP+ and CD133+ endothelial EPCs. Overall, significant and consistent increases in the angiogenic and MMP RNA transcripts assayed were observed in OVX + E2 mice (white columns) compared with OVX mice (gray columns). Columns, fold expression over the levels observed in control mammary glands without tumor; *, statistically significant differences.
between breast cancer and endothelial cells to generate a tumor-supportive environment.

In summary, our data suggest that E2 acts as a mobilizing agent for EPCs, which is tumor-responsive. This results in enhanced circulating EPCs that home to the tumor and initiate the neovascularization of tumors as well as participate in secreting endothelial and tumor-enhancing factors. The effect of antiestrogens on the E2 responsiveness of EPCs suggests the possible use of selective antiestrogens to target EPC mobilization, and hence, neovascularization of primary and metastatic breast cancers. Furthermore, because E2-mediated signaling pathways cross-talk with other receptor signal transduction pathways such as HER-2/neu, therapies directed against HER-2/neu may also serve as a novel target (19). Analogous to the use of liposomes and exosomes as delivery vehicles of toxic compounds to tumor tissue (20), EPCs can be perceived as a “Trojan horse”, whereby their mobilization to the tumor environment can be exploited for the specific delivery of anticancer compounds for breast cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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