The Stem Cell Mobilizer StemEnhance® Does Not Promote Tumor Growth in an Orthotopic Model of Human Breast Cancer

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Abstract. *Bone marrow-derived stem cells (BMDSC) have been implicated in tumor formation, though it is not clear whether they contribute to tumor growth. A novel mobilizer of BMDSC (StemEnhance®; SE) was used to investigate whether its daily administration promotes tumor growth. Forty mice were surgically transplanted with human MDA-MB-435-GFP breast cancer into the mammary fat pad of nude mice. The mice were gavaged for six weeks with 300 mg/kg of SE. Tumor growth was monitored using live whole-body fluorescence imaging. At the end of the study, tumors were excised and weighed. At the start of the feeding trial, tumor areas for both control and experimental group were statistically identical. Tumor growth rate was slower in the SE group (p=0.014) when compared to the control group. After 6 weeks, tumor areas were 40% larger in the control p<0.01) and mean tumor weight was 35% smaller in the SE-treated group (0.44 g vs. 0.68 g; p=0.031). Feeding of SE did not promote tumor growth but rather reduced the growth of human MDA-MB-435 breast cancer.*

The role of stem cells in tumor formation is being intensely investigated. Recently, Correa and Houghton (1) reported that chronic inflammation associated with *Helicobacter pylori* infection could attract bone marrow-derived stem cells (BMDSCs), which could be involved in the process of tumor formation. The link between chronic inflammation and cancer has long been recognized since Virchow speculated, in the 1860s, that cancer arises at the site of chronic inflammation (2, 3).

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Tumors appear to be able to attract peripheral blood stem cells (4). BMDSCs may enhance the development of existing tumors by forming blood vessels (5-7). Indeed, the growth rate of a tumor is intimately linked to the development of blood supply, whose growth could be supported by circulating BMDSCs. It was reported that incorporation of endothelial precursor cells (EPC) into tumor blood vasculature coincided with tumor growth (7, 8). If circulating stem cells were to contribute to tumor vasculature and tumor growth, then increasing the number of circulating stem cells should accelerate tumor growth.

A novel mobilizer of BMDSCs (StemEnhance®) was recently reported to increase the number of circulating CD34+ cells by 25% (9). We investigated the effect of daily consumption of StemEnhance® on the growth of implanted MDA-MB-435 human breast tumor in an orthotopic-implantation mouse model.

Materials and Methods

Animals. Forty female NCr *nu/nu* mice, 4 weeks old, were used in this study. Mice were kept in a barrier facility under HEPA filtration and were fed with autoclaved laboratory rodent diet (Tecklad LM-485; Western Research Products, Orange, CA, USA). All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

Expression vectors. The pLEIN and $pLNCX_2$ vectors were purchased from CLONTECH (Palo Alto, CA, USA). The pLEIN vector expresses enhanced green fluorescent protein (GFP) and the neomycin-resistance gene on the same bicistronic message that contains an internal ribosome expression site. The $pLNCX_2$ vector contains the neomycin-resistance gene for antibiotic selection in eukaryotic cells. The red fluorescent protein (RFP; DsRed2; CLONTECH) was inserted in the $pLNCX_2$ vector at the Egl II and *Not*I sites.

GFP vector production. For retroviral transduction, PT67, an NIH 3T3-derived packaging cell line expressing the 10 Al viral envelope, was purchased from CLONTECH. PT67 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10%

(v/v) heat-inactivated fetal bovine serum (FBS) (Gemini Biological Products, Calabasas, CA, USA). For vector production, packaging cells (PT67) at 70% confluence were incubated with a precipitated mixture of DOTAP transfection reagent (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; Roche Molecular Biochemicals, Mannheim, Germany) and saturating amounts of pLEIN for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h after transfection. For selection, the cells were cultured in the presence of 500–2,000 μg/ml of G418 increased in a step-wise manner (Life Technologies, Grand Island, NY, USA) for 7 days.

GFP gene transduction of human and animal tumor cell lines. For GFP gene transduction, 20-40% confluent human and animal tumor cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells (Clontech, Palo Alto, CA USA) and RPMI medium 1640 (Mediatech, Inc., Herndon, VA, USA) or other culture media containing 10% (v/v) FBS (Gemini Biological Products) for 72 h. Human breast MDA-MB-435-GFP cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI medium 1640 containing 10% (v/v) FBS (Gemini Biological Products) for 72 h. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 into selective medium that contained 50 μg/ml of G418. The level of G418 was increased to 800 μg/ml in a step-wise manner. Clones expressing GFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.

s.c. Tumor stock. To have growing tumor tissue stock for subsequent orthotopic implantation, six-week-old *nu/nu* female mice were injected *s.c.* with a single dose of 10^6-10^7 MDA-MB-435-GFP cells. Cells were first harvested by trypsinization, washed three times with cold serum-containing medium and then kept on ice. Cells were injected in the *s.c.* space of the flank of the animal in a total volume of 0.2-0.4 ml within 40 min of harvesting. The nude mice were killed to harvest tumor tissue 3 to 6 weeks after tumor cell injection for subsequent surgical orthotopic implantation (SOI) of tumor fragments (see below).

Surgical orthotopic implantation. Mice were anesthetized with isoflurane inhalation and put in a supine position. The right second mammary gland was chosen for orthotopic implantation. A small incision was made along the medial side of the nipple. The mammary fat pad was exposed through blunt dissection. A small cut was made on the fat pad and then bluntly expanded to form a small pocket. Four pieces of breast cancer tissue, previously prepared as described above, were sutured into the pocket using an 8-0 nylon suture. The skin was closed with a 6-0 silk suture. All procedures were carried out under a ×5 dissecting microscope (10).

StemEnhance® and treatment. StemEnhance® (SE), supplied by STEMTech HealthSciences, Inc. (San Clemente, CA, USA), was stored at room temperature and protected from light and humidity. The treatment was started on day-21 after SOI when the volume of primary tumors reached approximately 50-100 mm^3. Each cage was clearly marked for its group with one mouse per cage. Mice were assigned to treatments using a table of random numbers. For a duration of six weeks, experimental animals were gavaged qd with 300 mg/kg of SE dissolved in phosphate-buffered saline (PBS) while control animals were gavaged with PBS alone.

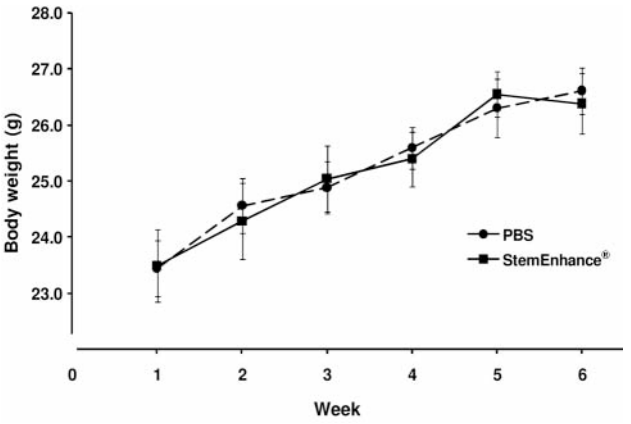

Figure 1. *Mouse body weight change during the course of the study. Body weight measured on a weekly basis did not differ between control (PBS; dotted line) and experimental (StemEnhance®; solid line) groups. Vertical bars indicate standard error of the mean.*

Data collection. The FluorVivo image system (Indec Systems, Santa Clara, CA, USA) was used for whole-body and open imaging. Whole-body optical imaging of GFP-expressing tumors was performed once a week after GFP-visible tumors were established. Weekly imaging data were analyzed to monitor tumor growth using PhotoShop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA), by quantifying the number of pixels the tumor mass covered (11, 12).

After six weeks (day-64 after SOI), animals in each group were sacrificed. At necropsy, open imaging was acquired for each animal. Imaging was conducted in the thoracic cavity and abdomen for metastasis to the lymph nodes and lungs.

Body weights of all the animals were measured weekly using an electronic scale. Primary tumors were weighed at the end of the study.

Statistical analysis used in the study. Tumor areas were adjusted for differences in the initial size of the implanted tumor by subtracting the pixel counts for an animal at week 0 from the pixel counts at all successive weeks. Other transformations of the data were also analyzed, including change in tumor growth weight between weeks and the percentage change in weekly growth of body weight, tumor area and tumor volume. All of these were analyzed using repeated measures analysis of variance, after transformation (logarithms, square-roots, or reciprocals) to achieve multivariate normality (Mardia skewness and kurtosis (13); Henze-Zirkler (14) test for multivariate normality). Tumor areas at week 0 were analyzed using a two-tailed *t*-test. The ratios of tumor areas at week 0 to week 6 were analyzed using a two-tailed *t*-test, after logarithmic transformation. Unless indicated otherwise, data are reported as mean±standard error.

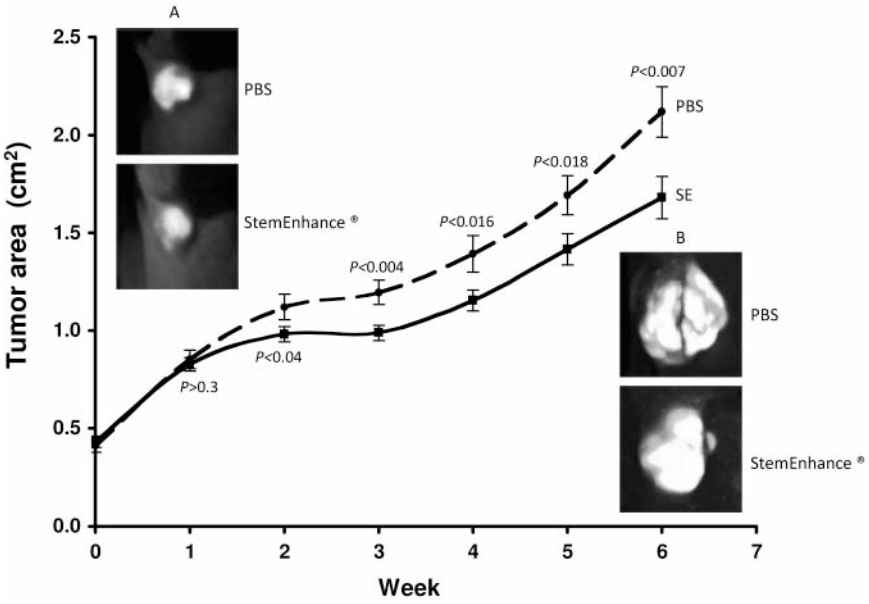

Figure 2. *Tumor area of MDA-MB-435-GFP, as measured using image analysis, in groups fed PBS (dotted line) and StemEnhance® (solid line). A and B are representative of the average size on the tumors found in the PBS (top) and StemEnhance® (bottom) groups at week 0 and week 6, respectively. Vertical bars indicate standard error of the mean.*

Results

Toxicity. There was no evidence of toxicity due to SE. No animals in either group died. Furthermore, as shown in Figure 1, animals in both groups showed identical body weight growth patterns (p=0.9). At each weekly time point, body weights and change in body weights for the two groups did not show any statistical difference. No visual or behavioral differences could be seen between the two groups.

Tumor growth. The effect of StemEnhance® on human breast cancer MDA-MB-435-GFP was evaluated by monitoring primary tumor growth. Feeding of StemEnhance® to the experimental group began when the average tumor volume reached approximately 50-100 mm^3. At the start of the feeding trial, tumor areas for both control (PBS; 0.41±0.03 cm^2) and experimental groups (SE; 0.43±0.03 cm^2) were statistically identical (p=0.63) (Figure 2).

For each animal, tumor growth in weeks 1 to 6 was determined by subtracting the area at week 0. Tumor growth data were analyzed using repeated measures analysis of variance. Tumor growth was approximately linear, as determined by orthogonal polynomial regression. Tumor growth rate was slower in the StemEnhance® group (p=0.014) when compared to the PBS group (Figure 2). The reduction in tumor growth was significant by week 2. At week 6, adjusted tumor areas were 40% larger in the control group (1.70±0.10 cm^2) than in the StemEnhance® group (1.25±0.08 cm^2) (p<0.01). Correspondingly, ratios of week-0/week-6 tumor sizes were much smaller in the control group (p<0.01).

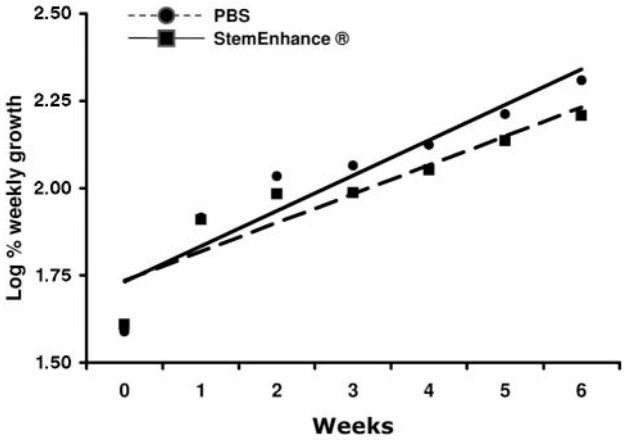

Figure 3. *Analysis of the logarithms of the percentage weekly tumor growth revealed less overall tumor growth in the StemEnhance® group (p=0.043), though growth curves were parallel. There was no week at which the growth difference between groups was significant. Therefore SE reduced the rate of tumor growth but did not alter the growth pattern.*

The weekly changes in unadjusted tumor areas, starting at week 0, were analyzed using the logarithms of the values to achieve univariate and multivariate normality. There were

significant reductions in mean log-tumor area by week 3 in the StemEnhance® group. At week 6, the mean tumor area was 2.12±0.13 cm^2 in the controls but only 1.68±0.11 cm^2 in the StemEnhance® group (p=0.01). Tumor growth rate over 6 weeks was slower (p<0.017) in the StemEnhance® group.

Analysis of the logarithms of the percentage weekly tumor growth revealed less overall tumor growth in the StemEnhance® group (p=0.043) but parallel growth curves (Figure 3). Because there was no week at which the growth difference between groups was significant, we conclude that StemEnhance® systematically reduced the rate of tumor growth but did not alter the actual growth pattern. For both groups, the rate of change (slope) decreased during weeks 2 and 3 and then leveled off. Metastasis was not seen in either group.

Tumor weight. At the end of the study, tumors were carefully excised and weighed. Tissue from one animal in the StemEnhance® group was damaged and was therefore eliminated from the analysis. Mean tumor weight in the StemEnhance®-treated group (0.44±0.05 g) was 35% lower than in the controls (0.68±0.09 g) (p=0.031). These results for tumor mass are consistent with the analyses of tumor area.

Tumors in the weekly images were nearly circular and excised tumors were oblate spheroid in shape. Using the radius of each tumor, we were able to mathematically estimate the volume using $V=4/3\ \pi a^2 b$, where a and b are the longitudinal and thickness radii, respectively. Tumor densities (W/V) were approximately constant, and were similar for the controls (1.07 g/ml) and SE-treated (1.08 g/ml) animals ($p_{t\text{-}test}$=0.92).

Discussion

Animals received 300 mg/kg qd x 6 weeks of StemEnhance® (SE), which is roughly 10 times the dose recommended for humans. At that level, growth was normal and animals showed no signs of toxicity. SE is a 5:1 concentrate of the cyanophyta *Apahnizomenon flos-aquae* (AFA). Our observations are consistent with Schaeffer *et al*. (15) who reported that consumption of 16,666 mg/kg of AFA (equivalent to >3,000 mg/kg SE) led to no sign of toxicity in mice.

Daily consumption of the stem cell mobilizer SE reduced the rate of tumor growth without affecting the growth pattern, notwithstanding the different tumor volumes and weights. No metastases were seen, at least under the conditions of this study.

Our data are consistent with those of Karnoub *et al*. (16) who reported that injection of MDA-MB-435 human breast cancer cells along with mesenchymal stem cells (MSCs) reduced tumor growth when compared to injection of cancer cells alone, though such reduction did not reach significance. However, Karnoub *et al*. (16) reported that simultaneous injection of breast cancer cells along with MSCs could increase metastasis to the lung for some tumor strains. In our study, increasing the number of circulating stem cells did not lead to metastasis. It is possible that the effect of MSCs on the metastatic process, as reported by Karnoub *et al*. (16), occurs upon co-injection of breast cancer cells and MSCs in close proximity.

Santarelli *et al*. (7) reported the incorporation of circulating endothelial progenitor cells (EPCs) into the blood vasculature of growing tumor in the mouse brain. In mice transplanted with GFP-expressing bone marrow cells, the authors reported that the 92-fold expansion of tumor size over a period of 9 days was accompanied by an expansion of vascular density and diameter of blood vessels, as well as an increasing number of GFP+ cells within the tumor. However, only a very small number of GFP+ cells were positive for the endothelial marker Flk-1, and such GFP+/Flk-1+ cells were found isolated, showing that they had not proliferated and had not contributed to the actual development of the tumor vasculature, but instead seemed to have been passively incorporated in the tumor vasculature tree.

Little data exist to suggest how circulating stem cells could contribute to reducing tumor growth. It is possible that after migrating in cancerous tissue, attracted by cytokines, and after proliferating and differentiating in cells of the target tissue, stem cells could secrete signals inhibiting cellular division.

It is important to mention that the antitumor properties of SE observed in this study could have also been in part due to the presence of specific compounds naturally found in *AFA* and concentrated in SE. For example, SE concentrates from *AFA* contains the photosynthetic blue pigment phycocyanin, a water soluble phycobiliprotein that was shown to have significant antioxidant (17) and anti-inflammatory (18, 19) properties, and even possible anticancer properties (20, 21). SE also contains a polysaccharide in AFA that was shown to stimulate NK cell activity (9) and support NK cell migration (22). The presence of these two compounds could have contributed to the overall antitumor effect observed with SE.

In conclusion, this study was conducted to investigate whether SE would enhance the growth of an existing tumor by contributing to the development of the tumor vasculature or the tumor mass itself. Instead of promoting tumor growth, SE actually inhibited tumor growth.

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