Flaxseed and Its Lignans Inhibit Estradiol-Induced Growth, Angiogenesis, and Secretion of Vascular Endothelial Growth Factor in Human Breast Cancer Xenografts *In vivo*

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Abstract **Purpose:** Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis, which is crucial in cancer progression. We have previously shown that estradiol (E2) increases VEGF in breast cancer. Phytoestrogens are potential compounds in breast cancer prevention and treatment by poorly understood mechanisms. The main phytoestrogens in Western diet are lignans, and flaxseed is a rich source of the mammalian lignans enterodiol and enterolactone.

Experimental Design: In the present study, ovariectomized mice were treated with continuous release of E2. MCF-7 tumors were established and mice were fed with basal diet or 10% flaxseed, and two groups that were fed basal diet received daily injections with enterodiol or enterolactone (15 mg/kg body weight).

Results: We show that flaxseed, enterodiol, and enterolactone counteracted E2-induced growth and angiogenesis in solid tumors. Extracellular VEGF *in vivo*, sampled using microdialysis, in all intervention groups was significantly decreased compared with tumors in the basal diet group. Our *in vivo* findings were confirmed *in vitro*. By adding enterodiol or enterolactone, E2-induced VEGF secretion in MCF-7 cells decreased significantly without agonistic effects. The increased VEGF secretion by E2 in MCF-7 cells increased the expression of VEGF receptor-2 in umbilical vein endothelial cells, suggesting a proangiogenic effect by E2 by two different mechanisms, both of which were inhibited by the addition of lignans.

Conclusions: Our results suggest that flaxseed and its lignans have potent antiestrogenic effects on estrogen receptor–positive breast cancer and may prove to be beneficial in breast cancer prevention strategies in the future.

The rate of breast cancer differs strikingly between various populations of the world, with highest incidence in countries with a Western lifestyle (1, 2). Studies of immigrant populations indicate that this difference is largely attributable to lifestyle factors, such as a diet containing large amounts of phytoestrogens rather than genetics (3, 4). The main class of phytoestrogens in the Western diet is the lignans, which are present in most plant food, with flaxseed being one of the richest sources (5). Flaxseed contains the main precursor of mammalian lignans, secoisolariciresinol diglycoside, which is converted by the colonic bacteria to the two major mammalian lignans enterodiol and enterolactone (6, 7). Dietary phytoestrogens have been studied as potential compounds in breast cancer prevention and treatment. However, phytoestrogens may elicit both weak estrogenic and antiestrogenic activities (8, 9). Cumulative exposure to estrogens is a risk factor of breast cancer, and the mechanisms by which phytoestrogens may protect against the disease are today poorly understood.

Angiogenesis, the process of forming new blood vessels from the existing vascular network, is a crucial step in cancer progression. One of the most potent stimulators of angiogenesis is vascular endothelial growth factor (VEGF; ref. 10). In breast cancer, high tumor levels of VEGF have been associated with poor prognosis and decreased survival (11, 12). The VEGF protein exists in several isoforms and is bioactive as soluble proteins in the extracellular space (13). Among the regulators of VEGF expression is hypoxia via hypoxia-inducible factor-1 but sex steroids have also been shown to regulate VEGF expression and an estrogen-responsive element in the gene for VEGF has been found (14, 15). We have previously shown that estradiol (E2) increases extracellular VEGF in breast cancer and normal human breast tissue *in vivo* (16–18). Moreover, we have validated microdialysis as an *in vivo* sampling technique for extracellular VEGF in breast tissue (17, 18).

In previous studies, we have found that diet supplementation with flaxseed or treatment with the phytoestrogen resveratrol to nude mice with established estrogen receptor (ER)-negative (ER$^-$) human breast cancer xenografts decreased tumor growth and extracellular VEGF (19–21). However, the

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majority of breast cancer expresses the ER and responds to antiestrogen treatment but the effects of phytoestrogens on ER^+ in breast cancer in the presence of E2 are very little explored.

In the present study, we found, using cell culture of ER^+ MCF-7 cells, human umbilical vascular endothelial cells (HUVEC), and established MCF-7 solid tumors in nude mice, that flaxseed, enterodiol, and enterolactone have the capacity to counteract E2-induced tumor growth, angiogenesis, and VEGF secretion both *in vitro* and *in vivo*.

Materials and Methods

Cells and culture conditions. The ER^+ MCF-7 cells (American Type Culture Collection, Manassas, VA) were used in all of the experiments. Cells were cultured in DMEM without phenol red supplemented with 2 mmol/L glutamine, 50 IU/mL penicillin G, 50 μg/mL streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Cell culture medium and additives were obtained from Life Technologies, Inc. (Paisley, United Kingdom) if not otherwise stated. Before experiments, cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into six-well plates (Costar, Cambridge, MA) at a density of $10{,}000/cm^2$. Cells were incubated for 1 day and then treated with or without 10^{-9} mol/L E2 (17β-estradiol; Apoteket, Umeå, Sweden), 10^{-6} mol/L enterolactone, 10^{-6} mol/L enterodiol (Sigma, St. Louis, MO), or combinations of E2 and enterodiol or enterolactone. Hormones were added in serum-free DMEM/F12 (1:1) without phenol red supplemented with 10 μg/mL transferrin (Sigma), 1 μg/mL insulin (Sigma), and 0.2 mg/mL bovine serum albumin (Sigma). The medium was changed every day, and the cells and medium were harvested after 7 days of hormone treatment.

Human umbilical vascular endothelial cells. Umbilical cords were donated anonymously after informed consent according to national ethical legislation. HUVECs were isolated from female donors by collagenase digestion at 37°C for 20 min as described previously (22). Cells were grown in medium consisting of DMEM without phenol red supplemented with nonessential amino acids, 1.6 mmol/L glutamine, 4 IU/mL penicillin G, 4 μg/mL streptomycin, 4 μg/mL insulin, 0.01 mol/L HEPES, 0.02 mg/mL endothelial cell growth factor (Roche Diagnostics, Bromma, Sweden), 16 IE/mL heparin (Apoteket), and 16% charcoal-filtered fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells used for experiments were from passages 2 to 3. Cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into six-well plates at a density of $20{,}000/cm^2$. Cells were treated with or without 10^{-9} mol/L E2, 10^{-6} mol/L enterolactone, 10^{-6} mol/L enterodiol, combinations of estrogen and enterolactone or enterodiol, MCF-7 supernatants, recombinant VEGF protein, recombinant VEGF protein (R&D Systems, Abingdon, United Kingdom), and VEGF antibodies (R&D Systems). Three days was chosen for the duration of treatment due to shorter survival time of HUVEC without the medium supplements described above. Hormones were added to the cultures in serum-free DMEM/F12 (1:1) without phenol red supplemented with 10 μg/mL transferrin, 1 μg/mL insulin, and 0.2 mg/mL bovine serum albumin.

Quantification of VEGF, soluble VEGF receptor-1, and VEGF receptor-2. VEGF, soluble VEGF receptor-1 (sVEGFR-1), and VEGFR-2 were analyzed using commercial quantitative immunoassay kits [QuantiGlo (human VEGF) and Quantikine (human sVEGFR-1 and VEGFR-2), R&D Systems] without preparation. According to the manufacturer, the VEGF kit detects VEGF165 and VEGF121 isoforms and the minimum detection limit is <1.76 pg/mL and intra-assay and interassay precision is 3% to 8%. The minimum detectable limit for sVEGFR-1 is 5.01 pg/mL and 4.6 pg/mL for the VEGFR-2 kit. The intra-assay and interassay precision for these kits is 3% to 8% and 3% to 7%, respectively, which was confirmed during our analyses. Protein content was determined using Bio-Rad DC Protein Assay (Bio-Rad, Sundbyberg, Sweden), and VEGF, sVEGFR-1, and VEGFR-2 concentrations were adjusted to total protein content.

Western blot analysis. Cell fractions of 12 μg protein were subjected to 15% SDS-PAGE under nonreducing conditions and transferred to a nitrocellulose membrane. Blots were incubated with an antihuman VEGF antibody (1:500; R&D Systems) followed by a horseradish peroxidase–conjugated antibody (1:1,000; DakoCytomation, Glostrup, Denmark). Bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Uppsala, Sweden). Recombinant human VEGF165 and VEGF121 were used as controls (R&D Systems).

Diets. Two different diets were prepared: basal diet, which is based on AIN93G (23) modified to contain 20% corn oil, or basal diet supplemented with 10% ground flaxseed (Linott variety; Omega Products, Melfort, Saskatchewan, Canada). The flaxseed diet was corrected for the contribution of flaxseed to fat, carbohydrate, and protein components so that the two diets were isocaloric as described previously (19, 20). Both diets were prepared by Dyets, Inc. (Bethlehem, PA) and sterilized by γ-irradiation by Isomedix Corp. (Whitby, Ontario, Canada).

Animals and ovariectomy of mice. Female athymic mice, BALB/c *nu/nu* (ages 6-8 weeks), from Taconic (Ry, Denmark) were housed in a pathogen-free isolation facility with a 12-h light/12-h dark cycle and fed chow and water *ad libitum*. The study was approved by Linköping University animal ethics research board. Mice were anesthetized with i.p. injections of ketamine/xylazine and ovariectomized; 3-mm pellets containing E2, 0.36 mg/90-day release (Innovative Research of America, Sarasota, FL), were implanted s.c. The pellets provide continuous release of E2 at serum concentrations of ~250 pmol/L, which is in the range of physiologic levels seen in mice during the estrous cycle. Twelve days after surgery, MCF-7 cells (5×10^6 in 200 μL PBS) were injected s.c. on both flanks. Tumor surface area was determined by measuring length and width and calculated by the following formula: $L / 2 \times W / 2 \times 3.14$. At a tumor area of 30 mm^3, the mice were divided into five subgroups such that the mean body weight and tumor size in each group were the same. One group was fed with the 10% flaxseed diet, and the other groups continued with basal diet and were given daily s.c. injections with either enterodiol (15 mg/kg body weight), enterolactone (15 mg/kg body weight), or vehicle. Enterodiol and enterolactone were dissolved in DMSO (5 mg of enterodiol or enterolactone dissolved in 50 μL DMSO suspended in mineral oil). Palpable tumor size was monitored weekly as before. At necroscopy, the body and tumor weights were recorded. Plasma was collected in heparin by cardiac puncture.

Microdialysis equipment and experiment. Tumor-bearing mice were anesthetized i.p. with ketamine/xylazine and kept anesthetized by repeated s.c. injections. A heating lamp maintained the body temperature. A small skin incision was made, and microdialysis probes (CMA/20, 0.5 mm in diameter; PES membrane, 10 mm in length, 100,000 molecular weight cutoff; CMA/Microdialysis, Stockholm, Sweden) were inserted into tumor tissue, connected to a CMA/102 microdialysis pump (CMA/Microdialysis), and perfused at 0.6 μL/min with saline containing 154 mmol/L NaCl and 40 mg/mL dextran 70 (Pharmalink, Stockholm, Sweden). After a 30-min equilibration period, the outgoing perfusate was collected on ice and stored at −70°C for subsequent analysis. We have previously validated 100,000 molecular weight cutoff membrane for VEGF measurement in murine tumors (18) and human breast tissue (16, 17).

Immunohistochemistry of tumor sections. Formalin-fixed, paraffin-embedded tumors were cut in 3-μm sections, deparaffinized, and subjected to antihuman VEGF immunohistochemistry (goat anti-human VEGF; 1:40 dilution; Calbiochem, Darmstadt, Germany) or anti–von Willebrand factor (rabbit anti-human von Willebrand; 1:1,000 dilution; DakoCytomation). Ki-67 was stained by using a MIB-1 antibody (mouse anti-human; 1:100 dilution; DakoCytomation). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high-power fields (×200 magnification) were examined in sections from three different tumors in each group. For VEGF scoring, the whole material was scanned to identify the range of intensity of the staining. Thereafter, the staining on the tumor sections was scored either as weakly or strongly positive. Vessel quantification of tumor sections was conducted as described previously (24) using a Nikon (Solna, Sweden) microscope equipped with a digital camera. Percentage of area stained positively for von Willebrand factor was assessed using Easy Image Measurement software (Bergstrom Instruments, Solna, Sweden). Ki-67 labeling index was calculated as percentage of positive cells over total cells counted. Three measurements were obtained from the border zone area at ×400 magnification of each section from five different tumors by a single investigator blinded to the treatment.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining. Detection of DNA fragmentation was done according to the manufacturer's instructions using an *in situ* cell death detection kit (Roche, Mannheim, Germany). Briefly, the sections were permeabilized with 0.01 mol/L citrate buffer (pH 6) and then incubated with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling reaction mixture, including enzyme solution and labeling solution (fluorescein-labeled nucleotides), and thereafter incubated with alkaline phosphatase–conjugated anti-fluorescein antibody. Fast Red (Roche) was added followed by counterstaining with hematoxylin. The apoptotic index was calculated as percentage of positive cells over total cells counted. Three measurements were obtained from the border zone area at ×400 magnification of each section from five different tumors by a single investigator blinded to the treatment group.

Statistics. The values represent the mean ± SE. Statistical analyses were done using Student's *t* test and ANOVA with Fisher's post hoc test where appropriate.

Results

Flaxseed, enterodiol, and enterolactone decreased tumor growth of estrogen-supplemented mice. MCF-7 tumors require E2 for continued growth and estrogen withdrawal after tumor establishment induced immediate tumor regression (data not shown). Therefore, no untreated control group was included in the experiment. Instead, the various treatments were added with a stable background estrogen supplementation at physiologic levels. Hence, this experimental model mimics breast cancer in premenopausal women. In all treatment groups, there were a significant decreased tumor growth compared with the basal diet group (ANOVA with Fisher's post hoc test; Fig. 1). Final tumor weights at sacrifice were 368 ± 43 mg in the basal diet group, 392 ± 44 mg in the flaxseed group, 326 ± 74 mg in the enterodiol group, and 340 ± 47 in the enterolactone group. Due to large tumor burden, the basal diet mice had to be euthanized at an earlier time point than the treated mice. No significant difference in initial body weight, food intake, or body weight change was recorded between the treatment groups, and final body weights were 21.7 ± 0.8 g in the control group, 20.8 ± 0.5 g in the flaxseed group, 20.7 ± 1.3 g in the enterodiol group, and 21.3 ± 0.7 g in the enterolactone group (P = 0.81, ANOVA).

***Flaxseed, enterodiol, and enterolactone decreased extracellular tumor VEGF* in vivo.** We have previously shown that secreted VEGF *in vivo* is dependent on tumor size; larger tumors exhibited increased VEGF levels (20, 25). This may be dependent on hypoxia in large tumors because hypoxia-inducible factor-1 is a potent regulator of VEGF (14). Therefore, microdialysis was done on size-matched tumors from the different treatment groups. Tumor weights at microdialysis were 322 ± 36 mg in the basal diet group, 374 ± 34 mg in the flaxseed group, 322 ± 47 mg in the enterodiol group, and 374 ± 66 mg in the enterolactone group, with no statistical differences between the groups (P = 0.7, ANOVA). No necrotic areas in the tumors were revealed by H&E staining. There were no differences in Ki-67 or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling in these size-matched tumors. Ki-67 in the basal diet group was 37.5 ± 1.7%, 36.5 ± 1.8% in the flax group, 34.6 ± 3.3% in the enterodiol group, and 35.7 ± 1.8% in the enterolactone group (P = 0.8, ANOVA). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling in the basal diet group was 4.7 ± 0.5%, 5.1 ± 0.6% in the flax group, 4.3 ± 0.3% in the enterodiol group, and 4.7 ± 0.9% in the enterolactone group (P = 0.82, ANOVA). Microdialysis was done on basal diet tumors at days 28 and 42 after tumor cell injection, revealing no difference in the secreted VEGF over this time frame in tumors of similar size. In the intervention groups, microdialysis was done after 3 weeks and there was a significant decrease of extracellular VEGF in all treatment groups compared with the basal diet group (ANOVA with Fisher's post hoc test; Fig. 2). All treatment groups had ~50% lower levels of secreted VEGF compared with the basal diet group.

Flaxseed, enterodiol, and enterolactone decreased tumor angiogenesis. To determine whether the decreased secretion of VEGF *in situ* was accompanied with decreased angiogenesis, tumor vessel area was counted after immunohistochemical staining of tumor sections using anti–von Willebrand factor. In all treatment groups, there was a decreased vessel area compared with the basal diet group: 3 ± 0.7% of total area in the basal diet tumors, 1.1 ± 0.2% in the flaxseed tumors, 1 ± 0.4% in the enterodiol tumors, and 0.6 ± 0.1% in the enterolactone tumors ($P < 0.01$, between all intervention groups compared with basal diet group, ANOVA with Fisher's post hoc test; Fig. 3).

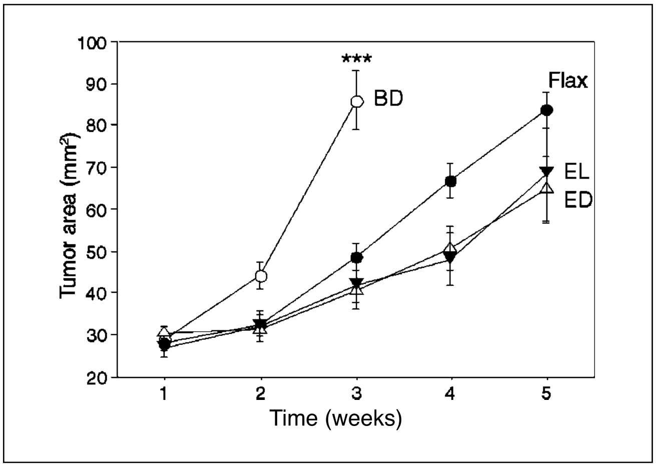

Fig. 1. Flaxseed (*Flax*), enterodiol (*ED*), and enterolactone (*EL*) decreased tumor growth. Mice were ovariectomized and supplemented with a physiologic level of E2 and fed basal diet (*BD*). MCF-7 cells were injected s.c. and tumors were formed on the flanks of the animal. At a tumor area of ~30 mm^2, one group of mice continued with basal diet, one group started with a diet of 10% flaxseed, one group started with daily s.c. injections of enterodiol (15 mg/kg body weight), and one group started with daily s.c. injections of enterolactone (15 mg/kg body weight). There was a significant decrease of tumor growth in all intervention groups compared with the basal diet group. ***, $P < 0.0.001$ (n = 7 in the basal diet group, n = 14 in the flaxseed group, n = 9 in the enterodiol group, and n = 8 in the enterolactone group).

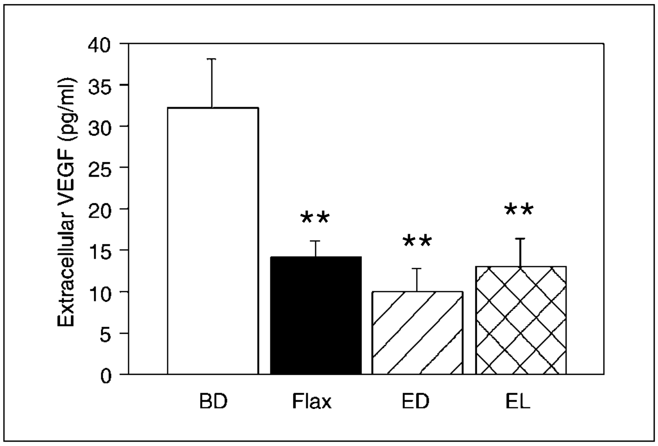

Fig. 2. Flaxseed, enterodiol, and enterolactone decreased extracellular tumor VEGF *in vivo*. Mice were treated as described in Fig. 1. After 3 wks of intervention, microdialysis was done on size-matched tumors as described in Materials and Methods. The perfusate was analyzed using ELISA. All intervention treatments caused a significant decrease of extracellular VEGF. **, $P < 0.01$, compared with the basal diet group ($n = 6$ in the basal diet group, $n = 6$ in the flaxseed group, and $n = 5$ in the enterodiol and enterolactone group).

***Enterodiol and enterolactone decreased E2-induced secretion of VEGF in MCF-7 cell culture* in vitro.** To further explore the regulation of VEGF secretion, cell culture experiments of MCF-7 cell were done. Enterodiol or enterolactone exposure for 7 days did not affect cell growth in culture; there were no differences in cell count or total protein levels of the cell pellet in the enterodiol-exposed and enterolactone-exposed cells compared with the control cells. E2 exposure increased cell count and total protein levels by ~25%. The total protein concentrations after 7 days in culture were 0.305 ± 0.01 mg in the control group, 0.388 ± 0.04 mg in the E2 group, 0.317 ± 0.2 mg in the enterodiol group, and 0.309 ± 0.03 mg in the enterolactone group ($P < 0.05$, between E2 and the control group, ANOVA with Fisher's post hoc test).

In the first experiment, VEGF secretion was determined after exposure of E2 and the lignans alone. We found that there was a 2-fold increase of VEGF in cell culture medium after E2 exposure compared with cell without added hormones in the medium ($P < 0.001$, ANOVA with Fisher's post hoc test; Fig. 4A). Enterodiol and enterolactone did not affect VEGF secretion compared with control cells (Fig. 4A). We then wanted to examine whether enterodiol and enterolactone had the capacity to inhibit E2-induced VEGF secretion, and indeed, we found that addition of enterodiol and enterolactone in combination with E2 decreased the secretion of VEGF significantly ($P < 0.01$ in the E2 + enterodiol and E2 + enterolactone groups, ANOVA with Fisher's post hoc test; Fig. 4B). The intracellular VEGF levels were significantly increased after E2 exposure (244 ± 57 pg/mg protein) compared with control cells (97 ± 13 pg/mg protein; $P < 0.01$), whereas enterodiol and enterolactone exposure did not affect the levels compared with control (75 ± 22 pg/mg protein and 53 ± 3 pg/mg protein, respectively, ANOVA with Fisher's post hoc test). Western blot revealed that the VEGF165 isoform is predominant in MCF-7 cells (Fig. 4C).

Up-regulation of VEGFR-2 in HUVEC after exposure of culture medium from MCF-7 cell. VEGF is known to exert its effects by acting on endothelial cells primarily via VEGFR-2. We therefore set up a series of experiments to investigate the effects of E2, enterodiol, and enterolactone on VEGFR-2 expression in HUVEC. In the first set of experiments, we exposed HUVEC with E2, enterodiol, and enterolactone directly and found no difference after direct exposure (Fig. 5A). However, in a tumor, secreted proteins from the tumor cells affect the surrounding stroma cell, including endothelial cells. We therefore treated HUVEC with cell culture medium from the hormone-treated MCF-7 cells. Using this approach, we found that the cell culture medium originating from E2-treated MCF-7 caused a significant increase of VEGFR-2 in HUVEC ($P < 0.01$, ANOVA with Fisher's post hoc test; Fig. 5B), whereas MCF-7 cell culture medium from enterodiol and enterolactone had no effect on VEGFR-2 in HUVEC (Fig. 5B). To explore if this increase was dependent on the increased VEGF levels in E2-treated MCF-7 cell, we first treated HUVEC with recombinant VEGF (7,000 pg/mL) alone or with a VEGF antibody (70,000 pg/mL). The levels of VEGFR-2

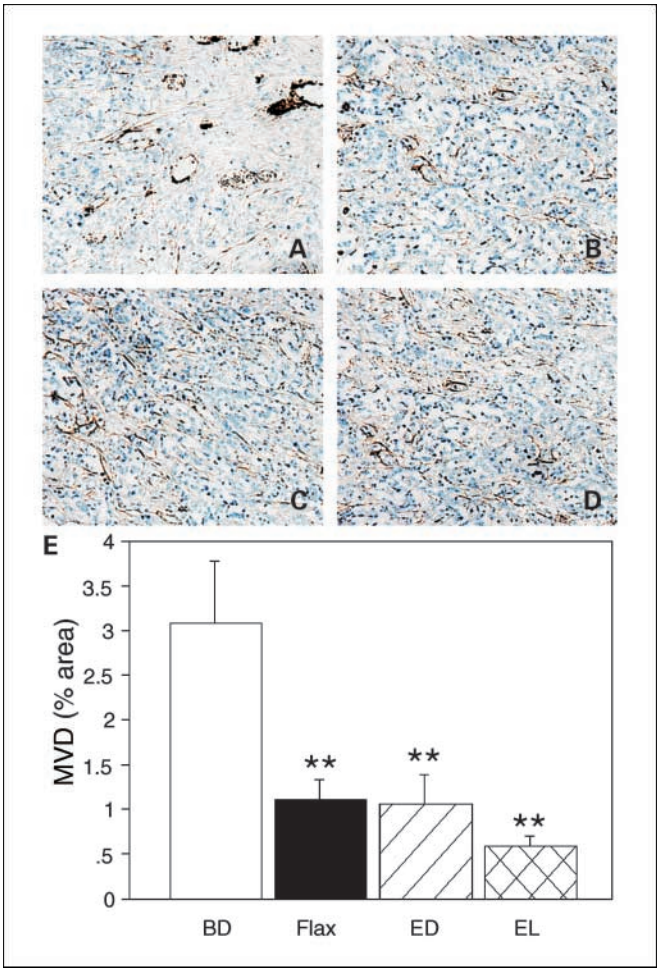

Fig. 3. Flaxseed, enterodiol, and enterolactone decreased tumor vasculature. Mice were treated as described in Fig. 1. Tumor sections were stained with anti–von Willebrand factor and vessel area was counted on tumor sections. Magnification, ×200. *A,* representative MCF-7 tumor from the basal diet group. *B,* representative MCF-7 tumor from the flaxseed group. *C,* representative MCF-7 tumor from the enterodiol group. *D,* representative MCF-7 tumor from the enterolactone group. *E,* tumor vessel density quantification was conducted in a blinded manner. Ten randomly selected high-power fields (×200 magnification) of three different tumors in each group were counted. **, $P < 0.01$, compared with the basal diet group.

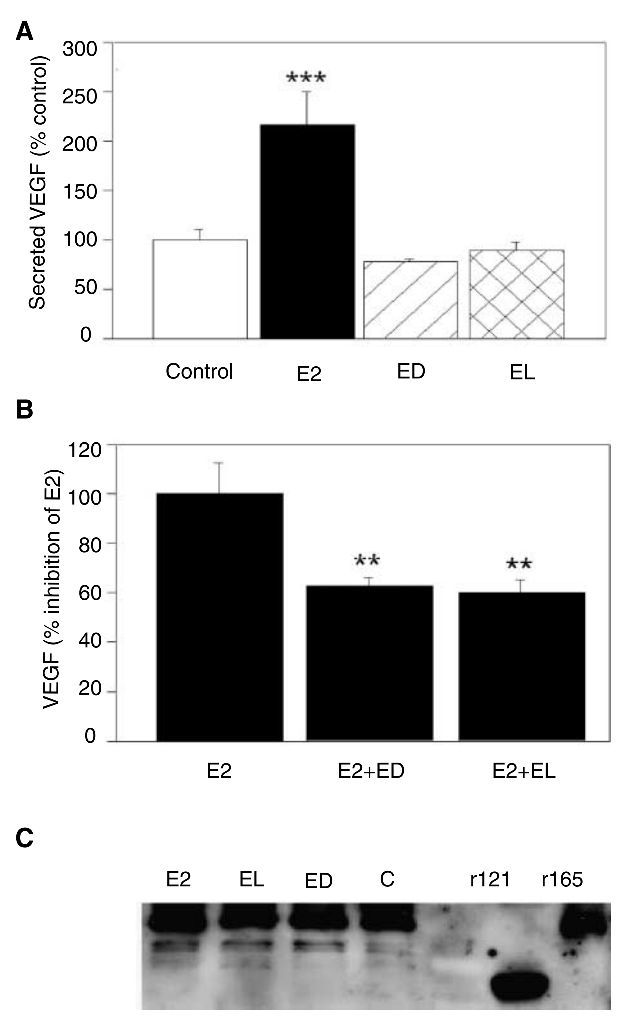

Fig. 4. Enterodiol and enterolactone decreased E2-induced secretion of VEGF in MCF-7 cell culture *in vitro. A,* MCF-7 cells were cultured without hormones (*C*) or in the presence of 10^{-8} mol/L E2, 10^{-6} mol/L enterodiol, 10^{-6} mol/L enterolactone, or a combination of E2 + enterodiol or E2 + enterolactone. The medium was changed everyday. VEGF was measured using ELISA 7 d in culture. E2 increased the VEGF secretion significantly compared with all other groups. $P < 0.001$ ($n = 6$ in each group). *B,* enterodiol and enterolactone added to the E2 treatment decreased the E2-induced secretion of VEGF significantly. **, $P < 0.01$ ($n = 6$ in each group). *C,* Western blot of MCF-7 cells.

decreased significantly after treatment with the antibody ($P < 0.01$, Student's *t* test; Fig. 5C). Cell culture medium from E2-treated MCF-7 cells in combination with the anti-VEGF antibody inhibited the increase of VEGFR-2 in HUVEC detected after the E2 MCF-7 culture medium. This strongly suggests that the up-regulation of VEGFR-2 seen after E2-treated MCF-7 medium was dependent on VEGF present in the medium ($P < 0.01$, Student's *t* test; Fig. 5C).

Discussion

In this study, we show that a dietary supplementation with 10% flaxseed or s.c. injection of the mammalian lignans enterolactone or enterodiol to ovariectomized E2-treated nude mice with established MCF-7 tumor explants decreased tumor growth and angiogenesis compared with mice on basal diet. Moreover, using microdialysis, we show that the extracellular levels of VEGF *in vivo* in the tumors from the intervention groups were significantly lower than in the basal diet group. This is the first study showing that flaxseed and its lignans enterodiol and enterolactone have the capacity to counteract the promoting effects of E2 on growth and angiogenesis in breast cancer *in vivo*. Our *in vivo* findings were confirmed

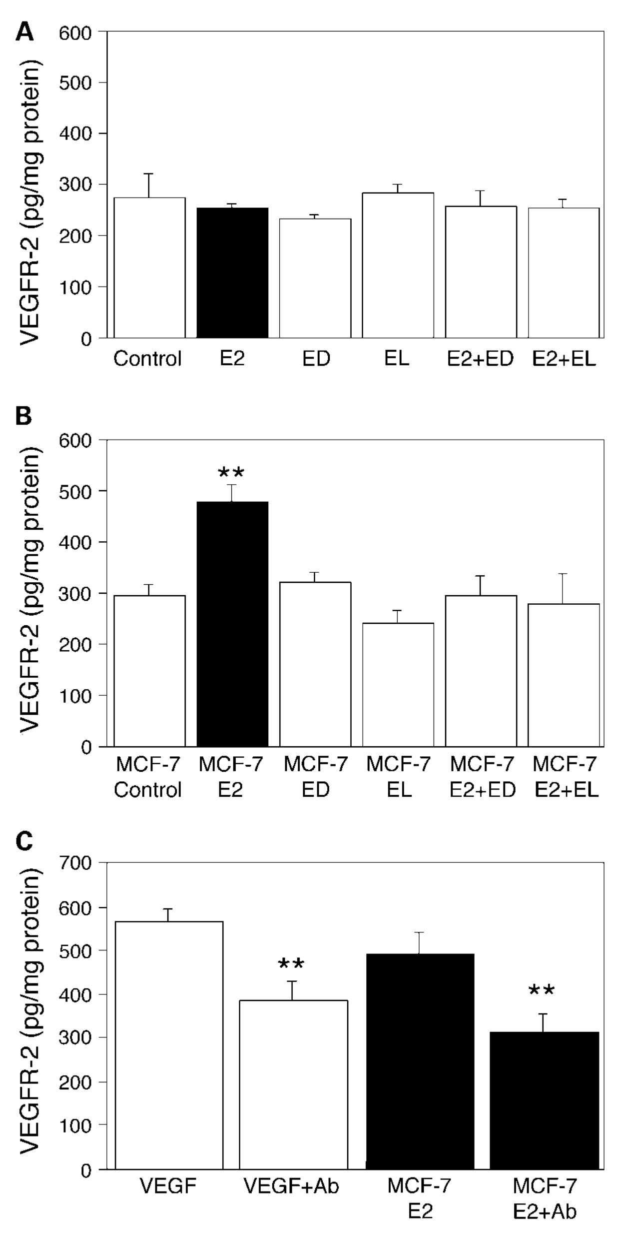

Fig. 5. VEGFR-2 in HUVEC after exposure to E2, enterodiol, and enterolactone and culture medium from MCF-7 cell. *A,* HUVECs were cultured without hormones (*C*) or in the presence of 10^{-8} mol/L E2, 10^{-6} mol/L enterodiol, 10^{-6} mol/L enterolactone, or a combination of E2 + enterodiol or E2 + enterolactone. The medium was changed everyday. Cell-associated VEGFR-2 was measured using ELISA. There was no difference between the treatments. *B,* HUVECs were exposed to hormone-treated MCF-7 cell supernatants as described in Fig. 4. There was a significant increase in VEGFR-2 after E2-treated MCF-7 culture supernatants. $P < 0.01$ ($n = 4$-6 in each group). *C,* treating HUVEC with recombinant VEGF and supernatants from E2-treated MCF-7 cells induced a similar level of VEGFR-2. Adding a VEGF antibody to the culture significantly decreased VEGFR-2. **, $P < 0.01$ ($n = 6$ in each group).

in vitro where both enterodiol and enterolactone decreased an E2-induced secretion of VEGF from MCF-7 cells. When HUVECs were incubated with cell culture medium from E2-treated MCF-7 cells, the VEGFR-2 levels increased significantly and in a similar fashion as with treatment recombinant VEGF to HUVEC. This increase was inhibited by adding an antibody against VEGF, suggesting that the increase in VEGFR-2 was mediated by the VEGF content in MCF-7 medium. By adding enterodiol and enterolactone, both the secretion of VEGF from MCF-7 cells was partly inhibited and the expression of VEGFR-2 on HUVEC was decreased compared with E2 treatment alone. Thus, E2 may increase angiogenesis both by increased VEGF secretion and by increased expression of VEGFR-2 in HUVEC, and both of these events were counteracted by the added phytoestrogens. Our data also suggest that flaxseed, enterodiol, or enterolactone does not exert any agonistic estrogen effects on ER^+ breast cancer.

The present results are in concordance with our previous findings in ER^- breast cancer showing that dietary supplementation with flaxseed reduced the tumor growth in nude mice by decreasing the proliferation rate, decreasing insulin-like growth factor and epithelial growth factor receptor, and reducing extracellular VEGF *in vivo* (19, 20). We have previously shown that a 6-week treatment of flaxseed to nude mice increased apoptosis and decreased cell proliferation in MCF-7 tumor xenografts (26). In the present study, mice were treated with phytoestrogens for 3 weeks and microdialysis was done on size-matched tumors without any differences in terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling or Ki-67 staining. The phytoestrogen-treated mice exhibited decreased levels of VEGF compared with basal diet mice, suggesting that a decrease in secreted VEGF may precede major changes in apoptosis and/or cell proliferation of the tumor cells. This is further supported by the *in vitro* results showing that, despite enterodiol and enterolactone did not affect cellular content, they inhibited E2-stimulated VEGF secretion.

Phytoestrogens may exert their effects through both ER-dependent and ER-independent mechanisms, and it has been reported that they bind preferentially to ER-β acting as selective ER modulators (27). They may also alter estrogen metabolism in postmenopausal women (28) and inhibit enzymes involved in the synthesis of sex steroids (29–31). Phytoestrogens are structurally similar to endogenous estrogens and may therefore exert a proestrogenic effect in breast cancer. In a previous study, using 10 times higher enterolactone concentrations than in our experiment, it was shown that enterolactone enhanced E2-stimulated DNA synthesis in MCF-7 cells (32). However, our previous *in vivo* studies have not shown any growth-promoting effects by enterolactone and enterodiol in MCF-7 xenografts in ovariectomized nude mice without estrogen supplementation (33). The present study confirmed that enterodiol and enterolactone do not exert any estrogenic effects on MCF-7 cell either in culture or in solid tumors *in vivo* in the presence of E2.

Angiogenesis is crucial in tumor development and progression (34). VEGF is one of the most potent proangiogenic proteins, and high VEGF levels have been shown to play a detrimental role in breast cancer progression and metastasis (11, 12). Several isoforms of VEGF exist as a result of alternative splicing of a single gene (35). The longer isoforms are bound in the extracellular matrix, whereas the shorter isoforms are freely diffusible and available to act on endothelial cells promoting angiogenesis (13, 36). Hence, the extracellular space is the bioactive site for VEGF, and by using microdialysis, it is possible to sample VEGF from this compartment. Using microdialysis among other techniques, we have previously shown that estrogen increases angiogenesis and VEGF secretion both in transgenic murine breast cancer, in human breast cancer cell and tumor explants *in vivo*, as well as in normal human breast tissue *in vivo* (16–18, 25, 37, 38). Moreover, we have shown that tamoxifen, an antiestrogenic compound and selective ER modulator, decreases VEGF secretion, although intracellular protein and mRNA levels increased in a similar fashion as with E2 exposure (25). Tamoxifen was shown to decrease the secreted VEGF possibly via an increase in the secretion of the antiangiogenic sVEGFR-1 (25, 38). As phytoestrogens also have been considered as selective ER modulators, enterodiol and enterolactone could have been expected to exert activities similar to that of tamoxifen on VEGF in our model system. However, we did not find any effects of either enterodiol or enterolactone on either intracellular or secreted VEGF nor were the levels of sVEGFR-1 affected after enterodiol and enterolactone stimulation (data not shown). Instead, our results indicate that both enterodiol and enterolactone blocked the E2-induced secretion of VEGF without estrogen-like properties as such. Estrogen may, in addition to a direct transcriptional control of the *VEGF* gene, also be involved in the alternative splicing resulting in the various VEGF isoforms, although in our model only the VEGF165 isoform was detected. Other estrogen-regulated pathways of VEGF may be indirect via hypoxia-inducible factor-1 or a regulation of cytokines, which in turn affect VEGF levels. Exactly by which pathway(s) phytoestrogens counteract estrogen-regulated VEGF secretion remains to be revealed.

Dietary phytoestrogens have been implicated in breast cancer prevention strategies, but there has been a concern about the potential estrogen-like properties of these compounds. Some phytoestrogens derived from soy, such as the isoflavone genistein, have indeed been found both *in vitro* and *in vivo* to function as an estrogen in breast cancer (39–41). The present study confirms previous studies suggesting that flaxseed, enterodiol, and enterolactone do not act as estrogens in ER^+ breast cancer. A beneficial effect of dietary flaxseed to women with breast cancer has also been shown in a randomized double-blind placebo-controlled clinical trial, where a diet supplementation with flaxseed to women diagnosed with breast cancer reduced tumor proliferation, decreased c-erbB2 expression, and increased apoptosis (42).

In summary, this study shows for the first time that flaxseed and its lignans enterodiol and enterolactone have the capacity to counteract E2-induced growth and angiogenesis in ER^+ breast cancer *in vivo* without estrogen-like action. The decreased angiogenesis by the lignans was mediated via decreased secretion of the E2-induced VEGF as shown both *in vitro* in cell culture and *in vivo* in tumor explants. The increased VEGF secretion by E2 in MCF-7 cells increased the expression of VEGFR-2 in HUVEC, suggesting a proangiogenic effect by E2 by two different mechanisms, both of which were inhibited by the addition of lignans. Our results suggest that flaxseed and its lignans have potent antiestrogenic effects on ER^+ breast cancer growth and may prove to be beneficial in breast cancer prevention strategies in the future.

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