Report

Estradiol promotes growth and angiogenesis in polyoma middle T transgenic mouse mammary tumor explants

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Summary

Estrogen is important for breast carcinogenesis and the majority of breast cancers maintain hormone dependency. Estrogen has the ability to stimulate both breast epithelial cell growth and angiogenesis, and a well-characterized *in vivo* cancer model where these functional interactions can be studied is lacking. We demonstrate estrogen dependent angiogenesis, growth *in vivo*, and proliferation *in vitro*, in explants from polyoma middle T transgenic mouse mammary tumors. Thus, in addition to genetic similarities, this model also exerts a sex hormone, and angiogenic phenotype similar to human breast cancer. This immune-competent animal model offers the opportunity to study molecular events in estrogen dependent breast cancer.

Abbreviations: PyMT: polyomavirus middle T antigen; MMTV: mouse mammary tumor promoter/enhancer; ER: estrogen receptor; ERE: estrogen responsive element; E2: estradiol

Introduction

Breast cancer is the most common form of cancer among women in the Western World. It accounts for almost 30% of all cancer cases in women and the incidence of the disease is still increasing [1]. The cumulative lifetime exposure to estrogen correlates with the risk of breast cancer. Prolonged exposure to endogenous sex steroids as a result of early menarche and late menopause increase the risk, while surgically induced menopause by bilateral ovariectomy before age 45 years markedly reduces the risk of developing the disease [2, 3]. Moreover, exogenous sex steroids, such as hormone replacement therapy increase the risk of breast cancer [4]. The mechanisms by which sex steroids promote cancer formation remain poorly understood. Estrogen has been shown to contribute to mammary carcinogenesis and continued cancer growth by increased cell division, production of growth factors, and by stimulating angiogenesis [5, 6]. Angiogenesis, the formation of new blood vessels from the existing vascular network, is essential

for continued tumor growth and spread to distant sites [7]. Vascular endothelial growth factor (VEGF), one of the most potent and specific angiogenic factors, is an endothelial specific mitogen that mediates developmental, physiological, and pathological angiogenesis [8]. In the female reproductive tract, where angiogenesis is a normal physiologic event, as well as in endometrial and breast cancer, VEGF is regulated by sex steroids and an estrogen responsive element (ERE) in the gene for VEGF has been identified [9, 10].

Approximately 70% of all breast cancers express the estrogen receptor (ER) and treatments designed to block estrogen-effects are important options in the clinic [11]. Angiogenesis inhibitors represent a new therapeutic approach for cancer and phase I clinical trials with these drugs are ongoing [12]. Thus, a breast cancer model that is well characterized according to its sex hormone and angiogenic phenotype, where the interaction between breast cancer cells and angiogenesis can be studied *in vivo*, could provide further insights into the mechanisms of how these events interact.

Transgenic mice carrying the polyomavirus middle T antigen (PyMT) under the control of the mouse mammary tumor promoter/enhancer (MMTV) develop mammary tumors that metastasize to the lungs at a frequency of >80% [13]. These tumors express activated oncogenes with constitutive tyrosine kinase activities refractory to normal cellular regulation, similar to genetic alterations such as HER-2 (*c-erbB2*) overexpression in human breast cancer [13]. A system where the primary tumors are excised and single-cell suspensions are cultured *in vitro* for subsequent injection into syngeneic mice has been developed and used in our laboratory for studies of immuno gene therapy of breast cancer. The advantages of this system are that a large number of animals can be derived from one single transgenic mouse and that the tumors grow in an immune-competent host. However, the sex hormone, and angiogenic phenotype of these tumor transplants have not been characterized.

Here we demonstrate that estradiol affects PyMT tumor growth *in vivo*, proliferation rate *in vitro*, and tumor angiogenesis. Thus, the PyMT tumor explants have an estrogen dependent phenotype according to growth pattern and angiogenesis, which is similar to the majority of human breast cancer.

Materials and methods

Animals and ovariectomy of mice

C57BL/6 and FVB/n mice (6–8 weeks old) were purchased from Charles River (Troy, NY). They were housed in pathogen free conditions with a light/dark cycle of 12/12 h and fed with rodent chow and water *ad libitum*. The McMaster University animal ethics research board approved all animal work.

Mice were anesthetized with isoflurane (Abbot), ovariectomized (OVX) and 3-mm pellets containing 17 β -estradiol (E2), 0.18 mg/60-d release, or placebo pellets (Innovative Research of America, Sarasota, Florida, USA) were implanted s.c. in the animal's back 7 days before tumor induction. The pellets provide continuous release of E2 at serum concentrations of 150–250 pM (confirmed by serum analysis), which is in the range of physiologic levels seen in mice during the estrous cycle.

Polyoma middle T (PyMT) tumor model

Tumor cells were derived from a transgenic mouse strain expressing PyMT antigen under the control

of the mouse mammary tumor virus (MMTV) long terminal repeat [13]. These mice develop spontaneous adenocarcinomas of all mammary epithelium by 8–10 weeks of age. These tumors were excised, minced, and incubated at 37°C with gentle stirring in collagenase/dispase solution (25 mg collagenase and 250 mg dispase, Roche, in 100 ml PBS) to generate a single-cell suspension, stored at –70°C. Thawed cells were placed in complete F-11 medium and were cultured until they reached confluence, usually 4–6 days. Thereafter, 0.5×10^6 cells in 200 μ l phosphate-buffered saline were injected s.c. into the right hind flank of a syngeneic female FVB/n host. Tumor volume was determined by measuring length, width, and depth of the tumor weekly using a caliper.

Cell proliferation assays

The primary cell culture of PyMT tumor cells were cultured in phenol-red free Dulbecco's modified Eagle's (Gibco BRL) medium supplemented with 1% penicillin/streptomycin, and 10% charcoal stripped fetal bovine serum and plated into a 96-well plate at a density of 5000 cells/well. Various concentrations of 17 β -estradiol (Sigma) were added and cells were allowed to grow for 3 days. Proliferation was measured by a MTT (tetrazolium blue) conversion test [14] and the absorbance at 540 nm was recorded using an ELISA plate reader. Mitotic cells/60 $\times$ High power field was counted on stained tumor sections.

ER and serum estradiol assays

Tumors were removed and snap frozen in liquid nitrogen and stored at –70°C and the receptor levels were measured by Abbot ER-EIA Monoclonal (Abbot park, IL, USA). Levels below 3 fmol/mg protein were considered receptor negative.

Formalin-fixed, paraffin embedded tumors were cut in 3 μ m sections, deparaffinized and subjected to anti-ER immunohistochemistry (Novocastra, Newcastle upon Tyne, UK with Envision detection, Dako). Sections were counterstained with H&E. Negative controls did not stain. Serum estradiol concentrations were assayed by Abbot AxSYM Systems (Abbot, IL, USA).

Anti-CD31 immunostaining of tumor vasculature and vessel quantification

Tumors were removed when they were approximately 150 mm³ in size, embedded in OCT compound, and

frozen rapidly in isopentane, and stored at -70°C . Frozen sections ($3\text{--}5\text{ }\mu\text{m}$) were processed and subjected to anti-CD31 immunohistochemistry (rat anti-mouse CD31; BD PharMingen, San Diego, CA). Vessel quantification of CD-31-stained tumor sections was conducted using a Leica (Deerfield, IL) Laborlux microscope equipped with a Sony (Tokyo, Japan) CCD digital camera. Ten medium power fields ($\times 200$) were examined per section in a blinded manner. Percent of tumor area stained with CD-31 was counted using Northern Exposure V2.9 imaging software (Empix Imaging, Mississauga, ON, Canada).

In vivo Matrigel bioassay for angiogenesis

Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) at 4°C was mixed with 150 ng/ml recombinant bFGF (Gibco BRL) and the mixture was injected s.c. into the abdominal midline of ovariectomized mice supplemented either with E2 or placebo. Seven days after injection, the Matrigel plug was removed, fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with Masson's Trichrome. Endothelial cell infiltration was determined using a Zeiss microscope equipped with a Sony CCD digital camera. Ten random fields were examined and endothelial cell infiltration was quantified using Northern Exposure imaging software.

Statistics

Data are expressed as mean $\pm$ SEM. Student's *t*-test or ANOVA were used as appropriate. A $p < 0.05$ was considered significant.

Results and discussion

In this study we demonstrate that PyMT tumor explants grew rapidly and extensively in OVX E2 supplemented mice compared to OVX mice without supplement (Figure 1(A) and (B)). Moreover, on gross examination these tumors showed a more aggressive growth pattern with invasion of the muscular tissue and local expansion into the peritoneal cavity (data not shown). Similar to the majority of human breast cancers, the PyMT tumors maintained a low-moderate expression of the ER as demonstrated by immunohistochemistry (Figure 2) and quantified by ER-EIA, $6.5 \pm 1\text{ fmol/mg protein}$, without difference between the treatment groups. This is in contrast to other transgenic mouse models of breast cancer, which appear to

lose the ER during cancer progression [15]. This loss could, however, be explained by estrogen treatment at pharmacological levels since high levels of estradiol down-regulate the ER [15, 16]. In our study, the E2 levels in intact normal cycling mice ranged between 30 and 345 pM and the OVX mice with E2 supplementation were maintained at a physiologic level of estradiol ($150\text{--}250\text{ pM}$) confirmed by serum analysis. Although the delivered E2 by the implants were within the physiologic range the levels were kept at a constant level without the cyclic variation seen in normal mice.

In vitro, the proliferation rate of the PyMT cells was enhanced by E2 in a dose response manner (Figure 1(C)), *in vivo* the mitotic index was 1.47 ± 0.14 in hormone exposed tumors and 1.26 ± 0.17 in tumors without hormone treatment. Estrogen dependent cell cycle regulatory mechanism(s) other than up-regulation of the transgene must be involved in this stimulation, since estradiol alone does not affect MMTV expression [17]. Although it has been shown that the MMTV promoter is enhanced by other steroids, such as progesterone and glucocorticoids, this promoter lacks an ERE [17]. However, it has been shown that in presence of ovarian hormones, polyomavirus infected mice have an increased tumor incidence in the mammary gland [18]. Whether this sex steroid dependency remains when the antigen is driven by the MMTV promoter/enhancer is not known.

The moderate increase in cell proliferation rate could not explain the extensive growth seen *in situ*. On gross examination the E2 treated tumors had a more developed vasculature compared to the placebo tumors. This finding led us to quantify the tumor vasculature, in tumors approximately 150 mm^3 in size, and indeed, we found that the vessel count was significantly greater in E2 treated tumors compared with placebo ($2.0 \pm 0.14\%$ of total area versus $1.2 \pm 0.07\%$, $p < 0.0001$, Figure 3(A)–(C)). In addition to increased microvessel density, the vessels in E2 exposed tumors also showed a more organized tubular formation (Figure 3(A) and (B)). Using the Matrigel assay, we could show that E2 treatment had a direct stimulatory effect on endothelial cell proliferation *in vivo* as E2 treated animals exhibited a massive infiltration of endothelial cells into the gel ($45 \pm 1\%$ of total area v.s. $10 \pm 0.7\%$, $p < 0.0001$, Figure 4(A)–(C)). This confirms previous data, showing that estrogen modulates angiogenesis via effects on endothelial cells, both under physiological and pathological conditions

Figure 1. Effect of estradiol (E2) on PyMT tumor cell growth. A. Tumor volume. PyMT tumor cells injected s.c. into ovariectomized FVB/n mice supplemented with E2, 150–250 pM (● $n = 20$), intact mice Δ ($n = 8$), or ovariectomized supplemented with placebo (○ $n = 20$). Tumor volume was measured weekly, * $p < 0.0001$. Inset shows the difference in volume between intact and OVX animals, * $p < 0.01$. B. Tumor weight at sacrifice, * $p < 0.0001$ E2 versus intact and placebo, # $p < 0.0001$ intact versus placebo and E2. C. PyMT tumor cell proliferation *in vitro*, in presence of various concentrations of 17 β -estradiol or vehicle, # $p < 0.0001$ control versus E2 (all groups), * $p < 0.01$ 10 nM E2 versus 0.1 nM E2.

Figure 2. Positive nuclear immunohistochemical staining for ER in PyMT tumor section.

[19]. The large difference in tumor outcome between E2 treated animals and normal cycling intact mice may be explained by the uninterrupted stimulation of angiogenesis and proliferation by constant E2 levels, within the physiologic range, compared to the cyclic pattern with days of lower hormone levels in intact mice.

Angiogenesis is important for tumor growth and anti-angiogenic compounds have the ability to attenuate tumor growth [20]. Various cells in the immune

system, such as T lymphocytes and macrophages, participate in the regulation of angiogenesis [21, 22]. Thus, immune-competent hosts are essential for investigation of tumor angiogenesis. This could be achieved by using transgenic mouse models of cancer, such as the PyMT. Breast cancer development is a multistage process and the balance between stimulation and inhibition on cell division, angiogenesis, and the immune system each contribute to which phenotype tumor cells will express *in vivo*. Estrogen has the ability to affect all of these systems, and studies of their interactions require physiologically relevant *in vivo* models that are well characterized according to their estrogenic phenotype.

In summary, we report that PyMT mammary tumors grew aggressively under physiologic E2 concentrations *in vivo*. *In vitro*, E2 stimulated proliferation of PyMT cells at a moderate level. Moreover, PyMT tumors exhibited an estrogen dependent angiogenesis *in vivo*. Taken together, these data demonstrate how a synergistic effect of E2 on cell proliferation and angiogenesis can result in extensive tumor growth *in vivo*. This *in vivo* model of breast cancer in an immune-competent host provides a useful tool to explore functional interactions between breast stromal, epithelial and endothelial cells in estrogen dependent breast cancer, as well as for studies of new anti-estrogen and anti-angiogenic therapies.

Figure 3. Effect of E2 on tumor angiogenesis *in vivo*. Mice treated in the same manner as in Figure 1. A. PyMT tumors exposed to estradiol stained with anti-CD31 showed organized microvessels. B. PyMT tumors grown without E2 showed only random staining with anti-CD31. C. Tumor vessel density quantification was conducted in a blinded manner. Ten randomly selected areas of four different tumors in each group, stained with anti-CD31, were counted, * $p < 0.0001$.

Figure 4. Effect of E2 on endothelial cell proliferation *in vivo*. Mice treated in the same manner as described in Figure 1. A. Matrigel plugs from estradiol treated mice showed a massive endothelial cell infiltration. B. Matrigel plugs from placebo mice showed some infiltration of endothelial cells. C. Quantification of endothelial cell infiltration into Matrigel. Ten randomly selected areas of three different Matrigel plugs in each group were counted, * $p < 0.0001$.

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