Effect of raloxifene on vascular endothelial growth factor expression in breast carcinomas of postmenopausal women

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Abstract

Objective: This study aimed to evaluate the effect of raloxifene on vascular endothelial growth factor (VEGF) expression in breast carcinomas of postmenopausal women.

Materials and methods: Sixteen postmenopausal patients with operable stage II, oestrogen receptor-positive, infiltrating ductal breast carcinoma were treated with raloxifene at a dose of 60 mg/day, for a period of 28 days prior to definitive surgery. Tumour size varied from 3 to 5 cm (mean 3.7 cm) and mean age of patients was 61.8 years (range 49–72 years). Tumour samples were obtained by incisional biopsy at the time of diagnosis and again at the time of surgery. Immunohistochemical evaluation of VEGF expression was assessed semiquantitatively based on fraction of stained tumour cells and on intensity of staining. McNemar's test of symmetry was used to evaluate agreement between positive or negative classification of VEGF expression prior to and following raloxifene treatment ($P < 0.05$).

Results: Fourteen of the 16 patients (88%) were classified as positive for VEGF expression prior to raloxifene treatment, while only 5 (31%) were classified as positive following treatment ($P < 0.007$).

Conclusion: Raloxifene significantly reduced VEGF expression in these oestrogen receptor-positive breast carcinomas of postmenopausal women.

Introduction

Formation of new blood vessels is essential for tumour growth and metastasis of various human neoplasias, including breast carcinoma (1). In addition, higher density of vessels in these tumours has been associated with

increase in aggressiveness and poor prognosis (1). These findings have stimulated studies on treatment options for breast cancer, focused on inhibition or blockade of tumour angiogenesis (2,3).

The term 'angiogenesis' means formation of new vessels from pre-existing ones, constituting a physiological process during human body growth and development. In adults, however, angiogenesis is restricted to healing of wounds, the menstrual cycle and neoplastic processes (4,5). Various factors regulate angiogenesis (5,6). Of these, the most important is vascular endothelial growth factor (VEGF) (5), which is able to stimulate practically all phases of angiogenesis and to maintain vascularization by inhibiting apoptosis of endothelial cells (6,7).

VEGF expression in breast tissue is affected by oestrogen and by neoplastic processes (8,9). Dabrosin (9) reported significantly greater VEGF expression in normal breast tissue of premenopausal women compared to that of postmenopausal women. Other authors have also reported higher tissue levels of VEGF in breast cancer compared to adjacent normal breast tissue, and change in VEGF expression following treatment with selective oestrogen receptor modulators (SERM) (2,10).

Tamoxifen is a first-generation SERM, approved by the US Food and Drug Administration for adjuvant treatment and chemoprevention of breast cancer in high-risk patients (11). Although some authors have reported an increase in VEGF mRNA expression in MCF-7 breast cancer cell lines exposed to tamoxifen (12), more recent studies have shown that tamoxifen inhibits VEGF secretion in breast cancer *in vivo* (2). However tamoxifen, when used for long periods of time may provoke undesirable effects, the principal of which is increased risk of endometrial carcinoma (13).

Raloxifene on the other hand, is a second-generation SERM approved in the USA and Europe for treatment of menopausal patients with osteoporosis in view of its efficacy in reducing bone mass loss and fracture risk (14). In addition, raloxifene also has a beneficial effect on breast cancer (15). Recently, a study of tamoxifen and raloxifene trial showed that raloxifene may be an alternative to tamoxifen,

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since it is equally effective in preventing invasive breast cancer (16), with the benefit of being associated with reduced incidence of endometrial carcinoma (16,17).

Some authors have reported that raloxifene reduces cell proliferation activity both in normal and neoplastic breast tissue (18,19); thus, confirmation of its effect on VEGF expression may provide an explanation for the mechanism of its antiproliferative effects. However, there are few publications of studies that have evaluated the effect of raloxifene on VEGF expression, particularly on breast cancer cells (20), a situation that led us to develop the present investigation.

Materials and methods

Patients

Sixteen postmenopausal patients with stage II, operable (larger than 3 cm), infiltrating, ductal, oestrogen receptor-positive carcinoma of the breast, receiving medical care at the Mastology Division of the Gynaecology Department of the Hospital Getúlio Vargas, Federal University of Piauí, Brazil, participated in this study. The investigation was approved by the Internal Review Board of the Federal University of Piauí and all patients gave their signed informed consent prior to initiation. All patients had been menopausal for at least 1 year and had no history of previous treatment for breast cancer. Tumour size varied from 3 to 5 cm (mean 3.7 cm), mean age was 61.8 years (range 49–72 years) and all patients had negative HER-2 expression (Table 1). Patients received 60 mg of raloxifene daily for 28 consecutive days prior to definitive surgery. Raloxifene therapy was initiated immediately after a patient

received the result of the diagnostic incisional biopsy. Two tumour samples were obtained per patient, by incisional biopsy during the study: one at the time of confirmation of the diagnosis of ductal, infiltrating carcinoma and evaluation of oestrogen receptor status, and the other 29 days later, at the time of surgery. Samples were fixed in 10% buffered formalin for 12–24 h, then were embedded in paraffin wax blocks. Following immunohistochemical staining, tumours in which semiquantitative evaluation of oestrogen receptor was classified as high ($\geq 10\%$ immunoreactive cells), were considered positive (21).

Immunohistochemistry for VEGF

For immunohistochemical evaluation of VEGF expression, paraffin wax blocks containing the samples were cut into 5- μ m-thick sections. Sections were deparaffinized and antigenic recovery was performed using 0.31% citric acid (pH 6) in a vaporizer for 60 min at 95 °C. Next, slides were incubated with primary anti-VEGF monoclonal antibody (immunoglobulin G, C-1 sc-7269, 200 mg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1 : 100, at 4–8 °C. Subsequently, slides were washed in phosphate-buffered saline containing Tween (PBS-Tween), and Novocastra Post Primary Block (Cod. 7161, Novocastra, Newcastle upon Tyne, UK) was instilled for 30 min; samples were then washed again in PBS-Tween and were incubated with diaminobenzidine tetrahydrochloride (Ref. D-5637, Sigma, St. Louis, MO, USA) for 5 min. Finally, slides were washed in distilled water, counterstained with haematoxylin, stained blue with ammoniacal water solution, dehydrated in absolute ethanol, passed through cubes of xylol and then mounted in Permount resin. Cells that expressed VEGF were identified by their dark-brownish cytoplasmic staining.

Quantification

VEGF expression was evaluated by two observers (blind to group identification), using light microscopy. These observers semiquantitatively assessed cells in which cytoplasm was positively stained ($\times 400$ magnification) using a system consisting of a light microscope (Nikon Eclipse E-400 Optical Microscope, Tokyo, Japan) connected to a video camera (Samsung Digital Camera SCC-131, Seoul, South Korea) with image capture, and transmission to a computer equipped with the Imagelab® software program (Softium Informatica LTDA, São Paulo, Brazil). Immunoreaction was assessed according to the criteria established by van Slooten *et al.* (22), taking the following parameters into consideration: the intensity of cell staining (I) and fraction of stained neoplastic cells (F). Intensity of staining was classified as 0 (negative), 1 (weakly stained), 2 (moderately stained) and 3 (strongly stained). Fraction

Table 1. Characteristics of patients

Characteristics	<i>n</i>	%
Age (years)		
40–49	1	6
50–59	4	25
60–69	9	56
≥ 70	2	13
Size of tumour (cm)		
3.0–3.9	11	68
4.0–5.0	5	32
Histological grade		
1	8	50
2	6	37
3	2	12
Lymph node status		
N0	9	56
N1	7	44
HER2 expression		
Negative	16	100

Figure 1. Photomicrographs of histological sections of breast cancer tissue sample from patient no. 9, stained with anti-VEGF antibody prior to treatment with raloxifene (a) showing a positive immunohistochemical reaction, as expressed by numerous cells with cytoplasm intensely stained in brown, compared to post-treatment (b) (original magnification $\times 400$).

of stained cells was classified as I (0–25%), II (25–75%) or III (75–100%). Final scores were the result of combination of the two parameters (I and F) and ranged from 0 to 6. Cases with a final score of ≥ 3 were classified as positive for VEGF. In all cases, brownish staining of cytoplasm was adopted as the standard for positivity. Assessment was initiated at the site with greatest quantity of stained cells, after which other microscope fields were selected at random, and staining intensity and percentage of stained tumour cells were assessed, resulting in the final score (22).

Statistical analysis

McNemar's test of symmetry (23) was used to evaluate level of agreement between positive and negative classification of VEGF expression prior to and following treatment with raloxifene, in patients with breast cancer. Statistical significance was established at $P < 0.05$.

Results

At light microscopy, a lower percentage of cells was seen to be stained brown using anti-VEGF antibody in the tumour samples obtained following raloxifene treatment, compared to higher concentration of cells with positive VEGF expression in samples collected prior to raloxifene treatment (Fig. 1). Quantitative analysis of classification of the 16 cases according to VEGF expression, showed that the 2 patients classified as VEGF-negative prior to treatment remained negative following treatment. Of the 14 patients who were classified as VEGF-positive prior to treatment, 9 became negative following treatment, while only 5 remained positive. Therefore, 88% of these patients (14/16) were classified as positive for VEGF prior to treatment, while only 31% (5/16) were classified as positive following raloxifene treatment ($P < 0.007$) (Table 2).

Discussion

Results of the present study show that raloxifene reduced VEGF expression in breast carcinomas of some post-

Table 2. Percentage of breast cancer cases with vascular endothelial growth factor-positive cells prior to and following raloxifene treatment

Pre-treatment	Post-treatment		Total
	Negative	Positive	
Negative	2	0	2 (12%)
Positive	9	5	14 (88%)
Total	11 (69%)	5 (31%)*	16 (100%)

*There was a statistically significant reduction in vascular endothelial growth factor expression following raloxifene treatment ($P < 0.007$).

menopausal women. The drug was administered for 28 days, since this is the mean length of time between diagnosis and definitive surgical treatment in our institute, thereby ensuring that no delays incurred as a result of the study. Dose used was 60 mg/day, this being the dose of raloxifene commonly used for prevention and treatment of osteoporosis, and in trials on chemoprevention of breast cancer. Incisional biopsy carried out in the present study both prior to and following raloxifene treatment reduces the chances of results that simply reflect tumour heterogeneity, which is a recognized problem linked to core biopsy sampling (24–26). Nevertheless, angiogenesis may be affected by previous surgical manipulation of the tumour as part of the process of wound healing, although the duration of such an effect is not known (26).

Raloxifene acts principally by binding to oestrogen receptors (27,28). Dowsett *et al.* (18) showed that raloxifene reduced proliferative activity only in oestrogen receptor-positive tumours, which led us to include only patients with this type of neoplasm in the present study. The mechanism of action of raloxifene, which acts as an anti-oestrogen in some tissues and an oestrogenic agonist in others, has yet to be fully clarified (27,28). Binding of this drug to oestrogen receptors appears to induce conformational changes in the receptor, permitting interaction with co-regulators and stimulating or inhibiting expression of elements responsive to oestrogen that are present in

cells' DNA (27,28). Moreover, different concentrations of oestrogen receptor subtypes alpha and beta, in various human tissues, as well as possible interactions between them, may also explain the different effects of raloxifene (29).

In the breast, where there is a predomination of oestrogen receptor alpha, raloxifene has an oestrogenic antagonist effect (27,28). Preliminary studies in premenopausal women have shown that raloxifene, used for 22 days, significantly reduced proliferative activity in normal breast tissue (4). Angiogenesis is a fundamental process in cell proliferation and in tumour growth and metastases (1,30). Control of gene expression of angiogenic factors, or blockade of their receptors, would be an effective alternative to other chemotherapies, for treatment of breast tumours (31). Nevertheless, it is unclear whether the anti-proliferative effect of raloxifene reported in some studies (4,18) may be due to alteration in expression of angiogenic factors, such as VEGF, directly in the neoplastic tissue.

VEGF, initially referred to as vascular permeability factor, is a 45-kDa homodimeric glycoprotein considered to be the principal angiogenic factor in human tissues, since it induces increased capillary permeability, proliferation and migration of endothelial cells (3). Of the factors that stimulate its expression, hypoxia is the most important. Four principal isoforms are known to have: 121, 165, 189 and 206 amino acids. In primary breast cancer, the 121 isoform is predominant, followed by 165 and 189 (32). Zhang *et al.* (33) showed VEGF121 to be the most angiogenic and tumorigenic isoform in MCF-7 breast cancer cells compared to isoforms 165 and 189. VEGF acts by binding to transmembrane receptors present predominantly in endothelial cells of blood vessels with tyrosine-kinase activity, promoting phosphorylation of various cytoplasmic proteins that initiate the process of angiogenesis (3,31). VEGF protein present in cytoplasm of cells of various tissues, may become available to endothelial cells by at least two different mechanisms: as freely diffusible proteins (VEGF121, VEGF165) or following protease activation and cleavage of the longer isoforms (5). Anti-VEGF antibody used in the present study is capable of detecting isoforms 121, 165 and 186, present in cell cytoplasm and ultimately revealing them in brown stain, therefore, covering the principal isoforms involved in adult human angiogenesis (3). On the other hand, to evaluate blood vessels it would be necessary to employ antibodies against VEGF receptors (3,5). Likewise, some authors have shown correlation between microvessel density and VEGF expression (34).

The effect of raloxifene on serum VEGF levels in menopausal women is controversial (35,36). Christodoulakos *et al.* (35) found no significant alteration in serum VEGF levels in menopausal women who had used raloxifene

continuously for 12 months. On the other hand, Lam *et al.* (36) reported that raloxifene significantly reduced serum VEGF levels after 12 and 36 weeks, compared to a placebo group. Nevertheless, evaluations based on serum VEGF levels are complex because a high proportion of VEGF found in serum is derived from platelets and activated on coagulation (37), a fact that led us to study VEGF expression directly in neoplastic breast tissue.

Only one *in vitro* study has evaluated the effect of raloxifene on breast cancer cell line MCF-7, and the results of that study showed that the drug had no effect on mRNA expression of VEGF121 and VEGF165 isoforms (20). However, to the best of our knowledge, no studies have been published evaluating the effect of raloxifene as a primary treatment for VEGF expression in human breast cancer cells. The results of the present study show that raloxifene reduced VEGF expression in some postmenopausal women's breast cancer; thus, this suggests that the drug may reduce neovascularization and further growth of oestrogen receptor-positive breast tumours.

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