Changes in vascular endothelial growth factor (VEGF) after chemoendocrine therapy in breast cancer

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Summary

Purpose: Angiogenesis has been proposed as a possible target for anticancer treatment, either by inhibition of the production of angiogenic factors or by inhibition of endothelial cell proliferation. The impact of preoperative chemoendocrine therapy is unknown in the regulation of angiogenic factors, but recent reports suggest that anticancer drugs have antiangiogenic activity.

Methods: The expression of two angiogenic factors VEGF and Angiopoetin-1 were quantified at different concentrations of doxorubicin, docetaxel, tamoxifen, exemestane and letrozol on MCF-7 and T47D cells.

Results: Low-drug concentrations led to increased VEGF-A gene transcription whereas high (10-fold increased) drug concentrations suppressed gene expression. A similar cell reaction was observed for VEGF protein with a smaller variety in the extent of modulation. Incubation of MCF-7 cells to different drugs showed a similar dose-dependent modulation of Angiopoietin-1 gene expression with enhancement at low-drug concentrations.

Conclusion: Treatment of breast cancer cells following a preoperative protocol showed a dose-dependent expression of VEGF and Angiopoetin-1. Only high-drug concentrations were followed by a decreased secretion of both factors whereas low concentrations induced up-regulation of VEGF and Angiopoietin 1.

Key words: Vascular endothelial growth factor; Angiopoietin-1; Breast cancer cells; Preoperative chemotherapy.

Introduction

Breast cancer is a very heterogeneous disease and it is well known that prognostic factors influence therapeutic conduct. Based on these, clinical trials have been performed in order to decide treatment, dose and the optimum sequence of different therapeutic agents to be used. The problems of defining the best therapy are immense and clearly this is an important area for research.

Tumor size, lymph node status, histological type, grade and progesterone and estrogen receptors are accepted as established prognostic and predictive factors valid for selection of treatments of patients with breast cancer. The role of other new factors like epidermal growth factor receptor gene (Erb-2), bcl-2, p53 and vascular endothelial growth factor (VEGF) alone or in association with the other classic factors to define the histological profile of the tumors could represent the framework for the therapy.

Tumor growth, infiltration and metastasis of solid tumors are dependent on angiogenesis. This process is mediated by a complex micro-environment of pro- and anti-angiogenic stimuli. Various angiogenic factors and cytokines induce neovascularization in tumors, namely members of the vascular endothelial growth factor (VEGF) and angiopoietin gene families. VEGF secretion by the tumor cells is a prerequisite of tumor development. It has been shown that VEGF is required for the initial stages of breast cancer tumorgenesis, and that this initial effect is related to the development of neovascular stroma [1]. VEGF and its endothelial cell-specific tyrosine kinase receptors are key regulators of tumor angiogenesis [2]. VEGF has been shown to be associated with poor prognosis and reduced survival [3]. Higher tumor levels of VEGF were associated in multivariate analysis as predictors for poor response to both chemotherapy (CMF) and tamoxifen [4].

Angiopoietin-1 is another ligand of an endothelial tyrosine kinase receptor. Together with VEGF it works as an endothelial cell survival factor [5]. In vitro Angiopoietin-1 protects against endothelial cell apoptosis, stabilizes endothelial tubules and induces vascular sprouting [overview in 6]. Angiopoietin-1 has been expressed in different breast cancer cell lines including MCF-7. Angio-poietin-1 caused retardation of tumor growth in a xenograft model [6]. Data from animal models reported that the rates of both cancer cell proliferation and apoptosis decreased significantly in the presence of Angiopoietin-1 [7].

VEGF expression is modulated by different factors, including hormones [8], probably by direct transcription of the VEGF gene by estrogen [9]. Few data about the influence of endocrine drugs on VEGF regulation are available. Therefore we selected tamoxifen and the aromatase inhibitors exemestane and letrozole to study their effect on VEGF expression. Different chemotherapeutic drugs are known to have anti-angiogenic effects [10-12], but details about regulation are rare. In locally advanced breast cancer, preoperative treatment is increasingly used. This concept allows us to observe tumor responsiveness

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to therapy directly in vivo. Reduction in microvessel density in primary breast carcinomas after neoadjuvant chemoendocrine therapy has been observed [13]. Mechanisms of modulation on angiogenesis are becoming more important since angiogenesis inhibitors are currently undergoing evaluation in clinical trials for the treatment of breast carcinoma [14, 15].

The aim of this study was to evaluate the responsiveness of breast cancer on VEGF and Angiopoietin-1 expression to different anticancer drugs used in a preoperative protocol.

Material and methods

Cell culture

The human breast cancer cell lines MCF-7 vector and T47D were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's MEM culture medium (Gibco, Invitrogen Corporation, Scotland, UK), supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria) was used. Cell lines were cultured in a humidified atmosphere of 5% CO and 95% air at 37°C.

Single-cell suspensions were seeded into six-well plates (6-well Tissue Culture Plate, Becton Dickinson Labware, NJ, USA) at $2x10^5$ cells/well and grown at 37°C for 24h. For the experiments 1 ml DMEM/well was added including substances at concentrations given in Table 1. One well without drug treatment served as a negative control; 24h later the cell culture superannuate was taken for later protein analysis using the ELISA method. Cells were used for RNA isolation.

Table 1. — *Drugs and concentrations used in vitro.*

Drug/Formulation	Concentration (nM)		
	X 0.1	X 1	X 10
Exemestane/Aromasin®	1.4	14	140
Doxorubicin/Adriablastin®	1.6	16	160
Tamoxifen/Nolvadex®	0.18	1.8	18
Letrozole/Femara®	100	1000	10000
Docetaxel/Taxotere®	4.2	42	420

RNA extraction and RT-PCR

RNA was extracted from the cells using the RNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA treated RNA was quantified by reading the absorbance at 260 nm. For DNA treatment 300 mM sodiummacetate pH 5.5, 5 mM magnesiumsulfate and 10 units RNA-free DNA (Applied Biosystems) were added and incubated at 23°C for 20 minutes. Two-thirds of the total volume 99% ethanol were used to precipitate the RNA at –80°C for 30 minutes. After centrifugation at 14,000 x g for 15 minutes RNA was washed with 70% ethanol and centrifuged at 14,000 x g for 5 min. Ethanol was removed and the reaction tube dried at room temperature. RNA was redissolved in RNA-free water.

Reverse transcription was performed using 3 µg RNA at a final concentration of 1 x PCR buffer, 5 mM of MgCI2, 1 mM dNTP, 2.5 µM random hexamer primers, 10 U of RNA inhibitor and 25 U of MuLV reverse transcriptase at a total volume of 10 µl. All reagents were purchased from PE Applied Biosystems Roche, Foster City CA, USA except the dNTP mix, which was purchased from Promega, Madison, WI, USA. Reverse transcription was performed at 23°C for 15 min. and at 42°C for 40 min using a Gene Amp PCR System 9700 (PE Applied Biosystems). After cDNA synthesis 5mM TrisHCI pH 7.5 (Dynal, Oslo, Norway) was added.

Table 2. — *Primers used for RT-PCR.*

Gene	Forward primer	Reverse primer
GAPDH	5'-GCCAAAAGGGTCATCATCTC-3'	5'-GTAGAGGCAGGGATGATGTTC-3'
VEGF-A (exon 2 and 4)	5'-AAGGAGGAGGGCAGAATCAT-3'	5'-GCTGTAGGAAGCTCATCTCT-3'
Angiopoietin-1	5'-CTAGATTTCCAAAGAGGCTGG-3'	5'-CCTCTGACTGGTAATGGCAA-3'
Angiopoietin-2	ACACACCACAAATGGCATCT	TAATTGTCCACCCGCCTC
sFLT	ACAATCAGAGGTGAGCACTG	CTGCTATCATCTCCGAACTC
FLT	ATCAGAGATCAGGAAGCACC	GGAACTTCATCTGGGTCCAT
Tie-2	TTCTGTCTCCCTGACCCCT	GTAAAAACCAGGGTGGCATG
KDR	GACTTCAACTGGGAATACCC	CATGGACCCTGACAAATGTG

Qualitative PCR was performed to examine the expression of GAPDH, VEGF, KDR, sFIt, FIt, Tie-2, Ang-1 and Ang-2 using the HotStarTaq Master Mix Kit (Quiagen, Hilden, Germany). PCR was performed using a cDNA equivalent of 0.3 µg total RNA at a final concentration of 1x HotStarTaq Master Mix (Quiagen), 10 nm sense primer and 10 nm antisense primer (MWG, Eggersheim, Germany) at a final volume of 25 µl. PCR conditions were 95°C for 15 min and 32 cycles: 95°C for 30 s, 55°C for 40 s, 72°C for 40 s. Primers are described in Table 2. PCR products were separated and visualized on a 1% agarose gel (LE Agarose, Boehringer Mannheim, Mannheim, Germany), stained with ethidium bromide. PCR products for GAPDH, VEGF and Angiopoietin-1 were purified using the QiaEx Kit from Qiagen (Hilden, Germany), quantified by optical density and used at 10-fold dilutions as standards in the quantitative PCR.

Quantitative PCR was performed in duplicates using a cDNA equivalent of 0.75 µg total RNA at a final concentration of 1 x SYBR Green Mix (PE Applied Biosystems), 300 nM forward primer and 300 nM reverse primer (Table 2). Primers for VEGF, Ang-1 and GAPDH were obtained from (MWG, Eggersheim, Germany). Primer concentration and PCR conditions were tested previously. TaqMan PCR conditions using an ABI PRISM 7700 Sequence Detector (PE Biosystems) were: 50°C for 2 min, 95°C for 10 min and in a total of 40 cycles 95°C for 15 s and 55°C for 1 min. The copy number of the mRNA were extracted from the CT (threshold cycle)/standard copy number plot. Fold change of the gene expression was calculated from the relation of the relative copy number of the mRNA of interest to that of GAPDH.

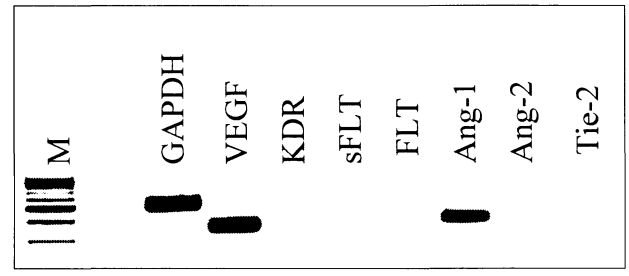

Figure 1. — Detection of VEGF mRNA and Angiopoietin-1 mRNA by RT-PCR.
Total RNA isolated from MCF-7 and T47D was amplified by RT-PCR with primers specific for GAPDH, VEGF-A family, FLT, KDR, sFLT, Tie-2 and Angiopoietin-1 and -2. The PCR products were separated on agarose gel. MCF-7 expresses VEGF and Angiopoietin-1 as demonstrated by the 140 bp and 150 bp long PCR product, respectively. M is a 100 bp ladder.

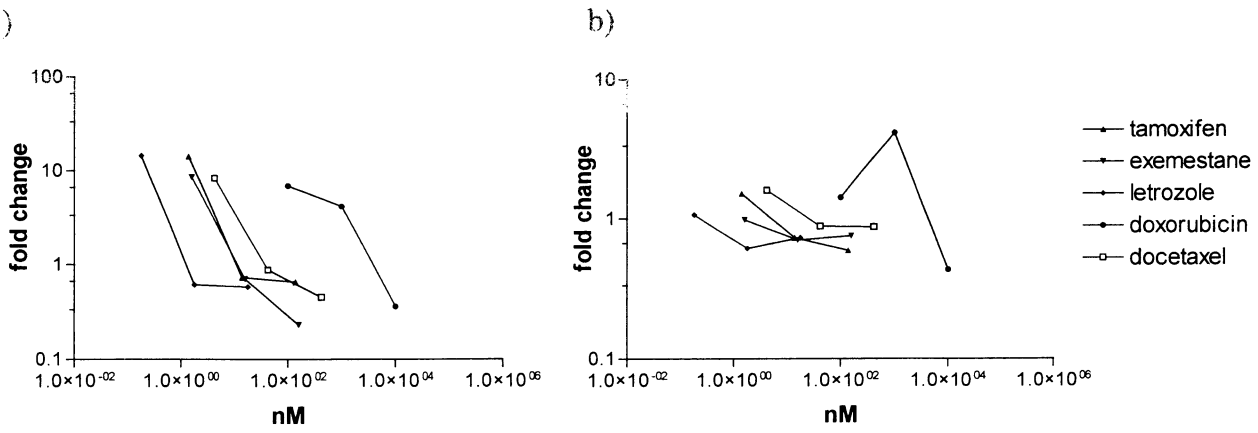

Figure 2. — Expression modulation of VEGF by chemotherapeutics.
Total mRNA from MCF-7 (A) and T47D (B) was subsequent for the quantitative RT-PCR with primers for VEGF-A and GAPDH. The copy number for mRNA encoding VEGF-A was related to the copy number of GAPDH and to the untreated culture. Shown are means and standard deviations from duplicate experiments. Low anticancer drug concentrations increased the copy numbers of VEGF, whereas high drug concentrations led to suppression of VEGF mRNA in both cells.

Enzyme-linked immunosorbent assay (ELISA)

For quantification of VEGF in cell culture superannuate we used Quantikine human VEGF immunoassay (R & D Systems Inc., Minneapolis, MN, USA). ELISA was performed according to the manufacturer's protocol. OD was determined at 450 nm with wavelength correction at 540 nm. Results were compared to the VEGF standard curve. Protein extracts from tissue (5 mg in 250 µl of 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.5 containing leupeptin (3 µg/ml), pepstatin (3 µg/ml) aprotinin (3 µg/ml) and PMSF (2 mM) were cleared by 15 min centrifugation at 12,000 x g. VEGF quantification was performed with the Quantikine assay according to the protocol for serum samples. Total protein was measured using the Bio-Rad protein assay (Munich, Germany) according to the manufacturer's protocol.

Results

Differential effect of anticancer drugs on VEGF expression

The breast cancer cells MCF-7 and T47D expressed the mRNA for VEGF-A as detected by RT-PCR (Figure 1). Exposure of these cells to various concentrations of anticancer drugs (Table 1) for 24 h led to differential modulations of VEGF-A mRNA as quantified by RT-PCR. Low doses of the used drugs increased up to 10-fold the copy number of VEGF-A mRNA in MCF-7 cells (Figure 2A) and moderately in T47D cells compared to non treated cells (Figure 2B).

In contrast, higher concentrations of the drugs suppressed the synthesis of mRNA coding for VEGF-A. A 10-fold increase of the drug concentration led to a switch in the VEGF-regulation from enhancement to suppression of the gene expression. Ten-fold more copies of VEGF-mRNA were detected in cultures of MCF-7 treated with 1.2 nM tamoxifen compared to untreated samples. In contrast, 12 nM tamoxifen inhibited up to 30% of the expression of VEGF-A. Exemestane had similar effects, however a 10-fold further increase of concentration led to further suppression of the VEGF-A copy number from 70% to 20% of the control.

The breast cancer cells MCF-7 produced VEGF protein as detected by ELISA (Figure 3). Incubation with different anticancer drugs for 24 h resulted in differential secretion of VEGF protein as quantified by ELISA. Incubation with low concentrations of all drugs was followed by an up to 10-fold increased protein concentration in the culture media of MCF-7 cells compared to untreated cells (Figure 3). A 100-fold increased concentration of tamoxifen, exemestane and letrozol did not change the VEGF-A protein concentration, whereas doxorubicin and docetaxel induced inhibition up to 24% of the control values. The responsiveness of the VEGF expression to different drugs was dose-dependent.

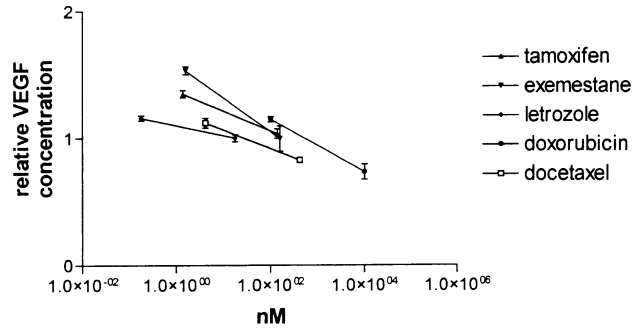

Figure 3. — Detection of VEGF protein in cultures of MCF-7 breast cancer cells.
VEGF protein was quantified in supernates of cell cultures 24 h after drug treatment using ELISA and shown as a relative concentration compared to untreated cell cultures. The VEGF protein showed a dose-dependent regulation due to different anticancer drug concentrations.

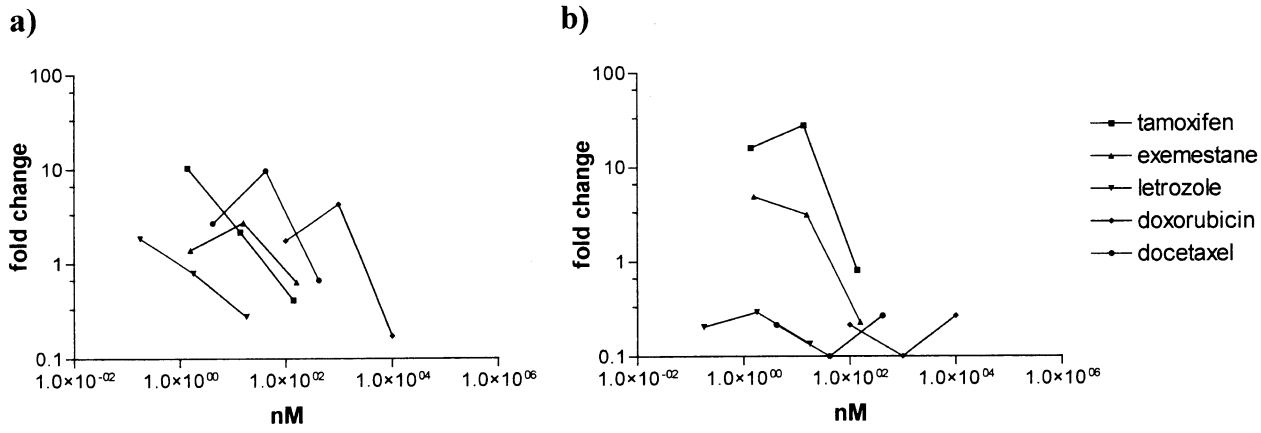

Figure 4. — Angiopoietin-1 expression in MCF-7 and T47D cells treated with chemotherapeutic drugs.
Total mRNA from MCF-7 (A) and T47D (B) was subsequent for the quantitative RT-PCR with primers for Angiopoietin-1 and GAPDH. The copy number for mRNA encoding Angiopoietin-1 was related to the copy number of GAPDH and to the untreated culture. Shown are means and standard deviations from duplicate experiments. Angiopoietin-1 mRNA was enhanced after low anticancer drug concentrations in MCF-7 cells. High drug concentrations resulted in suppression of Angiopoietin-1 expression in both cells. In T47D cells, low drug concentrations led to increased copy number in 2/5 anticancer drugs.

Differential effect of anticancer drugs on Angiopoietin-1 expression

The breast cancer cells MCF-7 and T47D expressed the mRNA for Angiopoietin-1 as detected by RT-PCR. Exposure of MCF-7 cells to various concentrations of anticancer drugs for 24 h led to differential modulation of Angiopoietin-1 mRNA as quantified by RT-PCR (Figure 4A). Low doses of the used drugs increased up to 10-fold the copy number of Angiopoietin-1 mRNA in MCF-7 cells. As has been seen on VEGF mRNA, a 10-fold drug concentration switched gene regulation from enhancement to a marked decrease of Angiopoietin-1 expression; 1.2 nM tamoxifen incubation led to a 10-fold increased copy number of Angiopoietin-1, whereas 12 nM tamoxifen decreased Angiopoietin-1 expression up to 70% compared to untreated cells; 1 µM doxorubicin led to a 6-fold increase of Angiopoietin-1 copy number, whereas 10 µM doxorubicin suppressed Angiopoietin-1 mRNA up to 80%. In T47D cells (Figure 4B), a dose-dependent switch in Angiopoietin-1 mRNA was observed only for tamoxifen and exemestane, whereas the other three drugs decreased Angiopoietin-1 expression irregardless of drug concentration; 1.4 nM tamoxifen led to an increased Angiopoietin-1 mRNA of 100% compared to a 10% suppression of Angiopoietin-1 expression after 140 nM tamoxifen; 10 µM doxorubicin and 420 nM docetaxel decreased the copy number of Angiopoietin-1 up to 100%.

Discussion

The present results achieved in an in vitro study of common breast cancer cell lines demonstrate that there is a dose-dependent modulation of angiogenic factors by different anticancer drugs. High drug concentrations led to decreased VEGF and Angiopoietin-1 in MCF-7 and T47D. In contrast, low concentrations of drugs resulted in an increased expression of both factors. To our knowledge, this is the first report about the interactions between Angiopoietin-1 and the different anticancer drugs.

Modulation of VEGF-A and Angiopoietin-1 gene expression were almost identical for all drugs, independent of their specific mechanism of action. Letrozol, doxorubicin and dexotaxel induced in this study a suppression of Angiopetin-1 irregardless of drug concentration. Furthermore, suppression of Angiopetin-1 was achieved only with a 10-fold drug concentration, but the extent was higher in comparison to VEGF. These findings suggest that there may be a similar mechanism in the regulation of both angiogenic factors.

Previous studies of different drugs in vivo and in vitro described changes in VEGF expression [10-12]. In patients with metastatic breast cancer receiving taxol chemotherapy, a decrease of VEGF has been observed [16]. Our study demonstrated that low concentrations of drugs increased VEGF, whereas high drug concentrations reduced VEGF expression in MCF-7 and T47D cells. Modulation of VEGF-A gene expression was identical for all drugs, independent of their specific mechanism of action. Low concentrations of anticancer drugs might modulate different cytokines and growth factors, which in turn induce an up-regulation of VEGF genes, as observed in our study. The factors which are involved in the dose-dependent switch of VEGF regulation remain to be determined.

Many different growth factors and cytokines like bFGF, TGF-ß, metalloproteinase-9, HER2, interleukin-18 are known to be involved in angiogenesis, including in breast carcinoma [overview in 18-21]. Cytokine secretion induced by paclitaxel has been described in human breast cancer cell lines [22]. Our data are in accordance with the report of increased VEGF mRNA observed at a 5 µM concentration of tamoxifen and toremifene in MCF-7 cells [23] although this concentration is known to inhibit cell

proliferation in MCF-7 cells [24]. In addition, tamoxifen and the antioestrogen toremifene did not inhibit an estrogen-induced increase of VEGF mRNA expression [23].

Of all the substances used in our investigation, most data about the influence of angiogenic factors are available for tamoxifen. Tamoxifen is known to have a number of estrogen-independent effects [25], including influencing the cytokine switch involved in the regulation of angiogenesis. It has been reported that two weeks of tamoxifen decreased preoperative serum VEGF by 30% in patients with breast cancer [26]. Tamoxifen directly reduced proliferation of a VEGF-dependent endothelial cell line in vitro. In vivo, orally administered tamoxifen reduced VEGF-mediated angiogenesis in rats [10]. In vitro studies have shown a dose-dependent effect: at nanomolar concentrations of tamoxifen, only growth arrest occurred whereas at micromolar concentrations, induction of cell death was observed in cell cultures.

This observation underlines the importance of drug concentration, as also seen in our experiments. To what extent tissue estrogen concentrations are involved in the VEGF switch, remains to be determined, since both aromatase inhibitors and both chemotherapeutics showed a similar modulation of VEGF like tamoxifen. Aromatase inhibitor anastrozole caused a significant suppression of tissue estrogen levels in neoadjuvant treated breast cancer patients [27]. Although VEGF is influenced by estrogen, probably by direct transcription of the VEGF gene [9], it has no crucial role for the observed VEGF modulation. Other factors like hypoxia, one of the probably most important stimuli for VEGF, may also influence VEGF expression, since a synergistic action of ß-estradiol and hypoxia on VEGF induction in MCF-7 and MCF-5C cells has been reported [17]. In addition, the VEGF gene is induced by tamoxifen as well as by estrogen in rat uteri [28]. No data about direct gene activation by aromatase inhibitors is available.

Hormone-independent drugs like taxane also interfere with angiogenesis. In experimental conditions it has been shown that docetaxel inhibits VEGF-induced angiogenesis at tumor sites [12]. Paclitaxel, a member of the taxane family, acted anti-angiogenically in transgene highly vascularized mouse breast cancer cells (Met-1) with a synchronous reduced expression of VEGF [11]. Experiments on mouse cell cultures showed a reduced de novo vascularization after docetaxel [29]. In metastatic breast cancer patients, a decline of serum VEGF in response to taxol therapy was associated at least with disease stabilization [16]. Our data confirm the angiogenic action of docetaxel, although suppression of VEGF was observed only with higher drug concentrations in vitro.

Angiopoietin-1 is another important regulator of angiogenesis. Different drugs resulted in a dose-dependent expression of the Angiopoietin-1 gene in MCF-7 cells, similar to the observed modulation on VEGF mRNA. In comparison to VEGF, suppression of Angiopoietin-1 was achieved only with a 10-fold drug concentration. This observed suppression occurred to a higher extent than that observed on VEGF. Both angiogenic factors are obviously regulated in a common pathway. To our knowledge this is the first report about the effect of chemoendocrine drugs on Angiopoietin-1 expression. In a study of 21 breast carcinomas, only three tumors expressed Angiopoietin-1, but none of the normal breast tissues [6]. Another investigation described a significantly reduced expression of Angiopoietin-1 in tumors compared with normal samples [30].

Our in vitro data suggest that low drug concentrations may support tumor growth due to enhanced angiogenesis instead of inhibiting further enlargement and metastasizing. Our results support the concept of sequential small doses of a drug instead of single high doses. Experimental data suggest that repeated low doses of chemotherapeutic agents might induce a stronger anti-angiogenic effect than single high doses [overview in 16]. In this study the drug concentration was for a long term, higher than required for VEGF and Angiopoietin-1 induction. A reduction in microvessel density was also observed after neoadjuvant chemoendocrine therapy in primary breast carcinomas [13]. The observed suppression of VEGF and Angiopoietin-1 in vitro supports the hypothesis of a direct effect on angiogenesis in contrast to a secondary effect on tumor regression. Because VEGF not only stimulates angiogenesis but also has immunosuppressive effects [31], knowledge about drug concentration-dependent angiogenesis is of clinical importance. Regulation of the endocrine therapeutics on VEGF is especially interesting, since tamoxifen is increasingly used as a cancer chemopreventive agent in high-risk women [32].

In conclusion, we were able to demonstrate that there is a dose-dependent modulation of the angiogenic-poetic factors by the different drugs. Chemo-endocrine substances, which are used in preoperative treatment of breast cancer, can regulate the expression of VEGF and Angiopoietin-1 in breast cells MCF-7 and T47D. High doses of drug concentrations were able to induce suppression of both factors, whereas low drug concentrations were followed by an increased expression of VEGF and Angiopoietin-1. Nevertheless more investigations are necessary to understand the mechanism of angiogenesis and especially for Angiopoietin-1 and maybe it will be possible to find an answer to the conflicting results.

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