Linköping University Post Print

Tamoxifen decreases extracellular TGF-beta 1 secreted from breast cancer cells - A post-translational regulation involving matrix metalloproteinase activity

Ulrika Nilsson, Jill A Jönsson and Charlotta Dabrosin

N.B.: When citing this work, cite the original article.

Original Publication:

Ulrika Nilsson, Jill A Jönsson and Charlotta Dabrosin, Tamoxifen decreases extracellular TGF-beta 1 secreted from breast cancer cells - A post-translational regulation involving matrix metalloproteinase activity, 2009, EXPERIMENTAL CELL RESEARCH, (315), 1, 1-9.

<http://dx.doi.org/10.1016/j.yexcr.2008.10.015>

Copyright: Elsevier Science B.V., Amsterdam

<http://www.elsevier.com/>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-16417>

Tamoxifen decreases extracellular TGF- β 1 secreted from breast cancer cells – a post-translational regulation involving matrix metalloproteinase activity

Ulrika W Nilsson, Jill A Jönsson, and Charlotta Dabrosin*

Linköping University, Department of Clinical and Experimental Medicine, Division of Oncology, SE-581 85 Linköping, Sweden

Running title: Tamoxifen decreases extracellular TGF- β 1 in breast cancer

*Correspondence to:

Charlotta Dabrosin MD, PhD

Division of Oncology,

University Hospital,

SE-581 85 Linköping,

Sweden

e-mail: chada@ibk.liu.se

Phone: +46 13 22 8595

Fax: +46 13 22 3090

Abstract

Transforming growth factor- β 1 (TGF- β 1) promotes cancer progression by regulating tumor cell growth and angiogenesis and high levels of TGF- β 1 have been associated with metastatic disease and poor prognosis in breast cancer patients. We have previously reported anti-angiogenic effects of the anti-estrogen tamoxifen in breast cancer, by increased matrix metalloproteinase-9 (MMP-9) activity and generation of endostatin. Here, we show that exposure of tamoxifen to ER-positive breast cancer cells for 7 days, decreased extracellular TGF- β 1. Intracellular TGF- β 1 levels were unaffected by tamoxifen treatment, indicating a post-translational regulation of TGF- β 1. Inhibition of MMP activity restored TGF- β 1 levels, suggesting an involvement of MMP activities in the down-regulation of TGF- β 1 by tamoxifen. Moreover, using an *in vivo* model of solid MCF-7 tumors in nude mice, we analyzed tumor levels of TGF- β 1 after *in vivo* treatment with estradiol and tamoxifen. Exposure of tumor-bearing mice to tamoxifen significantly decreased tumor TGF- β 1 protein levels, tumor growth and angiogenesis. In conclusion, our findings suggest a novel mechanism of action of tamoxifen in breast cancer via sex steroid dependent modulation of the proteolytic tumor microenvironment resulting in reduced extracellular TGF- β 1 levels.

Keywords: sex steroids, breast cancer, TGF- β 1, MMP, angiogenesis, nude mice

Introduction

Tumor progression is dependent on a number of cellular events, including cell proliferation, migration, resistance to host immune defense, and angiogenesis.

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional cytokine involved in the regulation of almost every aspect of cellular behavior, such as cell proliferation, differentiation, and migration [1, 2]. TGF- β 1 has also been widely implicated in the regulation of cancer; initially as a tumor suppressor and at later stages as a promoter of tumor progression [3-5]. Excess TGF- β 1 produced by tumor cells is suggested to act on the host to suppress immune surveillance, increase angiogenesis, and promote tumor cell survival and metastasis [6-9]. In breast cancer patients, an increased expression of TGF- β 1 has been shown to correlate with metastatic disease and poor prognostic outcome [10-12]. Moreover, TGF- β 1 activity may prime primary breast tumors for lung metastasis seeding [13].

Proteolytic remodeling of the extracellular environment is pivotal for the invasion and migration of cancer cells into the stroma. Matrix metalloproteinases (MMPs) play an important role in this remodeling and in the regulation of bioavailability of several growth factors, cytokines, and adhesion factors [14-16]. Depending on tissue distribution and expression levels of individual MMPs and endogenous inhibitors of their activity, these enzymes may either promote or inhibit tumor progression. Lately it has been shown that up-regulation of endogenous MMPs decrease angiogenesis in tumors by the release of matrix-associated anti-angiogenic factors such as angiostatin, tumstatin, and endostatin [17-21]. MMPs have been associated with a positive regulation of TGF- β activity and cell responsiveness to TGF- β [22]. However, it has been demonstrated that only cell surface-localized and not soluble extracellular MMP-2

and MMP-9 activity have the ability to promote tumor invasion and angiogenesis and to activate TGF- β s [23]. To our knowledge, it is unknown if also soluble extracellular MMP activity affects TGF- β 1 levels.

Sex steroids play an important role in breast carcinogenesis and progression and the majority of breast cancers are hormone dependent. Anti-estrogens, such as the synthetic, non-steroidal compound tamoxifen, and aromatase inhibitors, are cornerstones in the medical treatment of breast cancer. We have previously reported that tamoxifen treatment of experimental breast cancer, both *in vitro* and *in vivo*, induces an anti-angiogenic response [20, 24-26]. We recently showed that tamoxifen significantly up-regulated the expression and activity of MMP-9, whereas estradiol treatment decreased MMP-9 activity levels [20]. Moreover, over-expression of MMP-9 in solid MCF-7 tumors *in vivo* induced tumor regression and the release of extracellular endostatin [27].

In this paper, we investigated if the increased levels and activity of extracellular, soluble MMP-9 induced by tamoxifen, affected TGF- β 1 levels. We show that long-term tamoxifen treatment decreased the secreted levels of TGF- β 1 whereas estradiol increased these levels. *In vivo*, in solid breast cancer explants in mice, tamoxifen treatment significantly decreased TGF- β 1 protein levels and decreased angiogenesis. Moreover, *in vitro* experiments showed that inhibition of MMP-9 restored the TGF- β 1 levels, suggesting that MMP-9 activity is involved in the negative regulation of tumor TGF- β 1 by tamoxifen.

Materials and methods

Cell culture

MCF-7 (HTB-22; human breast adenocarcinoma, ER+ and PR+) cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in DMEM without phenol red supplemented with 2 mM glutamine, 50 IU/ml penicillin-G, 50 µg/ml streptomycin, and 10% fetal bovine serum, at 37°C in a humidified atmosphere of 5 % CO₂. Cell culture medium and additives were obtained from Invitrogen (Carlsbad, CA, USA) if not otherwise stated.

Hormone treatment of MCF-7 cells in culture

Confluent cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Costar, Cambridge, MA, USA), 2×10^4 cells/cm². Cells were incubated in the DMEM based culture medium for 24 hours and then treated with or without 1×10^{-8} M estrogen (17β-estradiol, Sigma, St. Louis, MO, USA), 1×10^{-6} M tamoxifen (Sigma), or a combination of estrogen and tamoxifen for 24 hours or 7 days. Hormones were added in serum-free medium (hormone medium) consisting of DMEM/F-12 (1:1) without phenol red, supplemented with 10 µg/ml transferrin (Sigma), 1 µg/ml insulin (Sigma), and 0.2 mg/ml BSA (Sigma). The hormone medium was changed every 24 hours. Control cells were incubated with hormone medium supplemented with vehicle, ethanol, and equivalent to the hormone treated groups (0.001%). After hormone treatment, cells and conditioned medium were harvested.

Total protein content was determined using Bio-Rad Protein Assay with bovine serum albumin as standard (Bio-Rad Laboratories, Stockholm, Sweden). Samples were stored at -70°C until subsequent analyses.

MCF-7 tumor explants in mice

Female athymic nude mice (6–8-weeks-old) were purchased from Taconic M&B (Ry, Denmark). They were housed in a pathogen free isolation facility with a light/dark cycle of 12/12 h and fed with rodent chow and water *ad libitum*. All animal work was approved by the Linköping University animal ethics research board. Mice were anesthetized with i.p. injections of ketamine/xylazine (Apoteket, Linköping, Sweden), ovariectomized and 3-mm pellets containing 17β -estradiol, 0.18mg/60-day release (Innovative Research of America, Sarasota, Florida, USA) were implanted subcutaneously in the animal's back 7 days before tumor induction. The pellets provide a continuous release of estradiol at serum concentrations of 150–250 pM, which is in the range of physiologic levels seen in mice during the estrous cycle. One week after surgery, MCF-7 cells (5×10^6 cells in 200 μ l PBS) were injected s.c. on the right hind side flank. Tumor volume was monitored by measuring length, width, and depth, of the tumor every 4 days using a caliper. At a tumor size of approximately 300 mm³ the mice were divided into two subgroups. One group continued with the estradiol treatment only ($n=12$), while two weeks of tamoxifen treatment (1 mg/every 2 days s.c.) was added to the estradiol treatment in the other group ($n=10$).

At the end of experiments, the mice were sacrificed and the tumors excised. The removed tumors were weighed, formalin-fixed and subsequently embedded in paraffin for immunohistochemical analysis, or snap frozen in liquid nitrogen and stored at -70°C until use.

Frozen tumor tissue was placed in 500 μ l ice-cold PBS and immediately homogenized for 30 s on ice at 24000 rpm by using a Polytron PT3000 (Kinematica AG, Littau, Switzerland). The tumor tissue homogenates was centrifuged at 11000 rpm for 5

minutes in an Eppendorf 5417R microcentrifuge (Eppendorf, Hamburg, Germany), and the supernatant was collected and stored in aliquots at -70°C Bio-Rad Protein Assay with bovine serum albumin as standard (Bio-Rad Laboratories, Stockholm, Sweden).

Quantification of TGF- β 1 and MMP-9

Supernatant from tumor tissue homogenates, as well as cell lysate and conditioned medium from hormone-treated MCF-7 cells, were analyzed for TGF- β 1 and MMP-9 proteins using commercial quantitative immunoassay kits (human TGF- β 1 Quantikine®, total human MMP-9 Quantikine®, R&D Systems, Minneapolis, MN, USA) without preparation. According to the manufacturers, the sensitivities of the assays were for the TGF- β 1 assay 4.61 pg/ml and <0.156 ng/ml for the MMP-9 assay, and the precision of the ELISA kits was confirmed during the experiments. The total MMP-9 assay measures both the active form and pro-form of human MMP-9. Assays were conducted according to manufacturer's guidelines and repeated on more than one cell harvest. In the TGF- β 1 assay, samples were analyzed both with and without acid activation. Acid activation was done by incubating 100 μ l of each sample to be assayed was incubated with 20 μ l of 1 N HCl for 10 minutes at room temperature to activate latent TGF- β 1, and thereafter neutralized by adding 20 μ l of 1.2 N NaOH/0.5 M HEPES. Optical density (OD) was measured using a VersaMax microplate reader (Molecular Devices, CA, USA) at 450 nm. TGF- β 1 and MMP-9 concentrations were correlated to total protein content in cell lysates.

Quantification of MMP-9 activity in vitro

The activity of MMP-9 was assayed using a gelatinase activity assay. Conditioned medium from hormone-treated MCF-7 cells was mixed 50:50 with 100 μ M of a quenched fluorogenic substrate specific for MMP-9 and MMP-2 (DNP-Pro-Leu-Gly-

Met-Trp-Ser-Arg-OH; Calbiochem, Merck Biosciences Ltd., Nottingham, UK), in a dark 96-well plate. The mixture was incubated at room temperature for 20 minutes, with gentle agitation. Fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., CA, USA), with λ_{ex} at 280 nm and λ_{em} 360 nm. All experiments were performed in a low-light or light-free environment. MMP-2/MMP-9 activity was expressed as relative fluorescence units (RFU)/mg total protein.

Inhibition of MMP-9

MCF-7 cells were treated with 1×10^{-6} M tamoxifen for 7 days in hormone medium. Hormone medium was changed every day. During the last 24 hours of hormone treatment, a synthetic inhibitor of MMP-2 and MMP-9 (MMP-2/MMP-9 Inhibitor II; 50 μM ; Calbiochem, Merck Biosciences Ltd., Nottingham, UK) or a specific mouse anti-human-MMP-9 antibody, 0.5 $\mu\text{g}/\text{ml}$ or 5 $\mu\text{g}/\text{ml}$ (Calbiochem, Merck Biosciences Ltd., Nottingham, UK) were added. Cells treated with tamoxifen in combination with vehicle (DMSO) or isotype murine IgG (R&D Systems, Minneapolis, MN, USA), served as control cells.

Immunohistochemistry of TGF- β 1 and MMP-9

Formalin-fixed, paraffin embedded tumor tissue biopsies were cut in 5 μm sections, deparaffinized and subjected to anti-TGF- β 1 and anti-MMP-9 immunohistochemistry (mouse anti-TGF- β 1, dilution 1:20, R&D Systems and rabbit anti-human MMP-9, dilution 1:50, Chemicon Int. Inc. CA, USA with HRP-DAB detection, R&D Systems). Sections were counterstained with Mayer's hematoxylin and coverslipped in Mountex. Negative controls (primary antibody omitted) did not show staining. For quantification of the staining all sections were first scanned to identify the range of intensity of the

staining. In a blinded manner ten high power fields (x200) per section were examined and scored either as weakly or strongly positive.

Quantification of tumor microvessel area

Formalin-fixed, paraffin-embedded tumors were cut in 3- μ m sections, deparaffinized, and subjected to anti-von Willebrand's factor (rabbit anti-human von Willebrand; dilution 1:1000; with Envision detection, DakoCytomation). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high power fields (x200) were examined by section of three different tumors in each group. Vessel quantification of tumor sections was conducted as described previously using a Nikon microscope equipped with a digital camera [28]. Percentage of area stained positively for von Willebrand's factor was assessed using Easy Image Measurement software (Bergstrom Instruments). Tumor sections were also subjected to H&E staining.

Statistical analysis. Statistical analysis was performed using SPSS software. One-way ANOVA, Fisher's exact test, and Student's *t*-test were used where appropriate. All statistical tests were two-sided. Results are expressed as mean $\pm$ SEM. Statistical significance was assumed at *P* values less than 0.05.

Results

Long-term treatment with tamoxifen decreased TGF- β 1 protein expression in conditioned media from cultured human breast cancer cells, but did not alter the intracellular levels

To examine the effects of estradiol and/or tamoxifen TGF- β 1 expression in breast tumor cells, hormone-responsive MCF-7 cells in culture were treated with estradiol and/or tamoxifen, or a combination of estradiol and tamoxifen, for 24 and 72 hours and 7 days respectively. The levels of TGF- β 1 protein secreted from the MCF-7 cells were quantified. As previously shown by others, we found that 24 hours of tamoxifen treatment gave a significant increase in TGF- β 1 protein levels in conditioned media [$P < 0.001$, as compared to control] (Fig. 1A), whereas estradiol or a combination of estradiol and tamoxifen had no significant effect. At 72 hours there were no differences between the groups (Fig. 1B). However, short term exposure does not represent clinical treatment with tamoxifen; therefore, we chose to expose MCF-7 cells to tamoxifen for a longer period of time, 7 days. Due to the growth inhibitory/ cytotoxic effects of tamoxifen, one week is the maximum time length for which it is possible to treat the tumor cells *in vitro*. Contrary to the short-term treatment, we found that treating MCF-7 cells with tamoxifen for 7 days significantly decreased TGF- β 1 protein levels in conditioned media, compared to control [$P < 0.001$] (Fig. 1C). Exposure to estradiol for 7 days induced a significant increase of TGF- β 1 [$P < 0.001$] (Fig. 1C). Also, adding tamoxifen to the estradiol treatment triggered a significant decrease in TGF- β 1 levels compared to treating cells with estradiol only [$P < 0.001$] (Fig. 1C). At 7 days TGF- β 1 was also analyzed without acid activation of the samples to distinguish between endogenous active and latent forms. Using this approach, very low levels of TGF- β 1

Figure 1. *Estradiol and tamoxifen regulate the extracellular levels of TGF-β1 protein secreted from cultured human breast cancer cells.* Human ER-positive MCF-7 breast cancer cells were cultured without hormones (*Control*) or in the presence of estradiol (*E2*; 10^{-8} M), tamoxifen (*Tam*; 10^{-6} M), or a combination of estradiol and tamoxifen (*E2+Tam*) for 24 hours or 7 days. Tamoxifen increased TGF-β1 levels compared to control after 24 hours, but following 7 days of treatment the levels secreted into the medium were significantly decreased. Estradiol induced a significant increase in TGF-β1 levels after one week. The intracellular levels of TGF-β1 were unaltered by treatments. (A) TGF-β1 protein levels in conditioned medium from MCF-7 cells after 24 hours of treatment. (B) TGF-β1 protein levels in conditioned medium from MCF-7 cells after 72 hours of treatment. (C) TGF-β1 protein levels in conditioned medium from MCF-7 cells after 7 days of treatment. (D) Intracellular levels of TGF-β1 protein in MCF-7 cells after 7 days of treatment. Error bars represent S.E.M.; ns, non-significant.

were detected without any differences between the groups; control group 14.13 ± 0.68 pg/mg protein, estradiol exposed group 12.7 ± 1.1 pg/mg protein, tamoxifen group 16.53 ± 1.0 pg/mg protein, and estradiol+tamoxifen 14.8 ± 1.5 pg/mg protein. This suggests that the major part of the extracellular TGF- β 1 secreted from MCF-7 cells is in latent form.

Further, to investigate if tamoxifen also affected the intracellular levels of TGF- β 1 protein after 7 days of exposure, we analyzed whole-cell lysates. As shown in Fig. 1D, we observed that the intracellular levels were not significantly altered following 7 days of treatment with neither tamoxifen nor estradiol, as compared to control.

Inhibiting extracellular MMP activity restored the tamoxifen-induced decrease in TGF- β 1

We have previously reported that prolonged tamoxifen treatment up-regulates MMP-9 expression in cultured MCF-7 cells *in vitro* and in solid MCF-7 tumors in nude mice [20, 26]. To further investigate if these previous findings of increased MMP-9 levels by tamoxifen were involved in TGF- β 1 regulation, experiments with this treatment were set-up. We confirm in the present paper that tamoxifen increased the extracellular protein levels of MMP-9 after 7 days [$P < 0.001$, compared to control] (Fig. 2C). In addition, Fig. 2A shows that tamoxifen altered the levels also after 24 hours, however the changes were not as profound as after one week [$P < 0.05$, compared to control] (Fig. 2A).

MMPs are tightly regulated at the post-transcriptional and/or the post-translational level, and it is therefore imperative to investigate these proteases in the extracellular space where they are biologically active. In this regard, we have previously shown tamoxifen may also alter the extracellular levels of natural inhibitors of MMPs [26].

Figure 2. Tamoxifen increases MMP-9 protein levels and activity.

Human ER-positive MCF-7 breast cancer cells were cultured with (*Tam*) or without (*Control*) tamoxifen for 24 hours or 7 days. 24 hours of tamoxifen treatment induced the extracellular levels but not the activity of MMP-9, whereas 7 days of tamoxifen significantly increased both extracellular protein levels and activity of MMP-9. (A) MMP-9 protein levels in conditioned medium from MCF-7 cells after 24 hours of treatment. (B) extracellular MMP-9 activity after 24 hours of treatment. (C) MMP-9 protein levels in conditioned medium from MCF-7 cells after 7 days of treatment. (D) extracellular MMP-9 activity after 7 days of treatment. Error bars represent S.E.M.; ns, non-significant.

Therefore, we verified if the altered MMP-9 protein levels also affected the extracellular physiological MMP activity using a substrate activity assay. Our results show that although tamoxifen affects the protein levels of MMP-9 after short-term treatment of 24 hours, this did not lead to an increase in extracellular activity of MMP-9 (Fig. 2B). On the other hand, confirming what we have previously shown, 7 days of treatment with tamoxifen caused a significant increase in activity [$P<0.001$, compared to control] (Fig. 2D). It should be stated that the fluorogenic substrate used in the activity assay may also be cleaved by MMP-2; however, as we have showed previously, the induced levels of active MMP-2 in MCF-7 cells are considerably lower than the levels of active MMP-9 [26].

Given the significant effect on the extracellular activity of MMP-9 and extracellular TGF- β 1 levels after one week of tamoxifen treatment, we thereafter tested the hypothesis of MMP-9 activity being involved in the down-regulation of TGF- β 1 by tamoxifen. MCF-7 cells were treated with tamoxifen in combination a synthetic inhibitor of MMP-9 (control cells were incubated with vehicle only) and thereafter measured TGF- β 1 in conditioned media. Indeed, as Fig. 3 shows, we detected a significant increase in TGF- β 1 protein levels in conditioned media following MMP-9 inhibition [$P<0.01$, as compared to control] (Fig. 3). To mimic the treatment situation in our *in vivo* model, cells were also treated with estradiol+tamoxifen together with the MMP-inhibitor. Inhibiting MMP-activity in this treatment combination also restored the TGF- β 1 levels in a similar fashion as in the tamoxifen exposed group; from 918 ± 78 pg/mg protein in the control group versus 1511 ± 151 pg/mg protein in the hormone exposed group, $P<0.01$. To rule out un-specific effects such as inhibition of other

Figure 3. *Inhibiting extracellular MMP-9 activity restores the tamoxifen-induced decrease of TGF-β1.*

Human ER-positive MCF-7 breast cancer cells were cultured in the presence of tamoxifen (*Tam*; 10^{-6} M) for 7 days. The last 24 hours of hormone treatment a synthetic MMP-9 inhibitor (*inhib*) was added in the concentration of 50 μ M or a specific anti-MMP-9 antibody was added at 0.5 and 5 μ g/ml. Cells treated with tamoxifen in combination with vehicle or isotype IgG served as control cells. In all treatment groups, TGF-β1 levels were increased by the addition of the various MMP-9 inhibitors. Error bars represent S.E.M.

MMPs or ADAMs by the synthetic inhibitor we confirmed our results using a neutralizing specific MMP-9 antibody, which inhibits the enzymatic activity of MMP-9. In a similar manner as the synthetic inhibitor the specific anti-MMP-9 at two different concentrations also restored the TGF-β1 levels significantly compared to control cells exposed to isotype IgG, Fig. 3.

Tamoxifen treatment decreased the levels of TGF- β 1 and increased the levels of MMP-9 in solid MCF-7 tumors growing in nude mice

To further explore the effects of tamoxifen on TGF- β 1, we extended our investigations to an *in vivo* model of solid MCF-7 tumors growing in nude mice. These tumors require estrogen for growth in nude mice; therefore, a non-treated control group is not possible to achieve *in vivo*. Moreover, maintaining the mice with a physiologic level of estradiol during tamoxifen treatment mimics a clinical situation in premenopausal women.

Following 2 weeks of treatment, tumors in the estradiol+tamoxifen group were significantly smaller than those in the estradiol group, where tumor volume kept increasing. After 2 weeks the animals were sacrificed and the tumors excised. Tumor tissue homogenates were analyzed for TGF- β 1 protein using quantitative ELISA. As Fig. 4A shows, there were significantly lower levels of TGF- β 1 protein in tumors of estradiol+tamoxifen treated animals than in animals treated with estradiol only [$P<0.01$]. Immunohistochemical staining of tumor tissue sections confirmed the presence of human, i.e. tumor cell-derived, TGF- β 1 in both treatment groups. A higher intensity of staining was observed in the estradiol treated tumors compared to the tumors derived from the estradiol+tamoxifen treated animals. This tumor model mainly consists of cancer cells and very little stroma cells; hence, staining mostly represents tumor cell TGF- β 1. In tumors from estradiol treated animals, 34 of 40 sections were scored as strongly positive, whereas 16 of 40 were strongly positive in the estradiol+tamoxifen group [$P<0.001$]. Representative sections are shown in Fig. 4B. Anti-MMP-9 staining of tumor section revealed increased levels of MMP-9 in estradiol+tamoxifen treated tumors where 31 of 40 areas were scored strongly positive

Figure 4. *Tamoxifen decreases TGF-β1 protein levels in solid MCF-7 tumors.*

Mice were oophorectomized and supplemented with a physiologic level of estradiol. Human ER-positive MCF-7 breast cancer cells were injected s.c., and tumors were formed on the right hind flank. One group of mice continued with estradiol only (*E2*), and in the other group tamoxifen treatment was added to the estradiol treatment (*E2+Tam*). (A) TGF-β1 protein levels in MCF-7 tumor tissue homogenates. Tamoxifen treatment significantly decreased TGF-β1 protein levels. Error bars represent S.E.M. (B) The immunohistochemical staining was quantified as described in the materials and methods section. In tumors from estradiol treated animals, 34 of 40 areas were scored as strongly positive, whereas 16 of 40 were strongly positive in the estradiol+tamoxifen group [$P < 0.001$]. Representative immunohistochemical TGF-β1 stained tumor sections from each treatment group.

for anti-MMP-9 staining compared to 5 of 40 areas in the estradiol alone group, [$P<0.001$], Fig. 5.

Figure 5. *Tamoxifen increased MMP-9 protein levels in solid MCF-7 tumors.* Mice were treated as described in Figure 4. Immunohistochemistry of MMP-9 was performed and quantified as described in the materials and methods section. In tumors from estradiol treated animals, 5 of 40 areas were scored as strongly positive, whereas 35 of 40 were strongly positive in the estradiol+tamoxifen group [$P<0.001$]. Representative immunohistochemical MMP-9 stained tumor sections from each treatment group.

Tamoxifen-treated tumors with low TGF- β 1 levels exhibited decreased tumor angiogenesis

To examine if tamoxifen-treated tumors, exhibiting low TGF- β levels, affected tumor angiogenesis we quantified vessel area stained with anti-von Willebrand's factor. We found that the vessel area was significantly lower on tumors treated with a combination of estradiol and tamoxifen, compared with estradiol treatment only in which TGF- β 1 levels were higher [$1.13 \pm 0.08\%$ of total area *versus* $3.6 \pm 0.45\%$; $P<0.001$], Fig 6.

Tumor sections did not reveal any necrotic areas upon H&E staining.

Figure 6. *Tamoxifen decreased microvessel area of solid MCF-7 tumors.*

Mice were treated as described in Figure 4. Tumor sections were stained with anti-von Willenbrand's factor and vessel area was counted on tumor sections. Representative sections from estradiol (E2) and estradiol+tamoxifen (E2+Tam) exposed tumors. Tumor vessel area quantification was conducted as described in the materials and methods section. Error bars represent S.E.M.

Discussion

In this study we show that the anti-estrogen tamoxifen, alone or in combination with estradiol, negatively regulate extracellular TGF- β 1 protein levels and extracellular MMP-9 activity in ER+ and PR+ human breast cancer cells (MCF-7). The intracellular protein levels of TGF- β 1 were unaffected by tamoxifen treatment, indicating a post-translational regulation of TGF- β 1. By inhibiting the activity of MMP-9 the extracellular TGF- β 1 levels were restored, suggesting that the down-regulation of TGF- β 1 by tamoxifen is mediated, at least in part, by extracellular MMP activities. Our *in vitro* findings were verified *in vivo* in nude mice bearing MCF-7 tumor cell explants, where tamoxifen treatment in combination with a physiological level of estradiol decreased TGF- β 1 levels compared with estradiol treatment only. Moreover, this was associated with reduced tumor growth as well as significantly decreased angiogenesis.

In addition to the inhibitory effects on proliferation/apoptosis, tamoxifen may also contribute to suppressing tumor growth by decreasing angiogenesis in breast cancer. We have shown in previous studies that tamoxifen decrease pro-angiogenic factors like VEGF and increase anti-angiogenic factors such as endostatin [20, 24]. It has been proposed that tamoxifen action in breast cancer may be mediated in part by regulation of transforming growth factor beta (TGF-beta) isoforms in tumor tissue; however, the pattern of this regulation are yet not clearly understood. TGF- β 1 functions as a tumor promoter by affecting both tumor cell survival and tumor angiogenesis [6, 9], and an increased expression of TGF- β 1 is often seen in tumors of breast cancer patients, which may correlate with poor prognostic outcome [12]. The levels of TGF- β in breast cancer patients treated with tamoxifen have been investigated in several previous studies [29-32]; however, most investigations do not provide strong evidence for a generalized

regulation of TGF- β 1 protein expression by tamoxifen in breast cancer patients. This may be attributable to the heterogeneity of human breast tumors, which are composed of a variety of cell types with distinct morphologies and behavior, and each with their associated clinical outcome. Furthermore, most studies report measurements of total tumor TGF- β mRNA levels and not of the extracellular pool of TGF- β protein. In this study we used a human cancer model with MCF-7 tumors growing in nude mice. These tumors consist predominantly of epithelial breast cancer cells. Two weeks of tamoxifen treatment to tumor-bearing mice resulted in a significant decrease in TGF- β 1 protein levels in the tumors, measured by ELISA, which was associated with reduced tumor vasculature and decreased tumor growth, compared to animals continually treated with estradiol only. These results are in line with reports of others, documenting decreased tumor angiogenesis and tumor growth when neutralizing/blocking TGF- β signaling [8, 33, 34], and suggest that down-regulating tumor cell-derived TGF- β 1 may be an additional mechanism by which tamoxifen acts in anti-tumorigenic and anti-angiogenic fashion in breast cancer.

Previous studies have shown that a short-term exposure of tamoxifen may induce an increase in TGF- β 1 mRNA and activity in MCF-7 cells [35, 36]. In the present study we confirm the results of others by showing an increased protein expression of TGF- β 1 after 24 hours of tamoxifen treatment, compared to untreated control cells. However, such short-term treatment poorly represents the clinical treatment with tamoxifen. Our data show that contrary to 24 hours of treatment, long-term treatment (one week *in vitro* and two weeks *in vivo*) with tamoxifen generates a decrease in TGF- β 1 protein levels, compared to cells not treated with tamoxifen. TGF- β 1 may be auto feedback regulated and the elevated 24 hours levels may therefore be involved in a down-regulation the

TGF- β 1 gene resulting in lowered levels after a period of time. However, after 72 hours of exposure to tamoxifen the levels of TGF- β 1 in tamoxifen exposed and control cells were equal. This suggests that, apart from an auto feedback regulation, additional mechanisms may be involved in the regulation of TGF- β 1 after long-term treatment with tamoxifen. Here, we show that long-term treatment with tamoxifen may down-regulate TGF- β 1 via MMP-9 activity.

MMPs have been shown to have multifunctional roles in breast tumor progression. Instead of merely functioning as key contributors to ECM degradation and thereby promoting tumor cell invasion and angiogenesis, MMPs are now understood to have much broader roles in tumor progression. Recent studies suggest that MMPs may also suppress tumor growth. In this regard we have recently shown that increasing the levels of soluble MMP-9 in human breast cancer *in vivo* results in increased generation of anti-angiogenic fragments, decreased angiogenesis, and decreased tumor growth, and that this may be among the mechanisms that explain the anti-tumorigenic properties of tamoxifen [20, 27]. Here, we show that long-term tamoxifen treatment affects the TGF- β 1 protein levels secreted from the breast tumor cells, whereas the intracellular levels of TGF- β 1 was unaffected. Interestingly, by inhibiting the extracellular activity of MMP-9, TGF- β 1 protein levels were restored. These results suggest a post-translational regulation of TGF- β 1 and may point to a novel role for MMPs in the feedback regulation of TGF- β 1 activity. Moreover, the down-regulation of TGF- β 1 levels may in turn be an additional anti-angiogenic effect of MMP-9 activity.

In summary, we have shown that tamoxifen negatively regulate TGF- β 1 protein levels in experimental breast cancer, which *in vivo* was associated with decreased tumor growth, and decreased angiogenesis. In addition, our *in vitro* results suggest that the

negative regulation of TGF- β 1 by tamoxifen is an extracellular event, which, at least in part, involves the activity of MMP-2 and MMP-9.

Our findings provide a novel mechanism by which tamoxifen may exert an anti-tumorigenic effect in breast cancer, which may be important both for the understanding of sex steroid regulation of breast cancer progression and for future therapeutic strategies against this disease.

Acknowledgements

This study was supported by grants from the Swedish Cancer Society, the Swedish Research Council, and Linköping University Hospital Research Funds.

References

- [1] J. Massagué, The Transforming Growth Factor-beta Family, *Ann. Rev. Cell Biol.* 6 (1990) 597-641.
- [2] H. Moses, R.J. Coffey, E. Leof, R. Lyons, J. Keski-Oja, Transforming growth factor beta regulation of cell proliferation, *J. Cell. Physiol. Suppl. Suppl 5* (1987) 1-7.
- [3] B. Bierie, H. Moses, Tumour microenvironment: TGF[beta]: the molecular Jekyll and Hyde of cancer, *Nat. Rev. Cancer* 6 (2006) 506-520.
- [4] P. Siegel, W. Shu, R. Cardiff, W. Muller, J. Massague, Transforming growth factor {beta} signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis, *Proc. Natl. Acad. Sci. U S A* 100 (2003) 8430-8435.
- [5] L. Wakefield, A. Roberts, TGF-[beta] signaling: positive and negative effects on tumorigenesis, *Curr. Opin. Genet. Dev.* 12 (2002) 22-29.
- [6] Y. Hasegawa, S. Takanashi, Y. Kanehira, T. Tsushima, T. Imai, K. Okumura, Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma, *Cancer* 91 (2001) 964-971.
- [7] J.-S. Nam, M. Terabe, M.-J. Kang, H. Chae, N. Voong, Y.-a. Yang, A. Laurence, A. Michalowska, M. Mamura, S. Lonning, J.A. Berzofsky, L.M. Wakefield, Transforming Growth Factor {beta} Subverts the Immune System into Directly Promoting Tumor Growth through Interleukin-17, *Cancer Res.* 68 (2008) 3915-3923.

- [8] M. Oft, K.-H. Heider, H. Beug, TGF[β] signaling is necessary for carcinoma cell invasiveness and metastasis, *Curr. Biol.* 8 (1998) 1243-1252.
- [9] Q. Yu, I. Stamenkovic, Transforming growth factor-beta facilitates breast carcinoma metastasis by promoting tumor cell survival, *Clin. Exp. Metastasis* 21 (2004) 235-242.
- [10] S. Desruisseau, J. Palmari, C. Giusti, S. Romain, P.M. Martin, Y. Berthois, Determination of TGF β 1 protein level in human primary breast cancers and its relationship with survival, *Br. J. Cancer* 94 (2006) 239-246.
- [11] A. Grau, W. Wen, D. Ramroosingh, Y.-T. Gao, J. Zi, Q. Cai, X.-O. Shu, W. Zheng, Circulating transforming growth factor- β -1 and breast cancer prognosis: results from the Shanghai Breast Cancer Study, *Breast Cancer Res. Treat.* [Epub ahead of print] (2007).
- [12] V. Ivanovic, M. Demajo, K. Krtolica, M. Krajnovic, M. Konstantinovic, V. Baltic, G. Prtenjak, B. Stojiljkovic, M. Breberina, Z. Neskovic-Konstantinovic, D. Nikolic-Vukosavljevic, B. Dimitrijevic, Elevated plasma TGF-[β]1 levels correlate with decreased survival of metastatic breast cancer patients, *Clin. Chim. Acta* 371 (2006) 191-193.
- [13] D. Padua, X.H.F. Zhang, Q. Wang, C. Nadal, W.L. Gerald, R.R. Gomis, J. Massagué, TGF[β] Primes Breast Tumors for Lung Metastasis Seeding through Angiopoietin-like 4, *Cell* 133 (2008) 66-77.
- [14] L.A. Liotta, W.G. Stetler-Stevenson, Metalloproteinases and cancer invasion, *Semin. Cancer Biol.* 1 (1990) 99-106.
- [15] J.D. Mott, Z. Werb, Regulation of matrix biology by matrix metalloproteinases, *Curr. Opin. Cell Biol.* 16 (2004) 558-564.

- [16] W. Powell, L. Matrisian, Complex roles of matrix metalloproteinases in tumor progression, *Curr. Top. Microbiol. Immunol.* (1996) 1-21.
- [17] L.A. Cornelius, L.C. Nehring, E. Harding, M. Bolanowski, H.G. Welgus, D.K. Kobayashi, R.A. Pierce, S.D. Shapiro, Matrix Metalloproteinases Generate Angiostatin: Effects on Neovascularization, *J. Immunol.* 161 (1998) 6845-6852.
- [18] Y. Hamano, M. Zeisberg, H. Sugimoto, J. Lively, Y. Maeshima, C. Yang, R. Hynes, Z. Werb, A. Sudhakar, R. Kalluri, Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin, *Cancer cell* 3 (2003) 589-601.
- [19] C. Lopez-Otin, L.M. Matrisian, Emerging roles of proteases in tumour suppression, *Nat. Rev. Cancer* 7 (2007) 800-808.
- [20] U.W. Nilsson, C. Dabrosin, Estradiol and tamoxifen regulate endostatin generation via matrix metalloproteinase activity in breast cancer in vivo, *Cancer Res.* 66 (2006) 4789-4794.
- [21] A. Pozzi, W. LeVine, H. Gardner, Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis, *Oncogene* 21 (2002) 272-282.
- [22] N. Dumont, C. Arteaga, The tumor microenvironment: a potential arbitrator of the tumor suppressive and promoting actions of TGF-beta, *Differentiation* 70 (2002) 574-582.
- [23] Q. Yu, I. Stamenkovic, Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis, *Genes Dev.* 14 (2000) 163-176.

- [24] S. Garvin, C. Dabrosin, Tamoxifen Inhibits Secretion of Vascular Endothelial Growth Factor in Breast Cancer in Vivo, *Cancer Res.* 63 (2003) 8742-8748.
- [25] S. Garvin, U. Nilsson, C. Dabrosin, Effects of oestradiol and tamoxifen on VEGF, soluble VEGFR-1, and VEGFR-2 in breast cancer and endothelial cells, *Br. J. Cancer* 93 (2005) 1005-1010.
- [26] U. Nilsson, S. Garvin, C. Dabrosin, MMP-2 and MMP-9 activity is regulated by estradiol and tamoxifen in cultured human breast cancer cells, *Breast Cancer Res. Treat.* 102 (2007) 253-261.
- [27] C. Bendrik, J. Robertson, J. Gauldie, C. Dabrosin, Gene Transfer of Matrix Metalloproteinase-9 Induces Tumor Regression of Breast Cancer In vivo, *Cancer Res.* 68 (2008) 3405-3412.
- [28] A. Schor, N. Pendleton, S. Pazouki, R. Smither, J. Morris, K. Lessan, E. Heerkens, L. Chandrachud, G. Carmichael, M. Adi, D. Chisholm, H. Stevenson, Assessment of Vascularity in Histological Sections: Effects of Methodology and Value as an Index of Angiogenesis in Breast Tumours, *Histochem. J.* 30 (1998) 849-856.
- [29] S. Brandt, A. Kopp, B. Grage, C. Knabbe, Effects of tamoxifen on transcriptional level of transforming growth factor beta (TGF-beta) isoforms 1 and 2 in tumor tissue during primary treatment of patients with breast cancer., *Anticancer Res.* 23 (2003) 223-229.
- [30] A. Butta, K. MacLennan, K.C. Flanders, N.P.M. Sacks, I. Smith, A. McKinna, M. Dowsett, L.M. Wakefield, M.B. Sporn, M. Baum, A.A. Colletta, Induction of Transforming Growth Factor {beta} 1 in Human Breast Cancer in Vivo following Tamoxifen Treatment, *Cancer Res.* 52 (1992) 4261-4264.

- [31] J. MacCallum, J. Keen, J. Bartlett, A. Thompson, J. Dixon, W. Miller, Changes in expression of transforming growth factor beta mRNA isoforms in patients undergoing tamoxifen therapy., *Br. J. Cancer* 74 (1996) 776-778.
- [32] A. Thompson, D. Kerr, C. Steel, Transforming growth factor beta 1 is implicated in the failure of tamoxifen therapy in human breast cancer., *Br. J. Cancer* 63 (1991) 609-614.
- [33] B. Tang, M. Vu, T. Booker, S. Santner, F. Miller, M. Anver, L. Wakefield, TGF- β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression, *J. Clin. Invest.* 112 (2003) 1116-1124.
- [34] J. Tuxhorn, S. McAlhany, F. Yang, T. Dang, D. Rowley, Inhibition of Transforming Growth Factor- β Activity Decreases Angiogenesis in a Human Prostate Cancer-reactive Stroma Xenograft Model, *Cancer Res.* 62 (2002) 6021-6025.
- [35] H. Chen, T. Tritton, N. Kenny, M. Absher, J.-F. Chiu, Tamoxifen induces TGF- β 1 activity and apoptosis of human MCF-7 breast cancer cells in vitro, *J. Cell. Biochem.* 61 (1996) 9-17.
- [36] C. Knabbe, M. Lippman, L. Wakefield, K. Flanders, A. Kasid, R. Derynck, R. Dickson, Evidence that transforming growth factor- β is a hormonally regulated negative growth factor in human breast cancer cells, *Cell* 48 (1987) 417-428.