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PRECLINICAL STUDY

MMP-2 and MMP-9 activity is regulated by estradiol and tamoxifen in cultured human breast cancer cells

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Abstract Sex steroids play a dominant role in breast carcinogenesis by still largely unknown mechanisms. Matrix metalloproteinases (MMPs) have been extensively studied in the context of matrix biology but it is not known if sex steroids affect MMPs in breast cancer. MMPs degrade extracellular matrix components enabling tumor cell invasion and metastasis, but may also regulate the bioavailability of a variety of biologically active molecules such as anti-angiogenic fragments, which may be beneficial for the host. This study shows that estradiol and tamoxifen regulate MMP-2 and MMP-9 as well as TIMP-1 and TIMP-2 in ER + PR + human breast cancer cells. The main finding was a significant effect of tamoxifen exposure, which increased intracellular and secreted protein levels whereas estradiol induced a significant decrease. The overall net effect of these alterations resulted in increased MMP-2/MMP-9 activity by tamoxifen treatment, which also significantly increased extracellular endostatin levels. We conclude that estradiol and tamoxifen have the ability to modulate MMP-2/MMP-9 activity, and endostatin levels in human breast cancer in vitro. The results suggest a possible role of MMP modulation associated with a generation of anti-angiogenic fragments in the therapeutic effect of tamoxifen in breast cancer.

Keywords Breast cancer · Endostatin · Estrogen · Matrix metalloproteinases · Tamoxifen · Tissue inhibitors of matrix metalloproteinases · MCF-7 cells

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Introduction

Breast cancer constitutes almost 30% of all cancer cases among women in the Western world today, and many of these women die from metastatic disease [1]. In order for invasion and metastasis to occur, the basement membrane and other extracellular matrix (ECM) components must be degraded [2]. MMP-2 and MMP-9, two members of the family of zinc-dependent endopeptidases known as the matrix metalloproteinases (MMPs), play a dominant role in these processes [3–6]. MMP-2 and MMP-9 have been shown to be involved in the regulation of the metastatic process in various cancers, including breast cancer [7–11]. MMPs have been implicated both as a positive and negative regulators of angiogenesis [12, 13]. Previous studies have shown that MMP-2 and MMP-9 may directly regulate angiogenesis by generating pro-angiogenic factors, such as VEGF, as well as the anti-angiogenic factors angiostatin, endostatin and tumstatin [14–18]. Hence, the regulation of the activity of these proteinases appears to be of great importance in tumor progression at different stages.

Sex steroids affect the risk of breast cancer occurrence and recurrence but the mechanisms are still unclear [19,20]. During the menstrual cycle and pregnancy, MMPs are key players in the vascular remodeling of the endometrium and ovaries, suggesting a sex steroid-dependent regulation of MMP activity [4, 21]. The normal breast is also a sex hormone dependent organ and the majority of breast cancer tumors maintain their hormone dependency. Anti-estrogen treatment such as tamoxifen is a cornerstone in breast cancer therapy today. If estrogen and anti-estrogen, in addition to their direct effects on cell cycle

control, also play a part in extracellular re-modeling by effects on MMPs is not known. In this paper we hypothesize that estrogen and tamoxifen affect the secretion and activity of MMP-2 and MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2, as well as the release of the bioactive anti-angiogenic peptide endostatin, in hormone-responsive breast cancer in vitro. We demonstrate a significant effect of tamoxifen exposure to MCF-7 cells, which increased both intracellular and secreted MMP-2 and MMP-9 protein levels, as well as the combined activity of MMP-2 and -9, whereas estradiol down-regulated the levels. Moreover, tamoxifen increased the extracellular levels of endostatin.

Materials and methods

Cells and culture conditions

MCF-7 (HTB-22; human breast adenocarcinoma, ER+ and PR+) cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were used in all experiments. MB-MDA-231 (HTB-26; human breast adenocarcinoma, ER–) (ATCC, Manassas, VA, USA) cells were used as a negative control cell line. All 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% CO_2. Cell culture medium and additives were obtained from Invitrogen (Carlsbad, CA, USA) if not otherwise stated.

Hormone treatment

Cells were trypsinized (0.05% trypsin and 0.02% EDTA) and seeded into Petri dishes (Costar, Cambridge, MA, USA), 10000 cells/cm^2. Cells were incubated for 24 h in the DMEM based culture medium and then for 7 days treated with or without 10^{-8} M 17-ß-estradiol (Sigma, St. Louis, MO, USA), 10^{-6} M tamoxifen (Sigma), or a combination of estradiol and tamoxifen of the various concentrations. Hormones were added to the MCF-7 cultures in serum-free medium consisting of a 1:1 mixture of nutrient mixture F-12 (HAM) and DMEM without phenol red, supplemented with 10 μg/ml transferring (Sigma), 1 μg/ml insulin (Sigma), and 0.2 mg/ml BSA (Sigma). The serum-free hormone medium was changed every 24 h. After hormone treatment, conditioned media was collected and cells were, after a PBS wash, lysed by repeated freeze-thaw cycles. This method of cell lysis may potentially activate some MMPs. However, the method was applied in all treatment groups and only relative comparisons between treatment-groups were made. The cells harvested were diluted in PBS and total protein content determined using Bio-Rad Protein Assay with BSA as standard (Bio-Rad Laboratories, Stockholm, Sweden). Conditioned media and cell lysates were stored at –70°C until subsequent analyses.

Quantification of MMP-2, MMP-9, TIMP-1, TIMP-2 and endostatin

Conditioned media and lysates of hormone treated MCF-7 cells were analyzed for MMP-2 (total), MMP-9 (active and total), TIMP-1, TIMP-2 and endostatin, by the corresponding quantitative ELISAs obtained from R&D. The MMP-2 assay detects both active MMP-2 and pro-MMP-2 (Quantikine®). Two different kits were used to measure endogenous active MMP-9 (Fluorokine™) and total MMP-9 (Quantikine®). The total MMP-9 ELISA recognizes both the active form and pro-form of human MMP-9. According to the manufacturer, sensitivities of the assays were as follows: total MMP-9 < 0.156 ng/ml, MMP-2 0.16 ng/ml (mean MDD), active MMP-9 0.005 ng/ml (mean MDD), TIMP-1 < 0.08 ng/ml, TIMP-2 0.011 ng/ml (mean MDD), and endostatin 0.023 ng/ml (mean MDD).

Assays were conducted according to manufacturer's guidelines. Typically 50–100 μl of sample was assayed and optical density (OD) was measured using a VersaMax microplate reader (Molecular Devices, CA, USA) at 450 nm. For the active MMP-9 Fluorokine™ immunoassay, relative fluorescence units (RFU) were measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., CA, USA). Excitation and emission wavelengths were 320 nm and 405 nm, respectively. Recombinant proteins standards provided by the manufacturer of each ELISA were included on each experimental plate as positive controls, and from this a standard curve was plotted, all according to manufacturer's guidelines. In the ELISA kit for active protein, active MMP was used as standard, accordingly ELISA for total MMP the pro-forms were used. Protein concentrations were determined in ng/ml, correlated to total protein content in cell lysate, and presented in diagrams as ng/mg total protein. All assays were repeated on more than one cell harvest.

MMP-2/MMP-9 activity assay

The combined activity of MMP-2 and MMP-9 was assayed using a MMP-2/MMP-9 activity assay. MCF-7 cells were cultured and seeded in Petri dishes as described above, and thereafter treated for 3, 5, or 7 days with estradiol or tamoxifen, or a combination of the two. Conditioned media was collected from the cells and mixed 50:50 with 100 μM of a quenched fluorogenic substrate specific for MMP-2 and MMP-9 (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 for 20 min at room temperature with gentle agitation. The fluorescent signal was monitored at room temperature on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., CA, USA), with λ_{ex} at 320 nm and λ_{em} 405 nm. Negative as well as positive controls were included in the assay. All experimental preparations and incubations were performed in a low-light or light-free environment to protect the substrate from degrading. MMP-2/MMP-9 activity was expressed as RFU/mg total protein.

Gelatin zymography

The activity of MMP-2 and MMP-9 in MCF-7 conditioned media (10× concentrated) was analyzed by gelatin zymography. Briefly, samples (× μg of proteins per lane; determined by total protein quantity) were prepared in a non-reducing loading buffer consisting of 63 mM Tris–HCl pH 6.8, 10% glycerol (v/v), 2% sodium dodecyl sulphate (SDS) (w/v), 0.0025% bromophenol blue (w/v), and electrophorezed on 10% SDS-polyacrylamide gels containing 0.1% gelatin. Recombinant active MMP-2 (66 kDa) and active MMP-9 (83 kDa) were used as positive controls, Calbiochem, Merck Biosciences Ltd., Nottingham, UK. After electrophoresis, gels were washed for 1 h in renaturing buffer (Novex® Renaturing Buffer; Invitrogen) to remove SDS and thereby restore enzyme activity, and then incubated for 24 h at 37°C in developing buffer (Novex® Developing Buffer; Invitrogen). Gels were then stained with 0.5% Coomassie Blue R-250 (w/v) in 40% ethanol (v/v) and 10% acetic acid (v/v), and de-stained in the same solution without dye. Proteinase activity was observed as clear bands against a blue background of undigested gelatin. Bands were compared with positive controls and molecular weight standards (Pre-stained, broad range; Bio-Rad) for accurate interpretation.

Quantitative gene expression analysis of MMP-2 and MMP-9

MCF-7 cells were hormone treated (10^{-8} M 17-ß-estradiol, 10^{-6} M tamoxifen) as described above. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) and following manufacturer's protocol. Briefly, cells were disrupted and lysed directly in the cell culture dishes using a lysis solution provided by the manufacturer. Samples were then homogenized using a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe, RNeasy spin column purified, and eluted in RNase-free water.

First-strand cDNA was synthesized from 5 μg of total RNA using 50 units of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with 150 ng of random hexamers according to manufacturer's instructions. Briefly, samples were incubated at 20°C for 10 min and 42°C for 50 min, and reverse transcriptase was inactivated by heating at 70°C for 15 min and cooling on ice for 5 min. Synthesized cDNA was treated with RNase H at 37°C for 20 min to remove the RNA template from the cDNA:RNA hybrid molecule, and thereafter diluted with nuclease free water 1:5 before stored at –20°C.

Real-time quantitative PCR was performed in triplicate on an ABI 7500 Fast Real-Time PCR System using the TaqMan® Gene Expression Assay for the human MMP-2 and MMP-9 genes (Applied Biosystems, Foster City, CA, USA). The final reaction volume (20 μl) included 10 μl TaqMan® Fast Universal PCR Master Mix, 5 μl of diluted cDNA, and 1 μl 20× TaqMan® Gene Expression Assay Mix containing 900 nM unlabeled primers and 250 nM FAM™ dye-labeled TaqMan® MGB probe. The reaction cycle consisted of a 95°C step for 20 s, followed by 40 cycles consisting of 3 s at 95°C and 30 s at 60°C. Human β-actin (TaqMan® Endogenous Controls, Applied Biosystems) was used as an invariant endogenous control to correct for minor experimental variations, and all fluorescent signals were compared to non-template controls to determine baseline noise.

Relative gene expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method [22]. An experimental plate with standard curves for MMP-2, MMP-9 and β-actin were initially run to validate the use of the $2^{-\Delta\Delta Ct}$ method. All data were calculated as means ± SEM from four samples per treatment group, and gene expression changes were confirmed on two separate reactions plates per sample.

Statistical analysis

Results are expressed as mean ± SEM. One-way ANOVA and Student's *t*-test were used to compare means between experimental groups. Statistical significance was assumed at *P* value less than 0.05.

Results

Effects of estrogen and tamoxifen on MMP-2/MMP-9 activity

We applied a MMP-2/MMP-9 activity assay, using a fluorogenic substrate specific for MMP-2 and MMP-9, to determine the effect of hormone treatment on MMP-2/MMP-9 activity. A dose-response relation was detected, as shown in Fig. 1, and the 10^{-8} M and 10^{-6} M concentrations of estradiol and tamoxifen, respectively, was chosen for all experiments. Conditioned media from 3, 5, or 7 days of treatment was incubated with the substrate and the generated fluorescence was measured. The MMP-2 and MMP-9 activity, expressed in RFU, was correlated with total protein content. As Fig. 2 shows, tamoxifen treatment induced a significant increase in MMP-2/MMP-9 activity [$P < 0.001$, as compared to control cells]. This increase was intensified by a longer time course of hormone exposure, with a largest effect observed after 7 days. Therefore, 7 days exposure was used in all subsequent experiments. Estradiol significantly decreased the MMP-2/MMP-9 activity [$P < 0.05$, as compared to control], this decrease was in part reversed by addition of tamoxifen to the estradiol treatment [$P < 0.01$, estradiol vs. estradiol + tamoxifen] (Fig. 2). No effect of estradiol or tamoxifen on MMP-2/MMP-9 activity was observed in ER-negative MB-MDA-231 cells [80089 ± 2227 RFU/mg protein, mean for all groups].

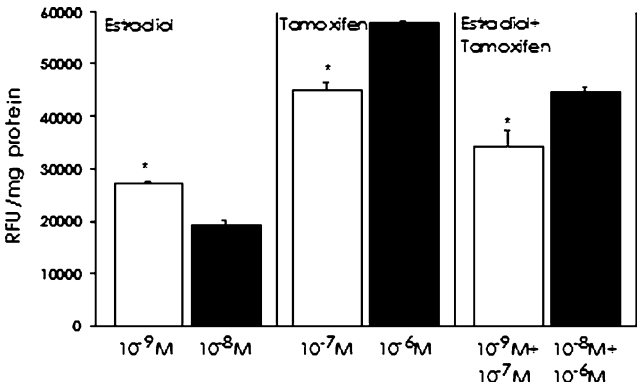

Fig. 1 Dose-response of estrogen and tamoxifen on MMP-2/MMP-9 activity. MCF-7 cells were cultured in serum-free media in the presence of estradiol and tamoxifen of various concentrations, and conditioned media was incubated with a MMP-2/MMP-9 specific fluorogenic substrate and the generated fluorescence measured. All values represent means ± SEM ($n = 4$ in each group). *$P < 0.05$

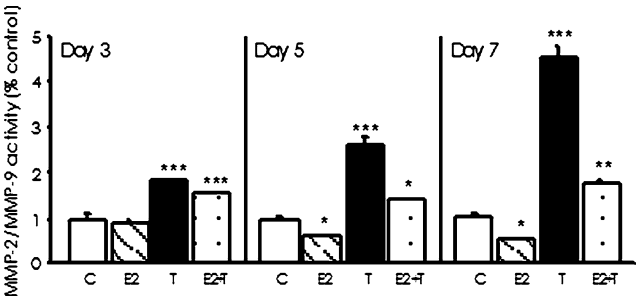

Fig. 2 MMP-2/MMP-9 activity in cell culture supernatant of hormone-treated MCF-7 cells. MCF-7 cells were cultured in serum-free media without hormones (control) or in the presence of estradiol (10^{-8} M), tamoxifen (10^{-6} M), or a combination of estradiol and tamoxifen for 3, 5 and 7 days. Conditioned media was analyzed for MMP-2/MMP-9 activity using a MMP-2/MMP-9 activity assay as described in Fig. 1. All values represent means ± SEM ($n = 4$ in each group). *$P < 0.05$ vs. control cells; **$P < 0.01$ vs. control cells; ***$P < 0.001$ vs. control cells

Zymographic analysis confirmed the results of the MMP-2/MMP-9 activity assay. Using this technique, both active and latent (pro-) forms of the MMPs can be visualized. Conditioned media from MCF-7 cells showed a significant increase of active MMP-9 after treatment with tamoxifen and estradiol + tamoxifen. Zymograms showed very low levels of MMP-2 activity. A representative zymogram and densitometry, of three independent experiments, is shown in Fig. 3A–C.

Effects of estrogen and tamoxifen on secreted and intracellular MMP-2 protein

In order to quantify protein levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and endostatin, quantitative ELISA was carried out on conditioned media and cell lysates of hormone treated MCF-7 cells. Figure 4A shows the total amounts of secreted MMP-2 protein (active and latent forms), which was significantly increased after tamoxifen treatment [$P < 0.01$, as compared to control]. A combination of estradiol and tamoxifen gave a similar increase in secreted MMP-2 [$P < 0.05$, as compared to control] (Fig. 4A). The amounts of secreted MMP-2 protein did not change with estradiol treatment. Intracellularly, exposure to tamoxifen as well as to estradiol + tamoxifen also triggered an increase in MMP-2 levels [$P < 0.001$, as compared to control] (Fig. 4B). Estradiol exposure significantly

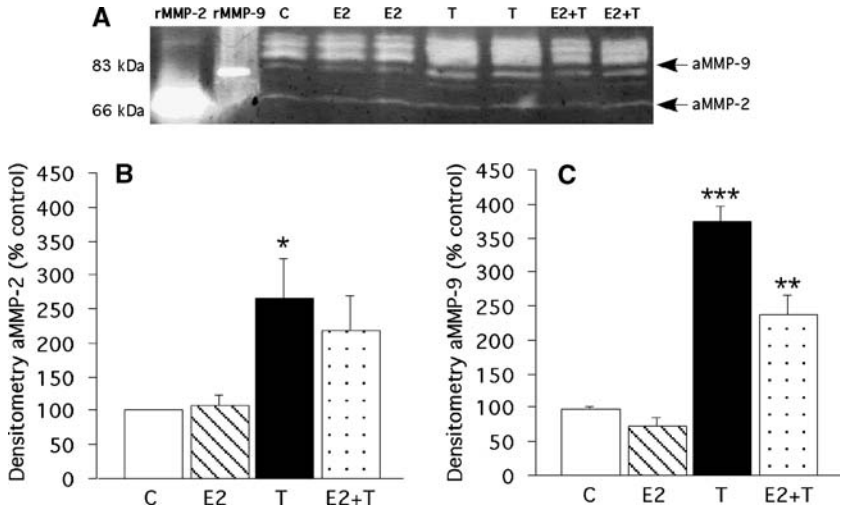

Fig. 3 Zymographic analysis of MMP-2/MMP-9 activity in media from hormone treated MCF-7 cells. MCF-7 cells were hormone-treated for 7 days as described in Fig. 2. Conditioned media was concentrated 10×, normalized for protein concentration and analyzed by gelatin zymography. (**A**) representative zymogram of three separate experiments (C, control; E2, estradiol; T, tamoxifen; E2 + T, estradiol + tamoxifen). (**B**) densitometry of active MMP-2 ($n = 3$)*$P < 0.05$ vs. control cells. (**C**) densitometry of active MMP-9 ($n = 3$), **$P < 0.01$ vs. control cells; ***$P < 0.001$ vs. control cells

lowered the intracellular levels of MMP-2 protein [$P < 0.01$, as compared to control] (Fig. 4B).

MMP-2 mRNA levels were low and no differences in gene expression in response to hormone-treatment could be detected.

Effects of estrogen and tamoxifen on secreted and intracellular MMP-9 protein, and MMP-9 mRNA levels

MCF-7 cells exposed to tamoxifen exhibited a significant increase of both secreted and intracellular MMP-9 protein levels [$P < 0.001$, $P < 0.01$, as compared to control] (Fig. 5A–B). Estradiol significantly decreased total MMP-9 protein, both intracellular and secreted [$P < 0.05$, $P < 0.001$, respectively, as compared to control] (Fig. 5A–B). Addition of tamoxifen to the estradiol treatment significantly increased both intracellular and secreted MMP-9 [$P < 0.001$, $P < 0.05$, respectively, estradiol vs. estradiol + tamoxifen] (Fig. 5A–B). The total levels of MMP-9 protein (pro- and active forms) were considerably higher than the total levels of MMP-2 protein (pro- and active forms) in the MCF-7 cells. This was equally observed in zymograms where only week bands of MMP-2 protein activity was detected.

MMP-9 mRNA levels were significantly down-regulated by estradiol and up-regulated by tamoxifen (Fig. 5C).

Effects of estrogen and tamoxifen on secreted active MMP-9

Most MMPs are synthesized as pro-enzymes and are activated by the proteolytic removal of the pro-domain. With the large increase seen in total MMP-9

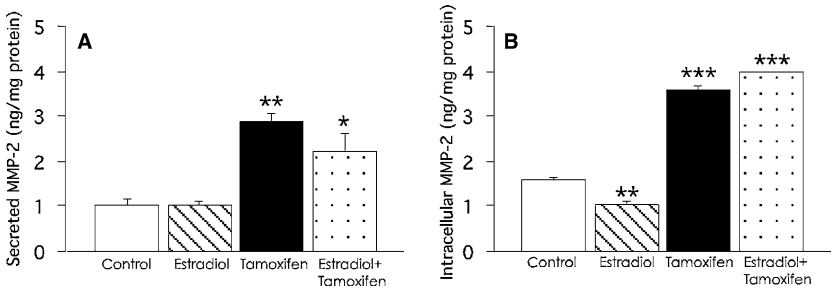

Fig. 4 Secreted and intracellular MMP-2 after hormone treatment of MCF-7 cells in culture. MCF-7 cells were hormone-treated for 7 days as described in Fig. 2. Conditioned media (**A**) and cell lysate (**B**) were analyzed for total MMP-2 using quantitative ELISA. All values represent means ± SEM ($n = 3$ in each group). *$P < 0.05$ vs. control cells; **$P < 0.01$ vs. control cells; ***$P < 0.001$ vs. control cells

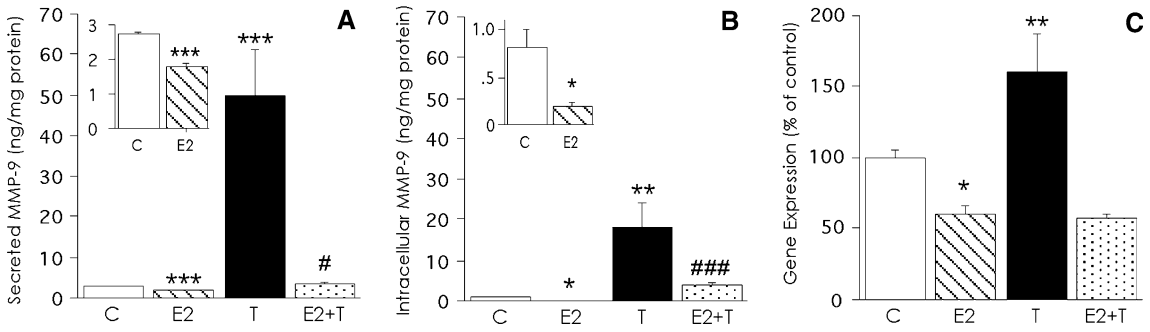

Fig. 5 MMP-9 protein levels and gene expression changes after hormone treatment of MCF-7 cells in culture. MCF-7 cells were hormone-treated as described in Fig. 3. Conditioned media (**A**) and cell lysates (**B**) were analyzed for total MMP-9 using quantitative ELISA. All values represent means ± SEM ($n = 6$ in each group). $^{*}P < 0.05$ vs. control cells; $^{**}P < 0.01$ vs. control cells; $^{***}P < 0.001$ vs. control cells; $^{\#}P < 0.01$ vs. estradiol-treated cells; $^{\#\#\#}P < 0.01$ vs. estradiol-treated cells. (**C**) MMP-9 gene expression changes in response to hormone-treatment were quantified using real-time PCR. All values represent means ± SEM ($n = 4$ in each group). $^{*}P < 0.05$ vs. control cells; $^{**}P < 0.01$ vs. control cells

after tamoxifen treatment, we were prompted to determine the amounts of active MMP-9 protein after the same treatments. As seen in Fig. 6A, there was a significant increase in the amounts of active MMP-9 [$P < 0.001$, as compared to control] in conditioned media of MCF-7 cells treated with tamoxifen. Similar results were seen after treating the cells with estradiol and tamoxifen in combination [$P < 0.01$, as compared to control] (Fig. 6A). There were also significantly higher levels of intracellular active MMP-9 in tamoxifen treated cells, compared to control cells [$P < 0.001$] (Fig. 6B). Furthermore, estradiol induced a significant decrease in the extracellular levels of active MMP-9 protein [$P < 0.01$, as compared to control] (Fig. 6B).

Effects of estrogen and tamoxifen on secreted TIMP-1 and TIMP-2

An imbalance between MMPs and their naturally occurring tissue inhibitors possibly plays a significant role in the invasive phenotype of malignant tumors. We applied a quantitative ELISA to investigate the effects of estradiol and tamoxifen on the secreted (extracellular) levels of TIMP-1 and TIMP-2, the tissue inhibitors of MMP-2 and MMP-9. Tamoxifen significantly increased TIMP-1 levels in conditioned media [$P < 0.05$, as compared to control], whereas estradiol significantly lowered the amounts [$P < 0.01$, as compared to control] (Fig. 7). Extracellular TIMP-2, as shown in Fig. 7, tended to increase with treatments. Tamoxifen exposure gave a significant increase in the amounts of TIMP-2 protein in cell culture media [$P < 0.05$, as compared to control] (Fig. 7).

Effects of estrogen and tamoxifen on the extracellular levels of endostatin

We further investigated whether MCF-7 cells produced the anti-angiogenic peptide endostatin, and whether the extracellular protein expression level was influenced by estradiol and/or tamoxifen. Endostatin protein was detected by quantitative ELISA in all samples, including untreated control cells (Fig. 8). The extracellular expression of endostatin was increased by

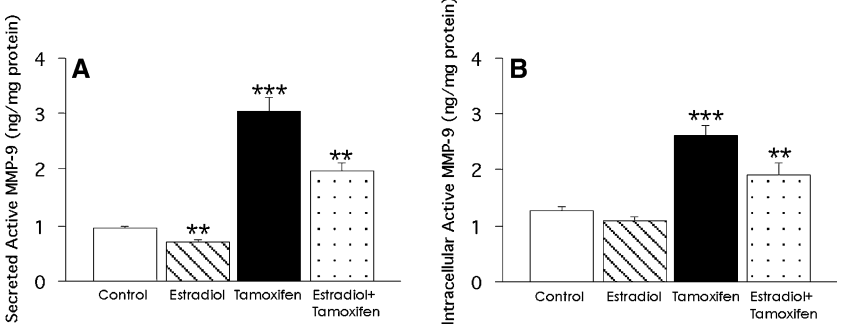

Fig. 6 Secreted and intracellular active MMP-9 after hormone treatment of MCF-7 cells in culture. MCF-7 cells were hormone-treated for 7 days as described in Fig. 2. Conditioned media (**A**) and cell lysate (**B**) were analyzed for active MMP-9 using quantitative ELISA. All values represent means ± SEM ($n = 6$ in each group). $^{**}P < 0.01$ vs. control cells; $^{***}P < 0.001$ vs. control cells

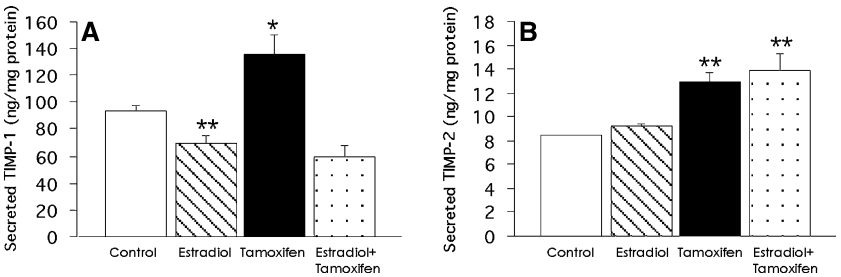

Fig. 7 Secreted TIMP-1 and TIMP-2 after hormone treatment of MCF-7 cells in culture. MCF-7 cells were hormone-treated for 7 days as described in Fig. 2. Conditioned media was analyzed for secreted TIMP-1 (**A**) and TIMP-2 (**B**), using quantitative ELISA. All values represent means ± SEM ($n = 6$ in each group). *$P < 0.05$ vs. control cells; **$P < 0.01$ vs. control cells

tamoxifen treatment [$P < 0.001$, as compared to control] (Fig. 8). In cells treated with a combination of estradiol and tamoxifen, a similar increase in extracellular endostatin protein levels was observed [$P < 0.001$, as compared to control] (Fig. 8).

Discussion

We show in this study that estradiol and the anti-estrogen tamoxifen regulate MMP-2 and MMP-9 as well as TIMP-1 and TIMP-2 in ER+ and PR+ human breast cancer cells (MCF-7) in vitro. The main finding was a significant effect of tamoxifen exposure, which increased both intracellular and secreted protein levels as well as the activity of MMP-2 and MMP-9, whereas estradiol exposure significantly down-regulated protein levels and MMP activity. Tamoxifen increased and estradiol decreased the endostatin protein levels in conditioned media.

Matrix metalloproteinases (MMPs) are active in many physiological tissue remodeling events in reproductive tissues, including endometrial regulation, ovulation, implantation, and uterine, and breast involution [21, 23], suggesting a sex steroid regulated activity under normal physiological conditions. However, this is the first study to show that sex steroids regulate MMPs and their tissue inhibitors in breast cancer.

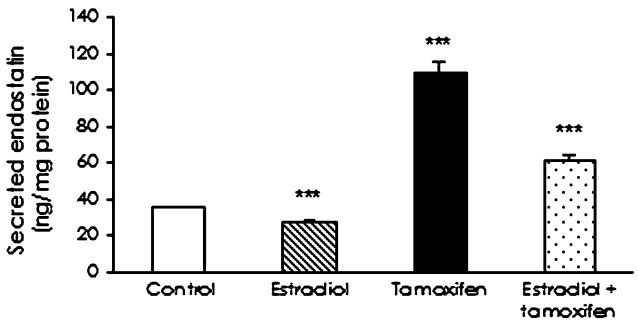

Fig. 8 Extracellular levels of endostatin after hormone treatment of MCF-7 cells in culture. MCF-7 cells were hormone-treated for 7 days as described in Fig. 2, and conditioned media analyzed for endostatin using quantitative ELISA. All values represent means ± SEM ($n = 6$ in each group). ***$P < 0.001$ vs. control cells

MMP-2 and MMP-9 are synthesized and secreted as pro-enzymes and require proteolytic removal of the pro-domain in order to become active [3, 24]. However, since uncontrolled activation of MMPs is potentially dangerous in a cellular environment, enzyme expression is tightly regulated at the transcriptional and/or post-translational level [24]. Furthermore, the extracellular activity of MMPs is controlled by endogenous tissue inhibitors such as TIMPs [25–28]. It is therefore important to investigate the activities of these proteases at the protein level, in the compartment where they are biologically active, the extracellular space. This is demonstrated by our results of mRNA expression levels of MMP-9. Although the changes were similar to the protein alterations with a decrease after estradiol and an increase after tamoxifen exposure the gene expression levels were much less pronounced than the detected change at the protein level. Moreover, it is important to measure the net-result and overall balance rather than a single MMP or TIMP. This is demonstrated by clinical studies which have shown an association with increased expression of TIMP-1 and TIMP-2 as well as increased MMP-2 and MMP-9 and poor prognosis and development of metastasis in several human cancers including breast cancer [29, 30].

To be able to measure the physiological result of the changes of both MMP and TIMP levels after hormone exposure, we applied a MMP-2/MMP-9 activity assay using a fluorogenic substrate specific for MMP-2 and MMP-9. We show that the end-result of tamoxifen exposure was a significant increase in extracellular MMP-2/MMP-9 activity whereas estradiol exposure resulted in a significant decrease of MMP-2/MMP-9 activity. In order to mimic the clinical situation, at least

in pre-menopausal women, cells were also exposed to a combination of estradiol and tamoxifen. This combination partly counteracted the decrease seen after treatment with estradiol alone.

Extensive research in the field of matrix biology has changed the view of matrix metalloproteinases. Instead of merely functioning as key contributors to ECM degradation and thereby contributing to tumor cell invasion, migration, and angiogenesis, they are now understood to play much broader roles in modulating tumor progression. As an example, MMPs act in an inhibitory fashion by releasing anti-angiogenic fragments such as angiostatin, endostatin, and tumstatin [17, 31, 32]. In situ carcinoma studies suggest that MMPs may be generated by the tumoral stroma and immune cells [33–35]. However, it cannot be ruled out that the cancer cells themselves contribute to the induction of pro-and anti-angiogenic events by producing MMPs. In fact, in this study we show that epithelial breast cancer cells in vitro produce MMP-2 and MMP-9. We also show that in addition to inducing an increase in the activity of these two proteinases, tamoxifen also increases the extracellular protein levels of endostatin. Taken together, these two events may partake in the potent anti-angiogenic response that we have previously seen with tamoxifen in MCF-7 tumor explants in nude mice [36].

Thus far, the effects of estrogen on MMP protein expression have been considered in only a few investigations with somewhat conflicting results, one reason being the use of various estrogens at different concentrations or use of serum-containing culture media [37–39]. Our present results are however, in line with one previous study, also using the naturally occurring estradiol [38]. In addition to the well known growth stimulatory effects of estrogen on breast cancer cells, estrogen has also been shown to positively regulate tumor angiogenesis in hormone-dependent breast cancer models by increasing VEGF levels [40–42]. The decrease of endostatin levels by estradiol shown in the present study, may further promote pro-angiogenic effects by estrogen in solid tumors.

In conclusion, we have shown that estradiol induces a significant decrease in the activity of MMP-2/MMP-9 whereas tamoxifen significantly increases MMP-2/MMP-9 activity in human breast cancer cells in vitro. In addition, TIMP-1, and to a lesser extent TIMP-2, seem to be regulated by sex steroids. Over expression of MMPs has previously been associated with breast cancer progression, but it is becoming increasingly clear that MMP activity also has biological consequences which may be beneficial to the host. Mapping the effects of sex steroids on carcinogenic events such as invasion and metastasis in established breast cancer is important, since the majority of breast cancers maintain their hormone dependency. This study provides evidence that estrogens and anti-estrogens have the ability to modulate the activity of matrix metalloproteinases in human breast cancer, and that this in turn may influence the angiogenic balance by affecting bioactive anti-angiogenic peptides such as endostatin. Moreover, given the complexity of the regulation of MMP activity we feel it is crucial to include all implicated components when predicting the biological outcome of it. Finally, we suggest a role for MMP modulation in the clinical therapeutic effect of tamoxifen in breast cancer. In vivo model systems are needed to further elucidate the link between sex steroids, MMP activities and the generation of anti-angiogenic fragments.

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