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Increased endostatin generation and decreased angiogenesis via MMP-9 by tamoxifen in hormone dependent ovarian cancer

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Abstract

There are several similarities between breast and ovarian cancer but anti-estrogen treatment is rarely used in ovarian cancer. We have previously shown that the most widely used anti-estrogen tamoxifen increased MMP-9 activity and endostatin generation in breast cancer. Here, we show that tamoxifen exposure of highly hormone responsive ovarian cancer cells decreased proliferation, and increased MMP-9 activity leading to increased levels of endostatin both in cell culture in vitro and in solid tumors of nude mice. Tamoxifen exposed tumors also exhibited significantly decreased tumor growth and tumor vascularisation. Moreover, in ascites from ovarian cancer patients, MMP-9 was undetectable in majority of cases but a significant correlation of MMP-2 and endostatin was found. The effects on MMPs and endostatin generation are previously unknown mechanisms of estradiol and tamoxifen in ovarian cancer, which may have therapeutic implications in future anti-cancer options of hormone dependent ovarian cancer.

1. Introduction

MMPs are a family of zinc-dependent endopeptidases important in extracellular matrix (ECM) remodelling processes [1,2]. Due to their ECM degrading capacity, MMPs have for long been viewed as key modulators of tumor progression and metastasis. Indeed, high expression levels of several MMP members have been correlated with tumor aggressiveness of various human cancers [3-5]. An increasing amount of observations, however, show that MMPs may have a dual role in tumor invasion and that MMP activity in fact may induce an anti-tumorigenic response [6,7].

Several studies show that up-regulated levels of endogenous MMPs decrease tumor angiogenesis, a crucial event for tumor growth, invasion and metastasis, by the proteolytic cleavage and release of anti-angiogenic factors, such as angiostatin, tumstatin, and endostatin from the tumor stroma, resulting in tumor regression [8-12]. Moreover, MMP inhibitors as anti-tumor therapy in clinical trials have been proven ineffective [13,14], further suggesting that MMP activities are not exclusively pro-invasive. The regulation of MMP expression and activity is complex and partly unknown, but tissue inhibitors of metalloproteinases (TIMPs) are considered to be the major endogenous inhibitors of MMP activity in the tissue [1,15]. The role of TIMPs in tumor biology is complex as they may control cell growth and apoptosis independent of MMPs [16] and high TIMP expression predicts poor prognosis in several malignancies, including breast and ovarian cancer [17-20].

We have previously shown that estradiol and tamoxifen, one of the most widely used endocrine therapies in the treatment of estrogen receptor (ER) positive breast cancer, regulate protein levels and activity of MMP-2 and MMP-9 in experimental breast cancer *in vitro* and *in vivo* [10,21]. In ER-positive breast cancer tamoxifen increased MMP activity, which resulted in increased generation of endostatin and tumor regression [10].

Ovarian tumors share several common characteristics with breast tumors, such as hormone-sensitivity and expression of hormonal receptors. Despite this fact, tamoxifen is rarely used as a treatment strategy in ovarian cancer. If estradiol and tamoxifen regulate MMPs, TIMPs, and endostatin levels in ovarian cancer is not known.

Here we show that tamoxifen increased MMP-9 activity and the generation of endostatin in endocrine-responsive ovarian cancer BG-1 cells. TIMP-1 and TIMP-2 protein levels were not significantly altered by exposure of either estradiol or tamoxifen. Ovarian tumor xenografts in nude mice treated with tamoxifen showed increased expression of MMP-9, increased generation of endostatin and decreased vessel density compared to tumors from mice treated with estradiol. Additionally, tamoxifen treatment decreased tumor growth significantly compared to estradiol treatment. In ascites from ovarian cancer patients TIMP-1 levels were high whereas the levels of MMP-9 were very low to undetectable. MMP-2 levels were 20-fold higher than those of MMP-9 and a significant positive correlation between MMP-2 and endostatin levels in the ascites confirmed the physiological relevance of MMP generation of endostatin in ovarian cancer patients.

2. Materials and Methods

2.1. Cells and culture conditions

Ovarian cancer BG-1 (kindly provided by Dr. Kenneth Korach (NIEHS, National Institute of Health, Research Triangle Park, NC, USA) and OVCAR-3 cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were used in all experiments. BG-1 cells were cultured in DMEM/F-12 (1:1) w/o phenol red, supplemented with 5 % fetal bovine serum, 1 % non-essential amino acids and antibiotics (50 IU/mL penicillin-G and 50 µg/mL streptomycin). OVCAR-3 cells were cultured in DMEM w/o phenol red, supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 1 µg/mL insulin, 10 mM HEPES, 1 % non-essential amino acids and antibiotics (50 IU/mL penicillin-G and 50 µg/mL streptomycin). Cells were kept at 37° C in a humidified atmosphere containing 5% CO₂. Cell culture medium and additives were obtained from Gibco/Invitrogen (Paisley, UK) if not otherwise stated.

2.2. Hormone treatment of ovarian cancer cells in culture

Ovarian cancer cells were grown to confluence, trypsinized (0,05% trypsin and 0,02% EDTA) and seeded into Petri dishes (Costar, Cambridge, MA, USA), at a density of 20 000 cells/cm². Cells were incubated for 24 hours and then treated with or without estrogen (17β-estradiol, Sigma, St. Louis, MO, USA) at 1 x 10⁻⁹ M, tamoxifen (Sigma) at 2 x 10⁻⁶ M, or a combination of estradiol and tamoxifen for 7 days. Hormones were added in serum-free DMEM/F-12 (1:1) w/o phenol red, supplemented with 10 µg/mL transferrin (Sigma), 1 µg/mL insulin (Sigma), and 0,2 mg/mL bovine serum albumin (Sigma). The hormone medium was changed every 24 hours. After hormone treatment, the conditioned media was collected and cells were lysed by repeated freeze-thaw cycles. 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.

2.3. *In vitro* cell proliferation assay

BG-1 and OVCAR-3 were grown to confluence, trypsinized and resuspended in complete medium, seeded into 96-multiwell plates (Costar, Cambridge, MA, USA), 3000 cells/well and incubated for 24 hours. Cells were then treated in serum-free hormone media (as described under hormone treatment above) with or without estradiol at 1×10^{-9} M, tamoxifen at 2×10^{-6} M, or a combination of estradiol and tamoxifen. The conditioned medium was changed every 24 hours and cells were treated for 72 hours. The number of viable cells was determined at day three by using the MTS assay (20 μ L/well) (Promega, Madison, WI, USA) and absorbance was determined at 490 nm after 2 hours of incubation at 37°C.

2.4. *Animals, oophorectomy of mice, and tumor establishment*

Female athymic nude mice, BALB/c *nu/nu* (ages 6-8 weeks), from Taconic (Ry, Denmark) were housed in a pathogen-free isolation facility, with a light/dark cycle of 12/12 hours and fed with rodent chow and water *ad libitum*. The study was approved by the Linköping University animal ethics research board. Mice were anesthetized with intraperitoneal (i.p.) injections of ketamine/xylazine and oophorectomised, and 3-mm pellets containing 17β -estradiol, 0.18 mg/60 day release (Innovative Research of America, Sarasota, FL, USA), were implanted subcutaneously. The pellets provide a continuous release of estradiol at serum concentrations of 150-250 pmol/L, which is in the range of physiologic levels seen in mice during the estrous cycle as previously shown. One week after surgery, BG-1 cells (5×10^6 in 200 μ L PBS) were injected s.c. on the right hind side flank. The tumors were measured every second day, and the surface area was calculated $(\text{length}/2 \times \text{width}/2) \times \pi$. At a tumor size of approximately 50 mm², the mice were divided into two subgroups. One group continued with the estradiol treatment only, whereas ten days of tamoxifen treatment (1mg/every 2 days s.c.) was added to the estradiol treatment in the other group. At the end of experiments, mice were

ethanized and tumors explanted, paraffin-embedded for immunohistochemical analysis, or snap frozen in liquid nitrogen. Frozen tumor tissue were homogenized for 30 s at 2900 rpm by using a Micro-Dismembrator S (B. Braun Biotech International, Melsingen, Germany) and resuspended in 1 ml ice-cold PBS. Total protein content of tumor homogenates 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.

2.5. Patients and ascites samples

The local ethical committee approved the study and all women gave their informed consent. Ascites was collected from 17 patients, diagnosed with advanced ovarian carcinoma stage 3 or 4. None of the women had any ongoing treatment at the time of collection. Ascites was analyzed for estradiol and progesterone levels by using commercial quantitative immunoassay kits (DRG Instruments GmbH, Germany). The sensitivity of the estradiol and progesterone assays was 16 pmol/L and 0.15 nmol/L respectively. The intra-assay variations were 5% for both kits. All samples were assayed in duplicates.

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

Ascites, tumor tissue homogenates, and conditioned media from hormone-treated ovarian cancer cells were analyzed for MMP-2, MMP-9, TIMP-1 and TIMP-2 proteins and endostatin using commercial quantitative immunoassay kits; (MMP-2 (total) Quantikine[®], MMP-9 (total) Quantikine[®], TIMP-1 Quantikine[®], TIMP-2 Quantikine[®] and Endostatin Quantikine[®], R&D Systems, Minneapolis, MN, USA) without preparation. Assays were conducted according to manufacturer's guidelines. The analyzed proteins in conditioned media from hormone-treated BG-1 cells or tumor homogenates were correlated and normalized to the total protein content and expressed as pg/mg protein or ng/mg protein. All assays were

repeated twice on ascites samples and tumor homogenates and on more than one harvest of BG-1 cells.

2.7. Quantification of MMP-9 activity in vitro

The activity of MMP-9 was assayed using a gelatinase activity assay. Conditioned media from hormone-treated BG-1 cells was mixed 1:1 with 100 μ M 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 substrate can be cleaved by both active MMP-2 and active MMP-9. The mixture was incubated at room temperature for 20 minutes, with gentle agitation. Fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA), with λ_{ex} at 280 nm and λ_{em} at 360 nm. All experiments were performed in a low-light or light-free environment. MMP-2/MMP-9 activity was determined in relative fluorescence units, correlated to total protein content in cell lysates, and presented in diagrams as percentage fluorescence of control treated cells.

2.8. Inhibition of MMP-9 in vitro

BG-1 cells were treated with 2×10^{-6} M tamoxifen for 7 days in hormone medium. Hormone medium was changed every day. During the last 24 h of hormone treatment, a specific mouse anti-human MMP-9 neutralizing antibody, 0.1 μ g/mL or 1 μ g/mL (Calbiochem, Merck Biosciences Ltd, Nottingham, UK) was added. BG-1 treated with tamoxifen in combination with isotype murine IgG (R&D Systems, Minneapolis, MN, USA) served as control.

2.9. Immunohistochemistry of MMP-9

Formalin-fixed paraffin embedded tumors were cut in 3- μ m sections, deparaffinized, and subjected to anti-MMP-9 immunohistochemistry (rabbit anti-human MMP-9; dilution 1:50; Chemicon Int. Inc. CA, USA) with Envision detection (Dako, Carpinteria, CA, USA). Sections were counterstained with Mayer's hematoxylin. Negative controls (primary antibody omitted) did not show staining. All sections were first scanned to identify the range of intensity of the staining. In a blinded manner, five high power fields (x200) per section were examined and scored either as weakly or strongly positive.

2.10. Intratumoral microvessel density

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; Dako, Carpinteria, CA, USA). Sections were counterstained with Mayer's hematoxylin. Negative controls did not show staining. Images of three hot-spot areas of three tumors in each treatment group were acquired on an Olympus BX41 microscope at 200x magnification. The images were digitally analyzed and percentage of area stained positively for von Willebrand's factor was quantified using ImageJ software version 1.39u (NIH, USA).

2.11. Statistical analyses

Data are expressed as means \pm SEM. Student's t-test, Fisher's exact test, and one-way ANOVA with Bonferroni post-hoc test were used where appropriate. Correlations were tested using Pearson's correlation coefficient. $P < 0.05$ was considered as statistically significant. Graphpad Prism 5.0 was used for all statistical analyses (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Tamoxifen decreased proliferation of ovarian cancer BG-1 cells in vitro

BG-1 cells are derived from a patient with stage III, poorly differentiated ovarian adenocarcinoma, and express high levels of ER (twice as much as estrogen-dependent breast cancer MCF-7 cells) [22] making this cell-line an excellent model for investigating hormone responsiveness of ovarian cancer tissue [23,24]. OVCAR-3, was established 1982 from the malignant ascites of a patient with progressive adenocarcinoma of the ovary and were originally described as ER-positive [25]. Later publications regarding ER expression and estrogen responsiveness are however contradictory, and some studies suggest that OVCAR-3 do not express a functional ER [26-28]. In our laboratory we could not detect ER α in OVCAR-3 by immunocytochemistry (data not shown). To investigate the sensitivity of BG-1 to estradiol and tamoxifen regarding proliferation, the number of viable cells in culture was determined using the MTS assay. The amount of viable cells were significantly increased in BG-1 after 72 hours of estradiol exposure (1×10^{-9} M), ($P < 0.001$, Fig 1). Tamoxifen (2×10^{-6} M) treatment alone significantly inhibited cell proliferation ($P < 0.001$, Fig 1) as compared to control treated cells. The addition of tamoxifen to the estradiol treatment counteracted the estradiol-induced increase in proliferation ($P < 0.001$, Fig 1). Estradiol treatment of OVCAR-3 did not enhance proliferation compared to control treated cells as determined by using the MTS assay (estradiol treatment; 98.37 ± 3.865 % vs. 100.0 ± 5.426 % of control treated cells, $n = 8$).

3.2. Estradiol decreased and tamoxifen increased MMP-9 protein in conditioned media of cultured human ovarian cancer BG-1 cells

The balance between MMPs and TIMPs in the tumor microenvironment may play an important role in cancer progression and invasiveness. In order to evaluate the effects of

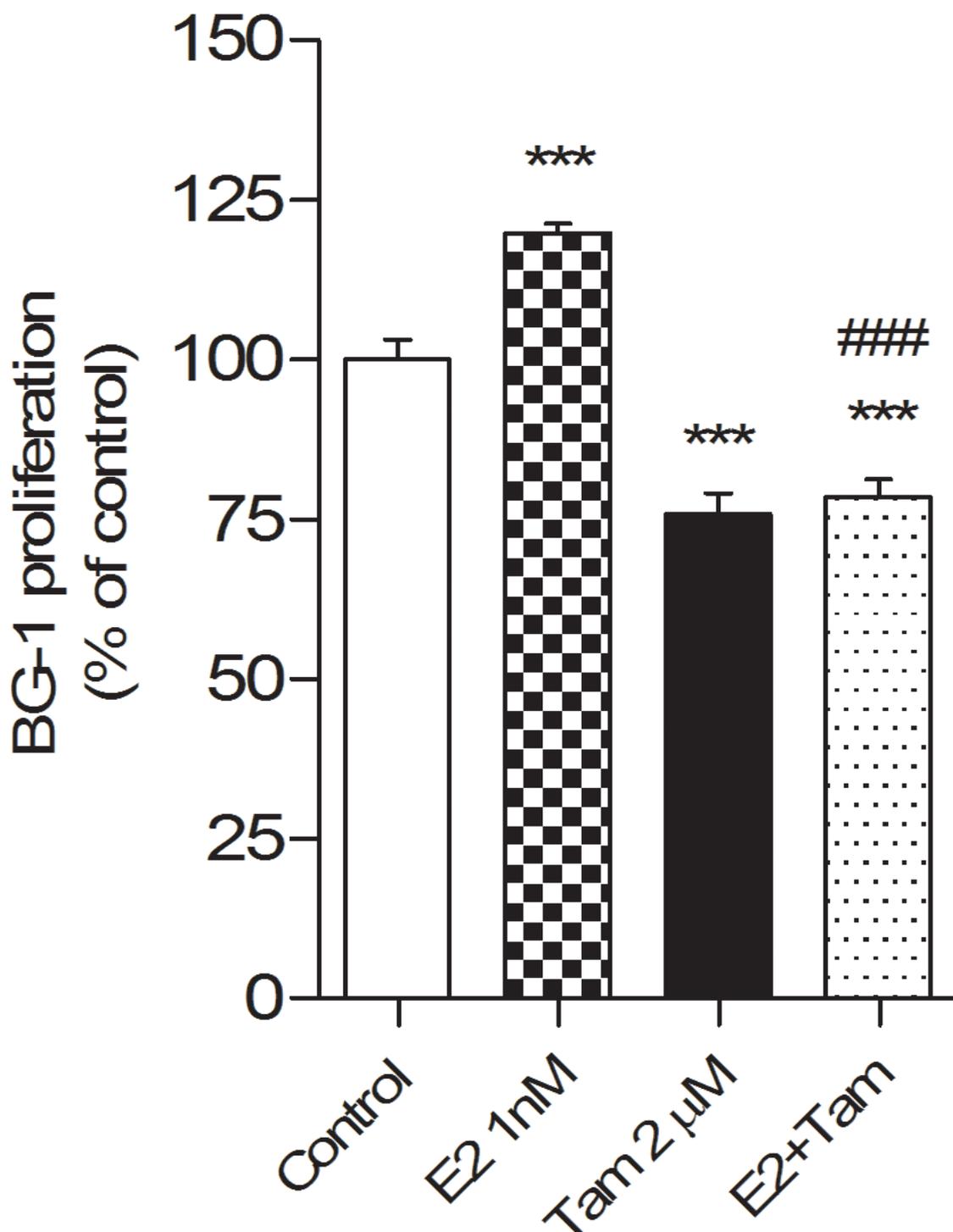


Figure 1. Tamoxifen decreased proliferation of ovarian cancer BG-1 cells. BG-1 were seeded into 96-multiwell plates and cultured in serum-free media without hormones (Control) or in the presence of 10^{-9} M estradiol (E2), 2×10^{-6} M tamoxifen (Tam), or a combination of estradiol and tamoxifen. MTS assay as described in materials and methods sections was performed after 72 hours of culture. Columns, mean ($n = 6-8$ in each group); bars, SEM. *** $P < 0.001$ vs control cells, ### $P < 0.001$ vs estradiol treatment.

estradiol and tamoxifen treatment on human ovarian cancer BG-1 cells regarding MMP-2, MMP-9, TIMP-1 and TIMP-2 protein levels, quantitative ELISA was carried out on conditioned media after 7 days of treatment. MMP-2 protein was not detectable in BG-1 supernatants in any of the treatment groups. TIMP-1 and TIMP-2 proteins were detected but expression levels were not significantly altered by either estradiol or tamoxifen treatment compared to control (Fig 2A and B). BG-1 cells exposed to estradiol at 1×10^{-9} M significantly decreased MMP-9 levels ($P < 0.01$), as compared to control (Fig 2C) whereas treatment with tamoxifen at 2×10^{-6} M alone exhibited a significant increase of secreted MMP-9 protein ($P < 0.001$) as compared to control. The addition of tamoxifen to estradiol treatment counteracted the estradiol-induced decrease of MMP-9 ($P < 0.001$), (Fig 2C). No altered expression was seen in estradiol-exposed (1×10^{-9} M) OVCAR-3 regarding the secreted levels of MMP-2, TIMP-1 or TIMP-2 compared to control-treated cells (MMP-2, 1199 ± 52.01 pg/mg protein (E_2) vs. 1275 ± 51.38 pg/mg protein (Control); TIMP-1, 20.22 ± 1.706 ng/mg protein (E_2) vs. 20.92 ± 1.349 ng/mg protein (Control); TIMP-2, 66.66 ± 3.811 ng/mg protein (E_2) vs. 66.30 ± 3.685 ng/mg protein (Control), $n = 5-6$ in each group). MMP-9 protein was not detected in supernatants from cultured OVCAR-3 of any treatment group.

3.3. Tamoxifen increased MMP activity in vitro

MMPs are secreted from the cell as inactive pro-enzymes and require proteolytic removal of the pro-domain in order to become active in the extracellular space. Additionally, activated MMP-2 and MMP-9 enzymes may be inhibited by their specific inhibitors TIMP-1 and TIMP-2. To determine the activity level of MMP-2/MMP-9 enzymes in conditioned media from estradiol- and tamoxifen-treated BG-1 cells, we used a fluorogenic substrate specific for MMP-2 and MMP-9. The substrate for MMP activity can be cleaved by both MMP-2 and MMP-9. However, as we show BG-1 cells do not contain any MMP-2 proteins, hence the

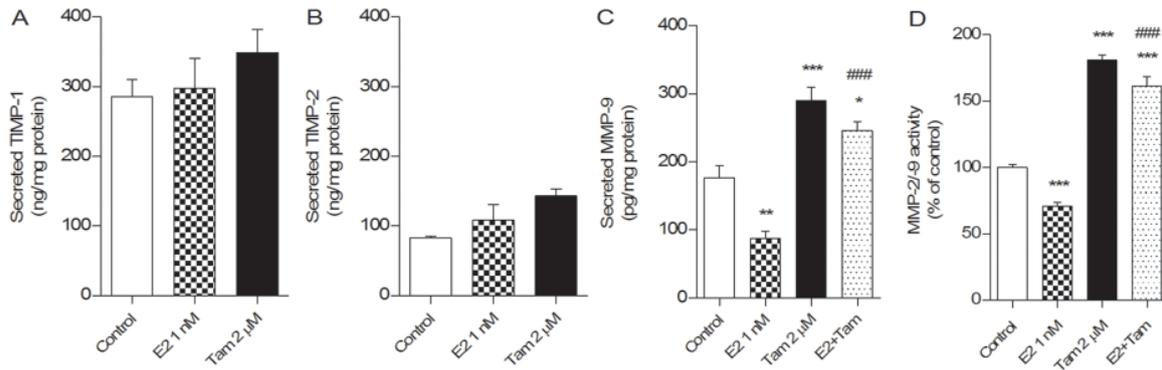


Figure 2. Secreted levels of TIMP-1, TIMP-2, MMP-9 proteins and MMP-2/-9 activity in conditioned media from hormone treated BG-1 cells. BG-1 cells were cultured in serum-free media without hormones (Control) or in the presence of 10^{-9} M estradiol (E2), 2×10^{-6} M tamoxifen (Tam), or a combination of estradiol and tamoxifen (E2+Tam) for 7 days. TIMP-1 (A), TIMP-2 (B), MMP-2 and MMP-9 (C) protein levels were investigated using quantitative ELISA. MMP-2/-9 activity (D) was investigated by using a fluorogenic substrate specific for both MMP-2 and MMP-9. MMP-2 protein could not be detected in conditioned media in any of the treatment groups. Therefore, the enzymatic activity reflects MMP-9 activity only. Columns, mean ($n = 6$ in each group); bars, SEM. * $P < 0.05$ vs control cells, ** $P < 0.01$ vs control cells, *** $P < 0.001$ vs control cells, #### $P < 0.001$ vs estradiol treatment.

measured activity reflects MMP-9 activity only. Conditioned media from 7 days of treatment was incubated with the substrate and the generated fluorescence was measured and correlated to total protein content. As Fig 2D shows, the enzymatic activity of MMP-2/-9 in conditioned media from estradiol-exposed BG-1 was significantly decreased ($P < 0.001$) compared to control treated cells, whereas tamoxifen treatment resulted in a significant increase in MMP-2/-9 activity ($P < 0.001$) vs control treated cells. The addition of tamoxifen to the estradiol treatment counteracted the estradiol-induced decrease of MMP activity ($P < 0.001$) as compared to estradiol treated cells, Fig 2D.

3.4. Tamoxifen induces endostatin generation via increased MMP-9 activity in vitro

Endostatin, the 20-kDa fragment of the carboxyl domain of collagen XVIII [29], is a potent endogenous inhibitor of angiogenesis and may be generated by active MMP-9 and to a lesser extent active MMP-2 by enzymatic cleavage [30]. Conditioned media from hormone-treated BG-1 cells were analysed for human endostatin using quantitative ELISA. As seen in Fig 3A,

tamoxifen treatment significantly increased levels of endostatin ($P < 0.001$) as compared to control, whereas conditioned media from estradiol-treated BG-1 cells exhibited significantly decreased levels of endostatin compared to control ($P < 0.01$). The addition of tamoxifen to the estradiol treatment counteracted the estradiol-induced decrease in endostatin levels ($P < 0.001$) as compared to estradiol treated cells, Fig 3A.

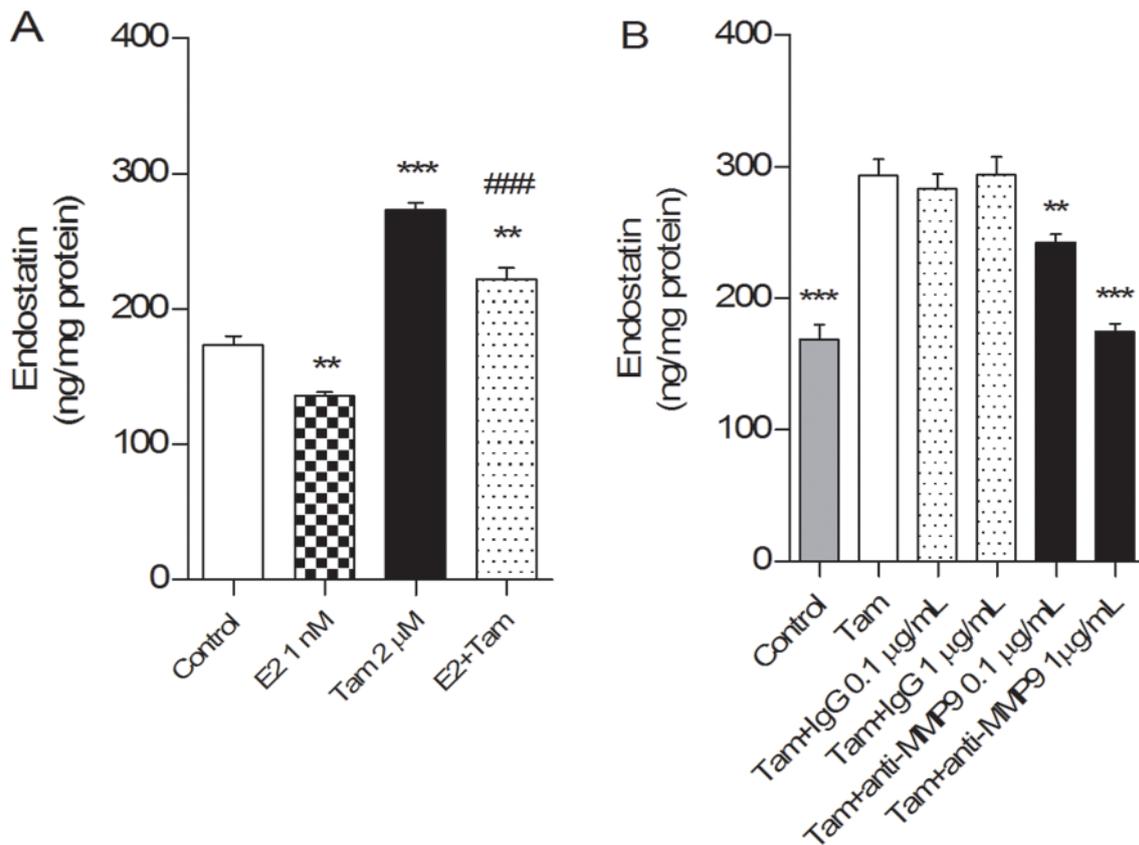


Figure 3. Increased endostatin generation of cultured BG-1 is mediated via tamoxifen-induced MMP-9 activity. BG-1 cells were hormone-treated for 7 days as described in Fig 2, and conditioned media analyzed for endostatin using quantitative ELISA. (A) Generated levels of endostatin in conditioned media of BG-1 treated with either estradiol, tamoxifen, or a combination of estradiol and tamoxifen. Columns, mean ($n = 6$ in each group); bars, SEM. ** $P < 0.01$ vs control cells, *** $P < 0.001$ vs control cells, ### $P < 0.001$ versus estradiol treatment. (B) Endostatin levels in BG-1 cultures after 7 days of tamoxifen exposure w/w/o the addition of a specific anti-human MMP-9 neutralizing antibody during the last 24 h. Columns, mean ($n = 6$ in each group); bars, SEM. ** $P < 0.01$ vs tamoxifen-treated cells, *** $P < 0.001$ vs tamoxifen-treated cells.

To explore whether MMP-9 induced the endostatin generation a neutralizing, specific anti-MMP-9 antibody was added to the last 24 h of tamoxifen treatment. Co-treatment of

tamoxifen and anti-MMP-9 (at 0.1 or 1 $\mu\text{g}/\text{mL}$) significantly decreased the levels of endostatin ($P < 0.01$ and $P < 0.001$, respectively) compared to tamoxifen treatment alone or in combination with isotype IgG (Fig 3B), indicating an MMP-9 dependent generation of endostatin by tamoxifen in these cells.

3.5. Tamoxifen treatment increased the expression of MMP-9 and endostatin levels in solid BG-1 tumors growing in mice

To further explore the effects of estradiol and tamoxifen on MMP-9 expression and endostatin generation, we extended our investigations to an *in vivo* model of solid BG-1 tumors growing in nude mice. These ovarian cancer cells require estrogen for tumor growth in mice; therefore, a non-treated control group is not possible to achieve. Following 10 days of treatment with estradiol w/wo tamoxifen, animals were sacrificed and tumors excised. Tumor tissue homogenates were analyzed for MMP-9 protein using quantitative ELISA. As Fig 4A shows, significantly higher levels of MMP-9 protein were found in tumors of estradiol+tamoxifen treated animals than in tumors from mice treated with estradiol only ($P < 0.01$). Immunohistochemical staining of paraffin-embedded tumor tissue sections confirmed the presence of human, i.e. tumor cell-derived, MMP-9 in both treatment groups. Tumors derived from estradiol+tamoxifen treated animals showed a higher intensity of staining compared to tumors from mice treated with estradiol. In tumors from estradiol-treated animals, 7 of 25 areas were scored as strongly positive, whereas 19 of 25 areas were strongly positive in estradiol+tamoxifen group ($P < 0.01$). Representative sections are shown in Fig 4B.

Tumor tissue homogenates were additionally analysed for endostatin using quantitative ELISA. As Fig 4C shows, the generated levels of endostatin were significantly enhanced in tumor tissue of estradiol+tamoxifen treated animals, compared to tumors from the estradiol-treated group ($P < 0.05$).

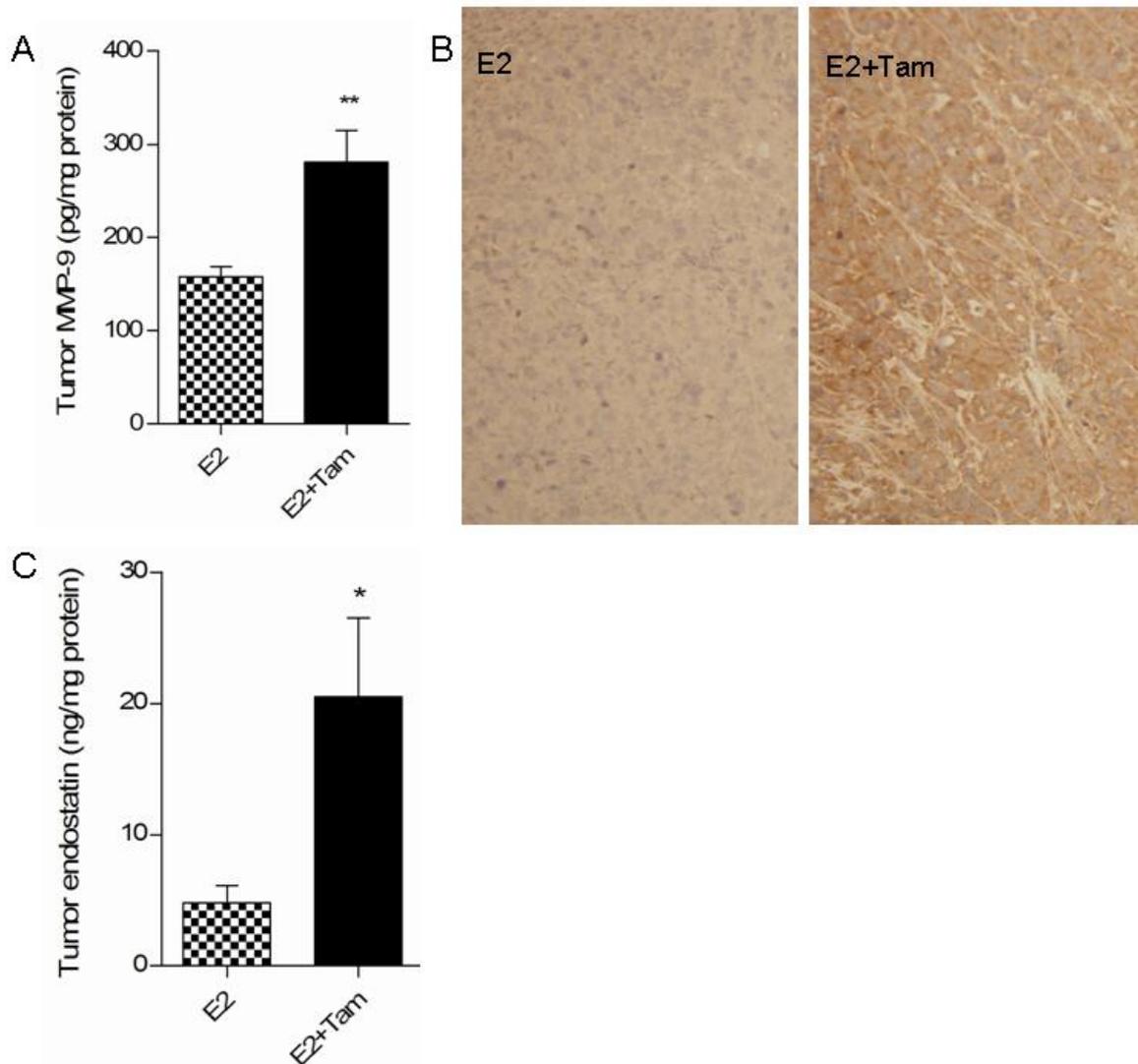


Figure 4. Tamoxifen enhanced MMP-9 protein and endostatin levels in solid BG-1 tumors. Mice were oophorectomised and supplemented with a physiologic level of estradiol. ER-positive BG-1 cells were injected s.c., and tumors were formed on the right hind flank. One group of mice continued with estradiol only (E2), and in the other group tamoxifen treatment was added to the estradiol treatment (E2+Tam). (A) MMP-9 protein levels in BG-1 tumor tissue homogenates. Tamoxifen treatment significantly increased MMP-9 protein levels. (B) Immunohistochemistry was performed as described in Materials and methods section. In tumors from estradiol treated animals, 7 of 25 areas were scored as strongly positive, whereas 19 of 25 areas were strongly positive in the estradiol+tamoxifen group ($P < 0.01$). Representative immunohistochemical MMP-9 stained tumor sections from each treatment group (magnification 200x). (C) Endostatin levels in BG-1 tumor tissue homogenates. Tamoxifen treatment significantly increased tumor endostatin levels. Columns, mean ($n = 5-6$ in each group); bars, SEM. * $P < 0.05$, ** $P < 0.01$ vs estradiol treatment.

3.6. Tamoxifen decreased tumor vasculature and tumor growth

To further investigate if tumors from the estradiol+tamoxifen treated mice, exhibiting increased MMP-9 and endostatin levels compared to estradiol treatment alone, affected tumor angiogenesis, we quantified vessel area of BG-1 tumors stained with anti-von Willebrand's factor. Fig 5A shows representative sections of both treatment groups. We found that the vessel density was significantly decreased in tumors from estradiol+tamoxifen treated mice compared to tumors from estradiol-treated mice ($P < 0.001$, Fig 5B). Tumor growth was monitored during treatment with estradiol and estradiol+tamoxifen. Tumor area of estradiol+tamoxifen treated mice were significantly lower than tumors from mice treated with estradiol only ($P < 0.05$), Fig 5C.

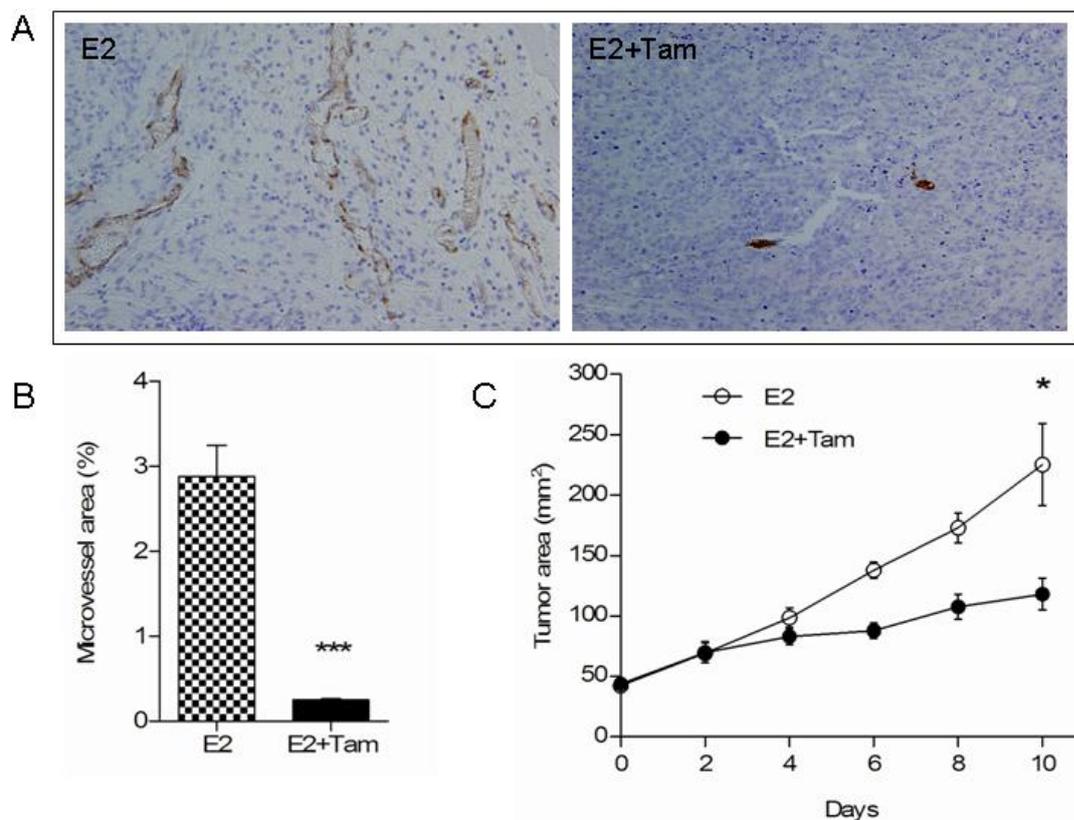


Figure 5. Tamoxifen decreased microvessel area and tumor growth of solid BG-1 tumors in nude mice. BG-1 tumors were formed and mice were treated as described in Fig. 4. Tumor sections were stained with anti-von Willebrand's factor and vessel area was counted. (A) Representative sections from estradiol (E2) and estradiol+tamoxifen (E2+Tam) exposed tumors stained with anti-von Willebrand's factor (magnification 200x). (B) Tumor vessel area (quantification was conducted as described in the Materials and methods section), Columns, mean ($n = 9$ in each group); bars, SEM, *** $P < 0.001$. (C) Tumor growth after treatment with estradiol (E2; unfilled circles) only, or estradiol+tamoxifen (E2+Tam; filled circles). Circles, mean ($n = 5-6$ in each group); bars, SEM, * $P < 0.05$.

3.7. Positive correlation of MMP-2 and endostatin in ascites from ovarian cancer patients

To investigate the physiologic characteristics of ovarian cancer patients, ascites was collected from ovarian cancer patients without any ongoing treatment, and analysed for estradiol, progesterone, MMP-2, MMP-9, TIMP-1, TIMP-2 and endostatin. Estradiol levels were low to absent (13 ± 7 pM); progesterone levels (0.35 ± 0.05 nM); MMP-2 levels (61 ± 1.4 nM); MMP-9 levels low to absent (1.1 ± 0.39 nM); TIMP-1 levels (3723 ± 296 nM); TIMP-2 levels (446 ± 35 nM) and endostatin levels (23 ± 1.2 nM). Patient characteristics are shown in Table I. A significant correlation was found between ascites MMP-2 and ascites endostatin ($r = 0.6605$, $P = 0.0039$, $n = 17$, Fig 6). No other correlations were found.

Table 1
Patients and ascites characteristic. All tumors demonstrated serous papillary adenocarcinoma histology.

Patient No.	Age	Stage	Estradiol (pg/mL)	Progesteron (ng/mL)	MMP-2 (ng/mL)	MMP-9 (ng/mL)	TIMP-1 (ng/mL)	TIMP-2 (ng/mL)	Endostatin (ng/mL)
1	77	3	37	0.6	54.6	0.0	5169	517	14.7
2	65	4	0	0.7	61.8	0.4	4486	319	24.9
3	68	4	0	0.8	52.7	0.0	3463	520	14.7
4	61	4	0	0.1	66.6	1.3	3793	497	20.9
5	72	3	0	0.2	62.8	0.2	3268	345	22.9
6	51	3b	39	0.2	61.3	6.1	1875	239	24.3
7	82	4	0	0.3	70.6	0.3	2848	319	26.4
8	67	4	0	0.3	62.0	2.7	4961	410	19.3
9	56	3c	0	0.4	56.2	0.0	3060	391	20.3
10	65	3	0	0.2	55.0	1.8	2151	395	19.5
11	56	3c	0	0.3	58.6	0.1	5771	809	23.5
12	71	3c	0	0.2	73.4	3.0	6110	626	34.6
13	79	4	0	0.2	57.4	1.2	2386	547	15.5
14	69	3	37	0.5	55.9	0.4	3210	444	24.2
15	66	3c	0	0.5	63.7	0.1	3884	562	24.2
16	59	3c	0	0.2	56.1	1.0	3590	361	28.5
17	34	3c	106	0.2	60.3	0.2	3264	270	25.4

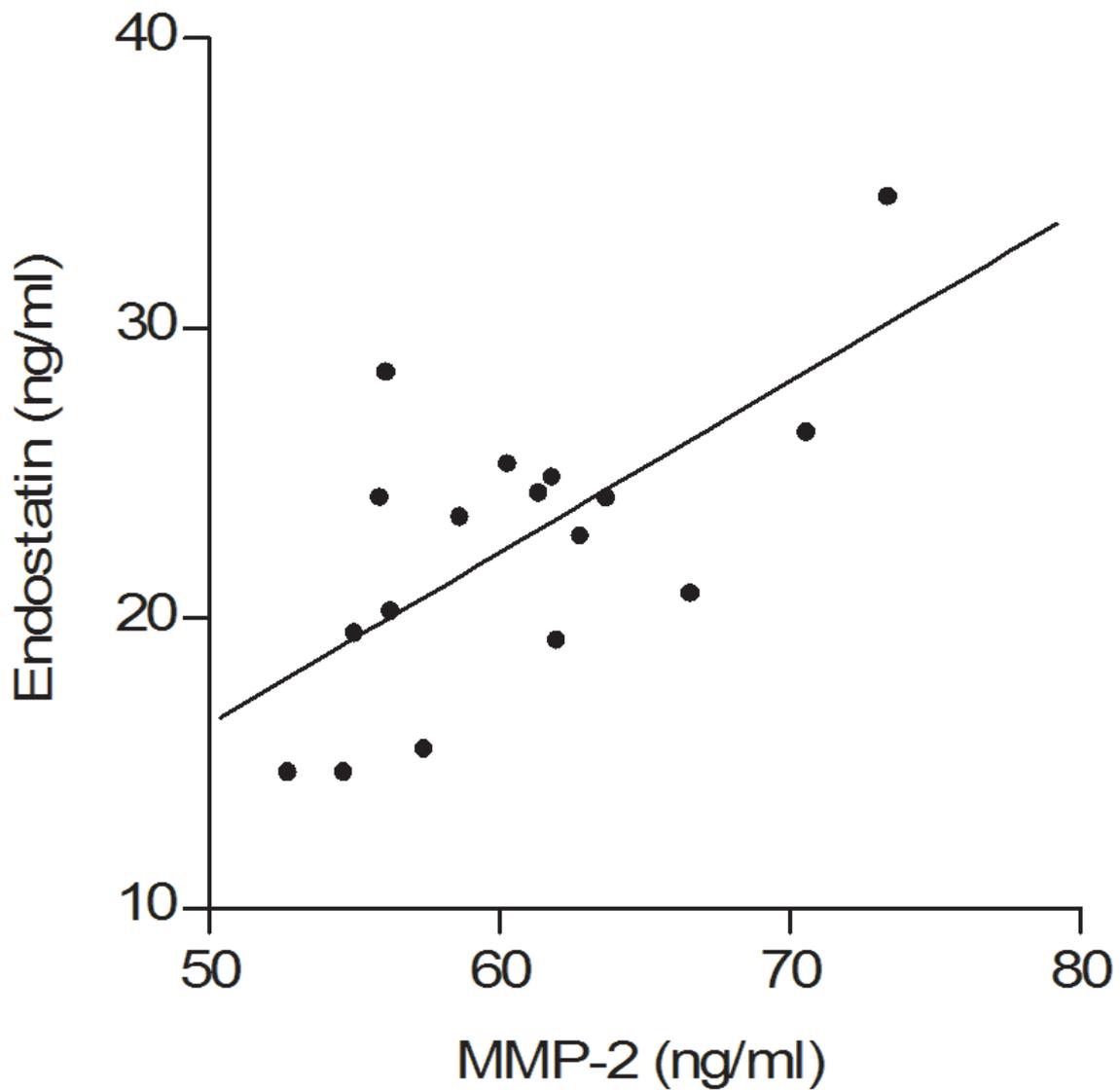


Figure 6. Positive correlation of MMP-2 and endostatin in ascites. Ascites collected from ovarian cancer patients without ongoing treatment was analysed for endostatin and MMP-2 using quantitative ELISA. Endostatin and MMP-2 correlated significantly ($r = 0.6605$, $P = 0.0039$, $n = 17$).

4. Discussion

In this study we show that tamoxifen increased and estradiol decreased extracellular MMP-9 protein level and activity of estradiol-responsive ovarian cancer BG-1 cells in culture. Further on, tamoxifen exposure of BG-1 significantly increased the generation of endostatin, an effect that could be reversed by the addition of a neutralizing MMP-9 antibody. TIMP-1 and TIMP-2 protein levels were not significantly altered by hormonal exposure. Ovarian BG-1 tumor explants in nude mice treated with tamoxifen showed significantly increased MMP-9 expression and endostatin levels, and exhibited reduced vascularity and tumor area compared to tumors treated with estradiol alone. In ascites collected from ovarian cancer patients there was a significant positive correlation between MMP-2 and endostatin whereas MMP-9 levels were low to absent.

Ovarian cancer is the gynaecologic malignancy with the highest mortality rate in the Western world [31]. The standard first-line therapy involves cytoreductive surgery followed by chemotherapy with platinum or platinum/taxane combination [32]. Despite initial high response rates, the majority of women will develop progressive disease and die of complications of their malignancy. In breast cancer, anti-estrogen therapy in the adjuvant setting has clearly improved overall survival of women and its activity has also been proved to be important in metastatic disease [33]. Although epidemiologic studies suggest that hormones play a key role in ovarian cancer, and that ER and PR are expressed in approximately 50% of cases [34-36], the therapeutic value of endocrine therapy as an adjuvant has never been assessed. Moreover, most trials of anti-estrogen therapy in ovarian cancer have been performed on heavily pre-treated patients, some of them refractory to chemotherapy, and in most cases with unknown receptor-status [37-40].

Angiogenesis is necessary for tissue growth, wound healing, and for a functional menstrual cycle. The angiogenic state in the tissue is highly regulated and maintained by the intricate

balance of pro- and anti-angiogenic mediators. Unregulated angiogenesis is involved in several pathological states [41]. As tumor growth beyond 1-2 mm³ is dependent on a vascular supply [42], pro-angiogenic proteins, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), need to exceed the local concentration of anti-angiogenic proteins in the tumor microenvironment. Endostatin is a powerful endogenous inhibitor of angiogenesis derived from collagen XVIII [29] that is generated by enzymatic cleavage. Endostatin upregulates anti-angiogenic genes and downregulates pro-angiogenic pathways leading to inhibition of endothelial cell proliferation and migration, induces apoptosis and causes G1 arrest of endothelial cells [43]. Increasing amounts of data show that several members of the MMP family may have tumor suppressing roles, mainly by decreasing tumor angiogenesis [6,7,9,11,12]. We have previously reported that tamoxifen increased MMP-9 activity in experimental hormone-dependent breast cancer [10,21], and that over-expression of MMP-9 by intratumoral gene transfer resulted in tumor regression via enhanced endostatin generation and decreased angiogenesis [11]. Hormonal treatments of ovarian cancer BG-1 cells in the present study confirm that they indeed are highly estrogen-sensitive as the cells respond to estradiol with increased proliferation, and to tamoxifen with decreased proliferation. Further on, we show that endocrine responsive ovarian cancer cells are regulated by estradiol and tamoxifen in a similar fashion as ER positive breast cancer regarding MMP activity and generation of endostatin.

These results supports previous studies, pointing out a therapeutic effect by increased MMP-9 levels and suggest that tamoxifen may decrease ovarian cancer growth by affecting MMP-9.

Ovarian cancer is characterised by accumulation of ascitic fluid providing the extracellular environment of the cancer cells. The ascites may also facilitate the spread of disseminated tumor cells to other sites in the peritoneal cavity. Our present data show that MMP-2 levels were high in the ascites of all patients suggesting that MMP-2 may be expressed

constitutively in ovarian cancer. Contrary to MMP-2, MMP-9 levels were low to absent in the ascites suggesting that MMP-9 may be an inducible protein in these patients. As we show in the present study, tamoxifen has the capacity to increase MMP-9 protein levels. This may result in increased generation of the anti-angiogenic protein endostatin leading to decreased angiogenesis. Thus, tamoxifen may have therapeutic effects in ovarian cancer via MMP-9 induced increase of endostatin generation that may tip the balance in favor of an anti-angiogenic milieu and by decreasing cell proliferation as shown in our present results. In this set of clinical patient samples, 17 women were included. There was a wide range of tumor stage, stage 3B to 4, among these patients. Moreover, some patients were radically operated with minor tumor burden, whereas other carried a massive tumor load. In this heterogenous patient material we could not detect any correlation between levels of endostatin and clinical prognosis. This does not rule out endostatin as a prognostic factor in a larger patient cohort. Taken together, we show that similar to estrogen dependent breast cancer, estradiol increased and tamoxifen decreased cell proliferation in endocrine responsive ovarian cancer cells. MMP-9 protein expression and activity were induced by tamoxifen leading to increased endostatin generation. By up-regulating MMP activity and endostatin levels with tamoxifen, tumor angiogenesis may be limited. Moreover, increased levels of MMPs may bind excess TIMPs and thereby inhibit growth-promoting activities that may be induced by TIMPs [16]. The physiological relevance of MMP activity and endostatin generation was further demonstrated in ovarian cancer patients where MMP-2 and endostatin levels in ascites correlated significantly. The low to absent levels of MMP-9 in ascites opens for a possibility to therapeutically increase MMP-9 by the use of tamoxifen, and thereby enhance the generation of anti-angiogenic fragments. This is a previously unknown mechanism of estradiol and tamoxifen in ovarian cancer and may have therapeutic implications in the treatment of ovarian cancer patients.

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Conflict of Interest

All authors state that there is not any financial support of this study that may pose conflict of interest.

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