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Estradiol Increases IL-8 Secretion of Normal Human Breast Tissue and Breast Cancer In Vivo

Christina Bendrik and Charlotta Dabrosin

IL-8 or CXCL8 has been associated with tumor angiogenesis, metastasis, and poor prognosis in breast cancer. Estrogen is crucial in breast carcinogenesis and tumor progression. Whether sex steroids affect IL-8 secretion of normal breast tissue or breast cancer is not known. Several cell types in a tissue secrete IL-8. Hence, regulatory mechanisms of IL-8 need to be investigated in whole tissue. We used microdialysis to sample IL-8 in normal human breast tissue in situ in pre- and postmenopausal women, preparatively in breast cancers of women, and in experimental breast cancer in mice. We found a significant positive correlation between IL-8 and estradiol in normal breast tissue and hormone-dependent breast cancer in vivo. Ex vivo, estradiol exposure increased the IL-8 secretion of normal whole breast tissue in culture. In experimental breast cancer, estradiol increased IL-8 whereas the anti-estrogen tamoxifen inhibited the secretion of IL-8 both in vitro and extracellularly in vivo in tumors of nude mice. An anti-IL-8 Ab inhibited endothelial cell proliferation induced by cancer cell produced IL-8 and tumors with low IL-8 levels exhibited decreased angiogenesis. Our results strongly suggest that estradiol has a critical role in the regulation of IL-8 in normal human breast tissue and human breast cancer. IL-8 may present a novel therapeutic target for estrogen driven breast carcinogenesis and tumor progression. The Journal of Immunology, 2009, 182: 371–378.

Sex steroids are crucial for development and function of the normal human breast but long-term exposure increases the risk of breast cancer (1–4). In addition to direct genetic cellular alterations of epithelial cells, the interaction with the tissue microenvironment is critical for cancer development. Events in the stroma such as inflammation, angiogenesis, and protease activities all contribute to tumor growth, invasion, and metastasis. Sex steroids have the capacity to affect all these events during the carcinogenic process in breast tissue, but the knowledge of this regulation is still limited.

IL-8, or CXCL8, originally described as a chemotactic factor for leukocytes, may contribute to cancer growth by its mitogenic and angiogenic actions (5). In breast cancer patients, high expression of IL-8 and its receptors and high serum levels have been associated with poor prognosis (6–8). Experimental studies of breast cancer cell lines and endothelial cells have revealed a strong correlation between the metastatic potential of breast carcinoma cells and the expression of IL-8 (9, 10), although inhibition of IL-8 alone did not lead to tumor regression but required a combination with anti-epidermal growth factor receptor for increased survival of mice (11). Several normal cell types and tumor cells express IL-8 and its receptors CXCR1 and CXCR2, and the expression may be regulated on a direct cellular level (8, 12, 13). However, tissue levels of IL-8 are dependent on the release by the various cell types present in the tissue and by microenvironmental factors, such as hypoxia, acidosis, and NO (5).

The effects of sex steroids on IL-8 expression seem to be complex and the existing data are conflicting; IL-8 expression by estrogen may also differ from one tissue to another. Estrogen has been shown to have an inhibitory effect on IL-8 production on epithelial cells, whereas immune cells may be stimulated to produce IL-8 by estrogen (14–16). In experimental breast cancer, IL-8 expression seems to be inversely correlated to estrogen receptor (ER) status, i.e., overexpressed in ER-negative invasive breast cancer cell lines compared with ER-positive breast cancer cell lines (14, 17). In one study, estrogen exposure to ER-negative cells transfected with the ER had no effects on IL-8 secretion (17). Yet, other studies have shown that estradiol in combination with progesterone or TNF enhance the induction of IL-8 secretion in ER-positive breast cancer cells (18, 19). In contrast, it has been shown in ER-expressing cornea cells that estradiol up-regulates IL-8 (20). These experiments were performed in cell culture with the known drawbacks as non-physiological conditions. In whole human endometrial tissue, which is entirely dependent on sex steroids for its function and where 100% of the cells express the ER, estradiol has been shown to indirectly up-regulate IL-8 (21). Moreover, in dendritic cells, estradiol significantly increased secretion of IL-8 (16). The above studies illustrates the complex regulation of IL-8, both at a cellular level and at a whole tissue level where all cell types interact with each other.

Microdialysis is an in vivo sampling technique, which reflects the extracellular space and molecules secreted by different cell types in a tissue. We have previously adapted this technique in human breast tissue, and we have also developed an ex vivo tissue culture model of whole normal breast tissue (22, 23). In the present study, microdialysis was used to sample IL-8 in vivo from normal human breast tissue in pre- and postmenopausal women, preparatively in breast cancer tumors, and in experimental breast cancer in nude mice. Whole normal breast tissue and breast cancer cells

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Abbreviations used in this paper: ER, estrogen receptor; PgR, progesterone receptor; VEGF, vascular endothelial growth factor.
were cultured and exposed to estradiol and progesterone. Our results revealed that estradiol has a significant effect on IL-8 secretion both in normal breast tissue and breast cancer in vitro and in vivo.

Materials and Methods
Subjects
The local ethical committee approved the study, and all women gave their informed consents. Of the healthy volunteer women, 12 were premenopausal (aged 22–30 years) and 6 postmenopausal (aged 52–55 years). All had been off sex steroid containing medication such as contraceptive medication both in normal breast tissue and breast cancer in vitro and

Microdialysis of healthy volunteers and breast cancer patients
A microdialysis catheter (CMA/Microdialysis AB), which consists of a tubular dialysis membrane (10 mm long × 0.52 mm in diameter, 100,000 atomic mass cut-off) glued to the end of a double-lumen tube (100 mm long × 0.8 mm in diameter), was used. The catheters were inserted guided by a catheter for i.v. use (Venflon 1.4 mm; BOC Ohmeda AB). The catheters were connected to a microinfusion pump (CMA 107; CMA/Microdialysis AB) and perfused with NaCl 154 mmol/L and dextran-70 40g/l, at a perfusion rate of 0.5 µL/min. The solution entered the catheter through the outer tube and left through the inner tube, from which it was collected. After a 90-min equilibration period, the outgoing perfusate was collected and stored at −70°C for subsequent analysis.

Mepivacaine (5 mg/ml) was administrated intracutaneously as a local anesthetic before the insertion of the microdialysis catheters. In the healthy volunteers, one microdialysis catheter was placed in the upper lateral quadrant of the left breast and directed toward the nipple as previously described (24–27). In the premenopausal women, a second catheter was inserted in abdominal s.c. fat. The breast cancer patients were investigated on the day before or the same day as surgery. In these women, the microdialysis catheters were inserted intratumorally in the center of the tumors. No complications occurred during or after the microdialysis experiments.

Microdialysis is a technique that allows continuous sampling of the extracellular fluid by passive diffusion of substances over a semipermeable membrane. The recovery, i.e., the amount of substances from the tissue that diffuses into the perfusion fluid, depends on the membrane properties, the flow rate, and the size of the compound of interest (28). Diffusion of low molecular substances over the dialysis membrane is almost complete at low flow rates using a 30-mm long dialysis membrane (29). However, for larger molecules, the recovery over the membrane decreases and the measured levels in the microdialysis sample will not be absolute concentrations in the tissue. The recovery of certain substances may be measured in vitro by putting a microdialysis in a vial containing the compound of interest, perfuse the catheter, and divide the concentration of the substance in the dialysate by the concentration in the vial. This in vitro recovery can only be an estimate of the in vivo recovery since other factors such as tissue pressure and temperature will affect the diffusion of substances. The in vitro recovery of IL-8 was 15.3 ± 0.35%. The microdialysis results in the present paper are, however, given as the raw data without any re-calculations based on the in vitro recovery.

Breast tissue biopsies
Biopsies from human breast tissue were obtained from premenopausal women, without ongoing hormonal treatment, undergoing routine reduction mammoplasty as previously described (23). Tissue biopsies containing epithelium, stroma, and adipose tissue were produced by using a 8-mm biopsy punch (Kai Europe) and placed in a 12-well plate (Costar). Serum-free medium was used consisting of a 1:1 mixture of nutrient mixture F-12 (Invitrogen) and DMEM without phenol red (Invitrogen) supplemented with transferrin (10 µg/ml; Sigma-Aldrich), insulin (1 µg/ml; Sigma-Aldrich), and BSA (0.2 mg/ml; Sigma-Aldrich) with or without physiological levels of estradiol (17β-estradiol; E2; Sigma-Aldrich) 10−8 M or a combination of 10−8 M estradiol and 10−8 M progesterone (E2 + P4; Sigma-Aldrich). Control biopsies were incubated with the vehicle, ethanol, equivalent to the hormone treated groups (0.001%). The biopsies were treated for 7 days at 37°C in a humidified atmosphere containing 5% CO2 and the medium was changed every day.

After the seventh day of incubation, the medium from each biopsy was collected and stored at −70°C and the biopsies were formalin fixed and embedded in paraffin for subsequent analyses.

Cells and culture conditions
MCF-7 (HTB-22; human breast adenocarcinoma, ER- and PR-positive) cells were obtained from ATCC. Cells were cultured in DMEM without phenol red supplemented with 2 mM glutamine, 50 IU/ml penicillin-G, 50 µg/ml streptomycin, and 10% FBS at 37°C in a humidified atmosphere of 5% CO2. Cell culture medium and additives were obtained from Invitrogen 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 6-cm dishes (Costar 20,000 cells/disc). Cells were incubated in the DMEM-based culture medium for 24 h and then treated with or without 10−8 M estrogen (17β-estradiol; Sigma-Aldrich), 10−6 M tamoxifen (Sigma-Aldrich), or a combination of estrogen and tamoxifen for 7 days. Hormones were added 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 transferrin (Sigma-Aldrich), 1 µg/ml insulin (Sigma-Aldrich), and 0.2 mg/ml BSA (Sigma-Aldrich). The hormone incubation was changed every day. After hormone treatment, the conditioned media was collected from a washed PBS wash. Total protein content was determined using Bio-Rad Protein Assay with BSA as standard (Bio-Rad). Samples were stored at −70°C until subsequent analyses.

Animals and ovariectomy of mice
Female athymic nude mice (6- to 8-wk old) were purchased from Taconic M&B. 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), ovariectomized, and 3-mm pellets containing 17β-estradiol, 0.18 mg/60-day release (Innovative Research of America) were implanted s.c. 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 × 104 cells in 200 µL PBS) were injected s.c. on the right hind side flank or in the mammary fat pad. Tumor volume was measured by length, width, and depth of the tumor every 4 days using a caliper. At a tumor size of ~300 mm3, the mice were divided into two subgroups. One group continued with the estradiol treatment only, while tamoxifen (1 mg/every 2 days s.c.) was added to the estradiol treatment in the other group.

Microdialysis experiments of breast cancer explants in mice
Tumor-bearing mice were anesthetized with an i.p. injection of ketamine/xylazine and thereafter kept anesthetized by repeated s.c. injections of ketamine/xylazine. A heating lamp maintained body temperature. A small skin incision was made and microdialysis probes (CMA/20, 0.5-mm diameter; PES membrane length 10 or 4 mm, 100 kDa cutoff; CMA/Microdialysis) were inserted into tumor tissue and sutured to the skin. The probes were connected to a CMA/102 microdialysis pump (CMA/Microdialysis) and perfused at 1 or 0.6 µL/min with saline containing 154 mM NaCl and 40 mg/ml dextran (Pharma- link). After a 90-min equilibration period, the outgoing perfusates (microdialysisates) were collected on ice and stored at −70°C for subsequent analysis. At the end of the experiments, the mice were euthanized and the tumors excised. The removed tumors were weighed, formalin-fixed, and subsequently embedded in paraffin for immunohistochemical analysis.

Determination of estradiol, progesterone, and IL-8
Plasma of women undergoing microdialysis was collected using a glass tube containing sodium citrate 3.8% as an anticoagulant, and were spun down and frozen at −70°C within 20 min of collection. Microdialysis, plasma, and tissue culture samples were analyzed for IL-8, estradiol, and progesterone using commercial quantitative immunonassay kits, IL-8 by using the Fluorokine MAP cytokine multiplex kits (R&D systems), and analyzed with the Luminex 100 analyzer. The sensitivity of IL-8 was 0.39 pg/ml and intra-assay variation 4.6–7.8%. Estradiol and progesterone were analyzed using ELISA kits (DRG Instruments). The sensitivity of the estradiol assay was 16 pmol/l and the intra-assay variation 5%. For progesterone, the cut-off level for luteal phase was 13 nmol/L, and the intra-assay variation 5%. The in vivo recovery since other factors such as tissue pressure and temperature will affect the diffusion of substances. The in vitro recovery of IL-8 was 15.3 ± 0.35%. The microdialysis results in the present paper are, however, given as the raw data without any re-calculations based on the in vitro recovery.
**Immunohistochemistry**

Formalin-fixed, paraffin-embedded normal breast tissue biopsies and tumors of nude mice were cut in 3-μm sections, deparaffinized, and subjected to anti-IL-8 immunohistochemistry (mouse anti-human IL-8, dilution 1/20 for breast biopsies and goat anti-human IL-8, dilution 1/20 for MCF-7 tumors (R&D Systems), with Envision detection (Dako)). Sections were counterstained with Mayer’s hematoxylin. Negative controls with isotype IgG did not show staining. All sections were first scanned to identify the range of intensity of the staining. Thereafter, the staining on each tumor or biopsy section was scored either as weakly or strongly positive. In a blinded manner, 10 high power fields (x200) were examined per section.

**Intratumoral microvessel density**

Formalin-fixed, paraffin-embedded tumors and breast tissue biopsies were cut in 3-μm sections, deparaffinized, and subjected to anti-von Willebrand’s factor (rabbit anti-human von Willebrand; dilution 1/1000; Dako). Sections were counterstained with Mayer’s hematoxylin. Negative controls did not show staining. In a blinded manner, 10 high power fields (x200) were examined of three different sections in each group. Vessel quantification of tumor sections was conducted as described previously using a Nikon microscope equipped with a digital camera (30). 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.

**HUVECs and in vitro cell proliferation assay**

HUVECs were isolated from female donors by collagenase digestion at 37°C for 20 min as previously described (31). Cells were grown in medium consisting of Medium 199 without phenol red supplemented with 1% non-essential amino acids, 1.6 mM glutamine, 4 IU/ml penicillin G, 4 μg/ml
media was measured using ELISA, supplemented with hormone solvent (Control) for 7 days. IL-8 in culture was mined at 490 nm after 2 h incubation at 37°C. The conditioned medium was changed every 24 h and HUVECs were treated for 72 h. The number of viable cells was determined at day 3 by the MTS assay (20). Tissue sections were stained with an antibody directed against IL-8 (Amgen) and a secondary antibody coupled to Cy3. The immunohistochemistry staining revealed that the epithelial cells showed most intense staining for IL-8 compared with the stroma. There was an increased intensity of staining and a greater

**FIGURE 2.** Secreted IL-8 after hormone exposure of whole normal breast tissue in culture. Breast biopsies were cultured in the presence of estradiol (E2; 10^{-7} M), a combination of estradiol and progesterone (E2 + P4; 10^{-8} M and 10^{-7} M, respectively), or serum-free medium alone supplemented with hormone solvent (Control) for 7 days. IL-8 in culture media was measured using ELISA, n = 6 in each group, ***, p = 0.0027 compared with control cells; *, p = 0.03 compared with control cells.

**Statistics**

Data are expressed as mean ± SEM. Student’s t test, ANOVA with Fisher’s post hoc test, and Pearson’s correlation coefficient with Fisher’s r to z test for the corresponding p values were used as appropriate. A p < 0.05 was considered as statistically significant.

**Results**

Significant positive correlation between E2 and extracellular IL-8 in normal breast tissue

There were no subsequent complications after the microdialysis experiments.

In the twelve women subjected to microdialysis in normal breast and s.c. fat, there was a positive significant correlation between extracellular local E2 levels and extracellular breast IL-8, r = 0.828, p = 0.0009, n = 12, Fig. 1A. There was also a significant correlation between local breast IL-8 and plasma E2, r = 0.716, p = 0.009, n = 12, Fig. 1B. There was no correlation between plasma P4 and local extracellular breast IL-8, r = 0.177, p = 0.6, n = 12, Fig. 1C. Microdialysis was also performed in abdominal s.c. abdominal fat. There was no significant correlation between extracellular fat IL-8 and extracellular local fat E2, r = 0.376, p = 0.23, n = 12, Fig. 1D. There was no significant correlation between extracellular fat IL-8 and plasma E2, r = 0.347, p = 0.3, n = 12, Fig. 1E; and no significant correlation was found between extracellular fat IL-8 and plasma P4, r = 0.043, p = 0.9, n = 12, Fig. 1F.

There was no correlation between plasma IL-8 and plasma E2 levels, r = 0.172, p = 0.6. Six postmenopausal women were investigated with microdialysis in normal breast tissue to include a group with very low endogenous estrogen levels. Fig. 1G shows a significant correlation between local E2 levels and local IL-8 levels in this larger cohort, r = 0.82, p < 0.0001, n = 18.

Increased secretion of IL-8 by estradiol incubation of normal human breast tissue biopsies

We have previously shown that breast biopsies maintain tissue morphology without developing necrosis during 7 days of culture analyzed with H&E staining and luminal cytokeratin 18 staining (23). We have also shown that ER and progesterone receptors (PgR) are preserved and that the breast epithelial cells proliferate i.e., express Ki67 when the biopsies are harvested after 7 days of culture with and without added hormones (23). In the breast biopsy experiments (n = 6 in each group), E2 exposure induced a significant increase of IL-8 secretion into the media, 1071 ± 170 pg/g tissue, compared with 407 ± 72 pg/g tissue in the control group, p < 0.01. E2 + P4 treatment to the tissue increased the IL-8 levels in a similar manner as E2 exposure only, 868 ± 55 pg/g tissue, p < 0.05 compared with controls, Fig. 2.

**IL-8 and von Willebrand’s factor immunohistochemistry of normal breast tissue biopsies**

The immunohistochemistry staining revealed that the epithelial cells showed most intense staining for IL-8 compared with the stroma. There was an increased intensity of staining and a greater

**FIGURE 3.** Immunohistochemical staining of anti-IL-8. Representative tissue sections from cultured breast tissue biopsy as described in Fig. 3. A. Breast tissue cultured without hormones. B. Breast tissue cultured with estradiol 10^{-7} M. C. Breast tissue cultured with estradiol 10^{-8} M and progesterone 10^{-8} M. Section from hormone treated tissue biopsies exhibited more intense and frequent staining compared with sections cultured without hormones. Three randomly selected areas of each section of six biopsies in each group were scored, 3/18 was scored positive in the control group and 17/18 in the E2 group and 16/18 in the E2 + P4 group, p < 0.05. Bars 100 μm.
number of epithelial cells that exhibited staining in the E2 and the E2+P groups compared with the control group, three random areas of each sections of six biopsies in each group was scored, 3/18 was scored positive in the control group, 17/18 in the E2 group, and 16/18 in the E2+P4 group, \( p < 0.05 \). Representative tissue sections are shown in Fig. 3, A–C. Staining with anti-von Willebrand’s factor exhibited a significant increase of microvessel area in the hormone-exposed biopsies compared with biopsies cultured without hormones, \( p < 0.001 \) in E2 and E2+P4 group compared with control biopsies, Fig. 4, A–D.

**Significant positive correlation of E2 and in vivo extracellular IL-8 in human breast cancer**

Ten postmenopausal breast cancer patients were subjected to microdialysis before surgery. All patients had palpable tumors, and the microdialysis catheter was inserted in the center of the tumor. Routine clinicopathological data such as content of ER and PgR, tumor histology and grade, and stage of the excised tumors were determined. All ten tumors expressed ER, and five tumors coexpressed ER with the PgR. There was a significant positive correlation with extracellular IL-8 and extracellular E2 in tumors which coexpressed ER and PgR, \( r = 0.881, p < 0.05 \), Fig. 5. When all ten patients were included, there were no significant correlations between extracellular IL-8 and estradiol or ER, PgR content, stage, tumor histology or grade, or size.

**Estradiol increased and tamoxifen decreased IL-8 secretion of breast cancer cells in vitro**

To further explore the effects of estradiol on IL-8 secretion, a cell culture experiment was set up. The ER/PgR-positive human breast cancer cells MCF-7 was cultured in presence of estradiol, tamoxifen, and a combination of the two hormones for 7 days. IL-8 was analyzed in the cell culture media and correlated to total protein content. Estradiol at \( 10^{-9} \) M increased the secretion significantly compared with control cells cultured without hormones, \( p < 0.01, n = 4 \). By increased concentrations of estradiol on the MCF-7 cells the levels of IL-8 increased further; 5.48 ± 0.95 pg/mg protein in \( 10^{-9} \) M exposed cells to 7.26 ± 0.5 pg/mg protein in \( 10^{-7} \) M exposed cells, \( p < 0.05 n = 4–5 \) in each group. Tamoxifen added to estradiol in the media counteracted the increase seen after estradiol exposure only, \( p < 0.01, n = 4 \) in each group, Fig. 6. Tamoxifen alone had no significant effects compared with control cells, Fig. 6.

**ABX-IL-8 decreased endothelial cell proliferation induced by cancer cell released IL-8**

It has previously been shown that IL-8 induces endothelial cell proliferation (32). In a first set of experiments, we cultured HUVEC with rIL-8 50 ng/ml, which induced an increased proliferation of the HUVECs to 0.147 ± 0.007 (OD at 490 nm) vs 0.115 ± 0.007 in the control cells, \( p < 0.01 \). The combination of r50 ng/ml IL-8 and ABX-IL-8 at 100 µg/ml decreased the proliferation rate significantly compared with rIL-8 alone, 0.147 ± 0.007 in the IL-8 exposed cells to 0.121 ± 0.003 in the combination group, \( p < 0.01 \). In a tumor, secreted proteins from the tumor cells affect the surrounding stroma including endothelial cells. To investigate whether the tumor cell derived IL-8 from MCF-7 cells...
had any physiological significance on endothelial cell proliferation, we treated HUVEC with cell culture media from E2-treated MCF-7 cells. ABX-IL-8 was either added to the MCF-7 cells during their culture or freshly added to the HUVEC cells together with the collected MCF-7 culture media. ABX-IL-8 decreased proliferation of HUVECs significantly compared with MCF-7 supernatant, \( p < 0.001, n = 6 \) in each group. Freshly added ABX-IL-8 was more efficient than ABX-IL-8 added during the MCF-7 cell culture, \( p < 0.001, n = 6 \) in each group, Fig. 7. Control IgG did not affect HUVEC proliferation compared with cells grown without IgG, data not shown.

**Tamoxifen decreased IL-8 secretion of solid breast cancer tumors in vivo**

To verify whether the inhibition of E2 released IL-8 by the anti-estrogen tamoxifen in vitro also were valid in whole tumor tissue, MCF-7 tumors were established in ovariectomized nude mice. One group of mice was treated with estradiol only and in the other group tamoxifen treatment was added. MCF-7 tumors require estradiol for growth in nude mice; therefore, a nonhormone treated group is not possible to achieve in vivo. Microdialysis of the tumors was performed to sample IL-8 from the extracellular space. In the s.c. tumors, a 10-mm microdialysis catheter perfused at 1 \( \mu l/min \) was used. The mammary fat pad tumors were smaller, dependent on the approval of the ethical committee, and these tumors were therefore investigated with a 4-mm long microdialysis catheter perfused at 1 \( \mu l/min \) to compensate for the shorter membrane. Similar to the in vitro results, tamoxifen treatment resulted in a significant decrease of IL-8 secretion compared with estradiol only exposed s.c. tumors, IL-8 16.3 \( \pm \) 2.4 pg/ml in E2 tumors compared with 10.3 \( \pm \) 0.47 pg/ml in the E2 + T tumors, \( p < 0.05 \), Fig. 8A. The level of decrease was similar, although the absolute levels were lower dependent on the shorter microdialysis membrane, in tumors grown in the mammary fat pad, 7.55 \( \pm \) 0.4 in the E2 exposed group and 3.98 \( \pm \) 0.3 in the E2 + T treated group, \( p < 0.001 \), Fig. 8B.

**Immunohistochemistry revealed a significant difference of intensity of staining after tamoxifen treatment.** In the estradiol group, seven out of nine tumors were scored as intensely stained in the E2 group whereas one out of six tumors showed intense staining in the E2 + T group, \( p = 0.04 \). Bars 100 \( \mu m \). D, Microvessel area in solid MCF-7 tumors, \( * \), \( p = 0.01 \).

**Discussion**

In this study, we show for the first time in normal human breast tissue that estradiol exhibit a significant positive correlation with
extracellular IL-8 levels in vivo, whereas no correlations were found in s.c. fat. Our in vivo results were confirmed ex vivo where estradiol treatment to normal human whole breast biopsies in culture increased IL-8 secretion significantly compared with biopsies cultured without added hormones. Progesterone had neither a counteracting nor additive effect on IL-8 secretion. In breast cancer tumors of women, extracellular IL-8 and local estradiol correlated significantly in tumors that were positive for both ER and PgR. This was further investigated in ER+/PgR+ MCF-7 breast cancer cells in culture, which increased their secretion of IL-8 after estradiol exposure. An Ab against IL-8, ABX-IL-8, inhibited cell proliferation of HUVEC exposed to media collected from MCF-7 cell culture. Moreover, the anti-estrogen tamoxifen inhibited the estradiol-induced IL-8 secretion. In solid MCF-7 tumors grown in nude mice, extracellular IL-8 in vivo was decreased after tamoxifen therapy compared with estrogen treatment only and these tumors also exhibited a decreased anti-IL-8 staining and decreased vessel density. Our results suggest that estradiol has a direct regulatory effect on IL-8 secretion in normal human breast tissue and in ER+/PgR+ breast cancer.

IL-8 has been shown to be up-regulated in several human cancers including breast cancer (33, 34). Higher levels of IL-8 in breast cancer compared with normal breast tissue has been demonstrated and higher serum levels of IL-8 in breast cancer patients have been associated with poor prognosis, increased tumor burden, and decreased postrelapse survival (6, 33). In experimental studies using breast cancer cell lines, a strong correlation between the metastatic phenotype and IL-8 expression has been shown suggesting a promoting role for IL-8 metastatic potential of tumor cells (9). In another tumor model, no proliferative activities of IL-8 in cancer cell culture were demonstrated but in tumor-bearing mice a neutralizing anti-IL-8 Ab induced decreased tumor growth and metastasis, and reduced angiogenic activity and vascular density (35). This suggests that IL-8 does not function as an autocrine growth factor but rather functions as a proangiogenic factor. IL-8 has also been shown to direct stimulate endothelial cell proliferation, survival, chemotaxis, and migration supporting the evidence of IL-8 as a proangiogenic factor (10, 32, 36).

Estrogens have been shown to increase angiogenesis and vascular endothelial growth factor (VEGF) expression in experimental breast cancer and normal human breast tissue (25, 37–39). Whether sex steroids are involved in the regulation of IL-8 is not known, and published data are somewhat contradictory. Generally, breast cancer cell lines expressing ER are of low metastatic potential and express very low levels of several cytokines and angiogenic molecules such as VEGF and IL-8, whereas ER-negative cell lines are more invasive and express high levels of these proteins. This does not, however, rule out an estrogen-dependent regulation of IL-8 in less metastatic breast cancer cells. We have previously shown that VEGF secretion is increased by estradiol in MCF-7 cells although these cells express VEGF at very low levels (40, 41). Very few studies have investigated direct effects on IL-8 secretion after estrogen exposure in hormone-dependent breast cancer models. Our present results suggest that estrogen has a role in the regulation of IL-8 secretion and the physiological consequence of this cancer cell released IL-8 on endothelial cells was proliferation, efficiently inhibited by a fully human anti-IL-8 Ab, ABX-IL-8.

In addition to promoting tumor angiogenesis, IL-8 attracts inflammatory cells, which contributes to tumor establishment by tissue remodeling (42). Stroma cells including immune cells contribute to a large part of the total tumor volume, and the close interaction between tumor cells and the surrounding cells has been shown in a coculture model of breast cancer cells and neutrophils; without the stimuli from cancer cells, the neutrophils did secrete very low levels of cytokine but after coculture with cancer cells the neutrophils started to produce high levels of cytokine (42). This emphasizes the need of whole tissue models and in vivo investigations for studies on the regulation of factors produced by various cells in a tissue or organ. Our novel results using microdialysis in situ in whole normal breast tissue as well as in whole breast cancers clearly show that IL-8 levels correlates with estradiol levels in vivo. That estradiol was the cause of the increased IL-8 levels in normal breast tissue was confirmed in our ex vivo experiments where estradiol exposure resulted in increased IL-8 secretion. We did not find any correlation between fat tissue IL-8 and estradiol, suggesting a tissue-specific hormone-dependent IL-8 secretion in the breast. Immunohistochemistry revealed that mainly the epithelial cells were expressing IL-8 and the stroma did only contribute to the IL-8 secretion to a minor extent. The epithelial staining was also increased in tissues exposed to estradiol and the combination of estradiol and progesterone, suggesting that these hormones also regulate the cellular content and not only the release of IL-8. The hormone-exposed biopsies also exhibited increased angiogenesis measured by microvessel area, suggesting a physiological response on the endothelial cells by hormone exposure.

The estrogen-dependent regulation of IL-8 was not limited to the normal breast since similar correlations were found in breast cancer tumors in women. Although the levels in cancerous tissue were lower than in the normal breast of premenopausal women, they were higher than in the normal tissue of postmenopausal women, suggesting that breast cancers indeed secrete higher levels of IL-8 compared with normal breast tissue when the background estrogen levels are similar. It has previously been shown that breast cancers are truly estrogen dependent and respond better to anti-estrogen therapy if the PgR also is expressed together with ER (43). This may be explained by PgR being an estrogen-dependent gene and that tumors not expressing the PgR have lost functionality of the ER (44). We found in the present study that tumors expressing a receptor profile indicating a functional ER by coexpressing PgR exhibited a significant correlation between extracellular estradiol and IL-8, whereas no correlation was detected in tumors expressing ER only and not PgR. This further supports our results of an estrogen-dependent regulation of IL-8 in breast tissues with functional ER as although only five patients with ER+/PgR− tumors were included a significant correlation was detected. The experimental data of MCF-7 cells in culture and solid MCF-7 tumors in mice further confirmed our patient data and showed that the estradiol induced increased IL-8 secretion could be counteracted by the anti-estrogen tamoxifen both in vitro and in vivo.

In conclusion, the control of extracellular IL-8 seems to be dependent on estradiol in normal human breast and breast cancer in vivo. The increase of IL-8 by estrogen in experimental breast cancer caused endothelial cell proliferation, which was efficiently inhibited by an anti-IL-8 Ab, suggesting a physiological role of the estrogen induced IL-8.

The incidence of breast cancer is increasing in the Western world and it has long been known that sex steroids increase the risk, whereas an early oophorectomy reduces the risk, of breast cancer by up to 60% (45, 46). Ovarian ablation is, however, associated with osteoporosis and cardiovascular disease as well as vasomotor symptoms and urogenital atrophy. Anti-estrogen therapies such as aromatase inhibitors and tamoxifen are effective therapies against breast cancer, and tamoxifen may also reduce the incidence of new breast cancers by more than 40% (47–49). However, these treatments may induce severe side effects such as endometrial cancer, thromboembolism, osteoporosis, and vaginal atrophy. Hence, there is an urgent need for studies of the
mechanisms of sex steroids on normal breast tissue and breast cancer to develop novel, more selective preventive and therapeutic strategies against this disease. IL-8 may be one potential target in estrogen dependent breast carcinogenesis and breast cancer progression.

Disclosures

The authors have no financial conflict of interest.

References


