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Estrogen Decreases Chemokine Levels in Murine Mammary Tissue

Implications for the Regulatory Role of MIP-1 Alpha and MCP-1/JE in Mammary Tumor Formation

Peter Fanti, Michael Nazareth, Robert Bucelli, Michael Mineo, Kathleen Gibbs, Michael Kumin, Kevin Grzybek, Lisa Raiber, Kristin Poppenberg, Kelly Janis, Catherine Schwach, and Susan M. Aronica

Department of Biology, Canisius College, Buffalo, New York

Estrogen contributes to the development of breast cancer through mechanisms that are not completely understood. Estrogen influences the function of immune effector cells, primarily through alterations in cytokine expression. Chemokines are proinflammatory cytokines that attract various immune cells to the site of tissue injury or inflammation, and activate many cell types, including T lymphocytes and monocytes. As an initial step toward ultimately determining whether regulation of chemokine expression and/or biological activity by estrogen could potentially be a contributing factor to the development and progression of mammary tumors, we evaluated the effect of estrogen on the expression of specific chemokines in murine mammary tissue. We also evaluated whether exposure of female mice to various chemokines could alter the growth of mammary tumors in the presence of estrogen. We report here that estrogen significantly decreases levels of the chemokines MIP-1α and MCP-1/JE in murine mammary tissue. Co-treatment with 4-hydroxytamoxifen partially reverses the suppressive effect of estrogen on MIP-1α levels. Estrogen increases the growth of CCL-51 cell-based tumors in the mammary glands of female mice. Co-treatment with the chemokine MIP-1α or MCP-1/JE substantially decreases the ability of estrogen to stimulate the formation of CCL-51 cell-based tumors. Our results show that estrogen might influence the bioactivity of specific chemokines through alteration of chemokine expression in mammary tissue, and further suggest that decreases in murine chemokines evoked by estrogen exposure could contribute to the promotion of mammary tumor growth.

Key Words: Chemokine; estrogen; mammary; mammary tumor.

Introduction

Estrogen has been recognized and characterized as a risk factor for the development of breast cancer in women *(1)*. The exact cellular and molecular mechanisms through which estrogen might contribute to changes in mammary cells toward a malignant phenotype or promote the uncontrolled growth of tumor cells are not completely understood. In addition to stimulating growth and development of female reproductive tissues *(2)*, estrogen has also been shown to affect the growth and differentiation of several classes of immune and/or hematopoietic cells *(3)*. Estrogen has been shown to alter recruitment of myeloid progenitor cells in rodents, and suppress B cell lymphopoiesis in murine bone marrow *(4,5)*. Estrogen down-regulates the expression of several cytokines produced by hematopoietic and immune cells, including interleukins (IL) IL-1, IL-2, IL-4, and IL-6 *(6,7)*. Given the reliance of immune cell communication on cytokine release and activity *(8)*, it seems reasonable that estrogen may be intimately involved in regulation of immune cell development and function.

Members of the chemokine superfamily, a distinct subset of proinflammatory cytokines, are released by epithelial and endothelial cells upon tissue injury and infection, and from monocytes, macrophage, and other immune cells at the sites of tissue inflammation *(9)*. Chemokines serve as chemoattractants for a number of cell types, and serve to activate a number of mature immune effector cells as well *(10)*. Experimental evidence has shown that estrogen can suppress mRNA expression of the chemokine monocyte chemoattractant protein 1 (MCP-1/JE) in both murine macrophage cells and in stromal cells isolated from human endometrium *(11,12)*. This suppressive effect of estrogen on MCP-1 mRNA levels is reversed by the selective estrogen receptor modulator (SERM) tamoxifen, suggesting that the suppressive effect of estrogen may occur at the level of the estrogen receptor *(11,12)*.

The overall goal of our research is to determine whether alteration of chemokine levels by estrogen could be a contributing factor to the development of breast cancer. The role of chemokines in promoting chemotaxis and activation of immune cells may be relevant to decreasing or eliminating the spread of breast cancer at the level of the mammary tissue itself, especially since a number of immune cells

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Author to whom all correspondence and reprint requests should be addressed: Susan M. Aronica, PhD, Department of Biology, Canisius College, 304 Health Science Center, 2001 Main Street, Buffalo, New York 14208. E-mail: aronicas@canisius.edu

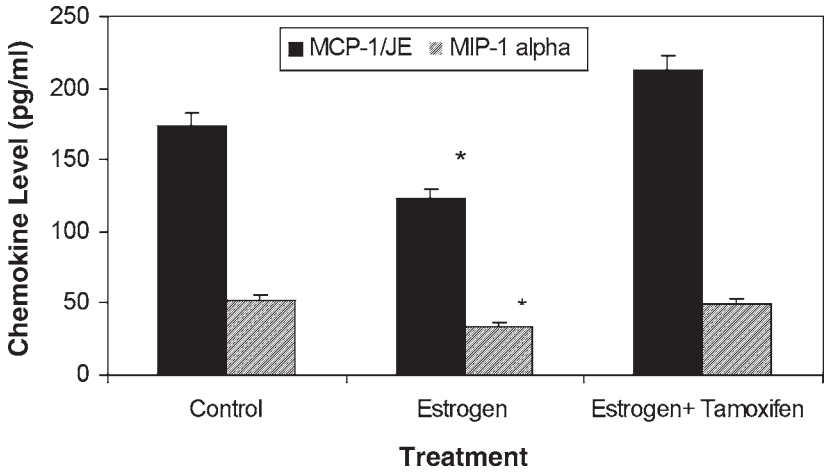

Fig. 1. Effects of estrogen and tamoxifen on the level of MIP-1α and MCP-1/JE protein in cultured mammary cells. Cultures of murine mammary cells were treated with vehicle alone (control), 1 n*M* E_2, or with E_2 in combination with 10 n*M* 4-hydroxytamoxifen (tamoxifen) and harvested after 24 h. Cell lysates were assayed for MIP-1α and MCP-1/JE content by ELISA, as described in *Materials and Methods*. Each bar represents the mean chemokine level, expressed as pg/mL, detected in the lysates of four treated mammary cell cultures, assayed in duplicate and obtained in separate experiments. *Denotes significant difference ($p < 0.05$) from basal chemokine level detected in the control treatment group.

have been shown to infiltrate into normal breast tissue as well as mammary tumors *(13)*. We have hypothesized that, in addition to directly stimulating cell growth and proliferation, estrogen could promote the survival and growth of malignant cells within mammary tumors by suppressing the production and/or action of chemokines within mammary tissue, leading to decreased attraction of monocytes and other immune cells to sites of tumor development. By evaluating whether estrogen regulates the expression and/or activity of chemokines or chemokine receptors in murine mammary tissue, we may be able to discern whether potential modulation of local immune cell function is related to the action of estrogen in promoting breast cancer cell growth. Toward that end, we set out to investigate in the studies reported here whether estrogen treatment, in the presence or absence of 4-hydroxytamoxifen, could alter the expression of specific chemokines in murine mammary tissue. Moreover, we evaluated whether the presence of specific chemokines within murine mammary tissue could disrupt the promotion of mammary tumor cell growth by estrogen.

Results

Basal Expression and Hormonal Regulation of MIP-1α and MCP-1/JE in Cultured Mammary Cells

We first examined the effects of estrogen on chemokine levels in primary cultures of murine mammary cells. Both MIP-1α and MCP-1/JE were detected in lysates obtained from mammary cells treated with vehicle alone. In all experiments, the basal level of MIP-1α (51.4 pg/mL) was substantially lower than the basal level of MCP-1/JE (174 pg/mL) detected in control mammary cell lysates (Fig. 1). We observed that estrogen exposure significantly decreased the levels of both MIP-1α and MCP-1/JE in cultured mammary cells 25–30% within 24 h (Fig. 1). In contrast, co-treatment of cells with 4-hydroxytamoxifen tended to reverse the suppressive effects of estradiol on MIP-1α and MCP-1/JE levels, increasing levels of MCP-1/JE above control levels in some experiments (Fig. 1). While not significant, the levels of MIP-1α and MCP-1/JE in the conditioned media from estrogen-treated cells tended to be lower than those present in the media from vehicle treated cells (data not shown).

Basal Expression and Hormonal Regulation of MIP-1α and MCP-1/JE in Murine Mammary Tissue

Based on what had been reported previously for the presence of specific chemokines in murine and human tissues, we evaluated murine mammary tissue for the presence of the chemokines MIP-1α and MCP-1/JE. In order to assess basal chemokine levels, mammary gland tissue was removed from intact female C3H mice that had been treated with a corn oil/0.1% ethanol vehicle (control vehicle), and whole cell lysates were prepared from the mammary tissue. Lysates were analyzed for MIP-1α and MCP-1/JE protein levels by ELISA, as described in *Materials and Methods*. We were able to detect MIP-1α in the mammary tissue of female C3H mice at a basal level of 68.9 pg/μg total protein (Fig. 2A). We were also able to detect murine MCP-1/JE at a basal level of 100.6 pg/μg total protein (Fig. 2B). In order to evaluate hormonal regulation of chemokine levels, female mice were treated with vehicle alone (control), or with the vehicle containing estrogen, tamoxifen, or estrogen in combination with 4-hydroxytamoxifen for 72 h, as described in

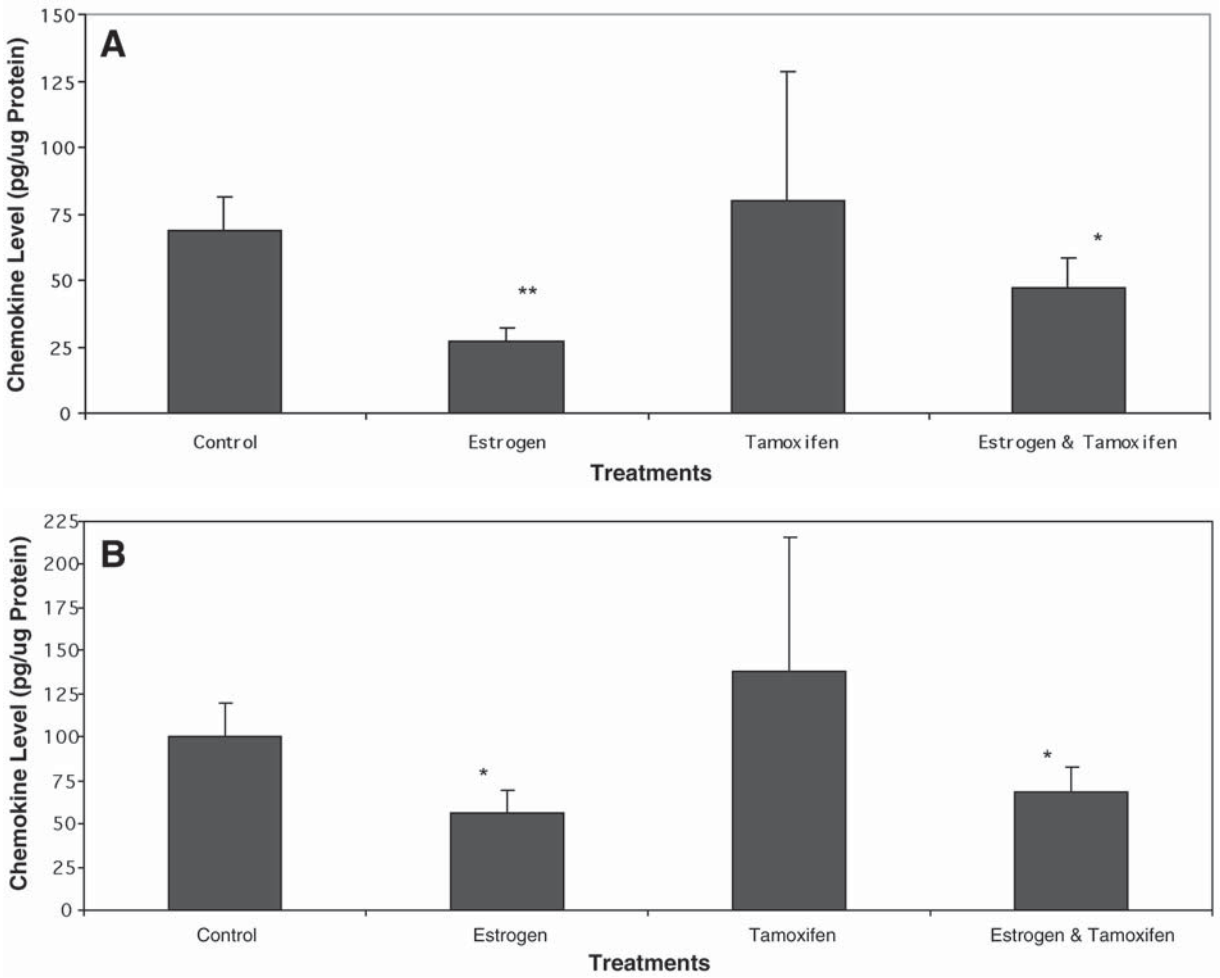

Fig. 2. Effects of in vivo estrogen and tamoxifen treatment on the level of MIP-1α and MCP-1/JE in mouse mammary tissue. Female C3H mice were treated with corn oil vehicle alone (control), or corn oil containing 5 μg estrogen, 5 μg 4-hydroxytamoxifen (tamoxifen), or estrogen in combination with 4-hydroxytamoxifen for 72 h. Mammary tissue was removed, and the levels of MIP-1α (**A**) and MCP-1/JE (**B**) protein were determined by ELISA, as described in *Materials and Methods*. Each bar represents the mean ± SD level of MIP-1α (**A**) and MCP-1/JE (**B**) protein, expressed as pg/μg protein, in the mammary tissue obtained from nine animals (one animal/treatment condition) in separate, independent experiments. *Denotes significant difference ($p < 0.05$) from basal chemokine level detected in the control treatment group. **Denotes significant difference ($p < 0.001$) from basal chemokine level detected in the control treatment group.

Materials and Methods. Analysis of whole tissue lysates for the expression of chemokines by ELISA revealed hormonal regulation of MIP-1α and MCP-1/JE by estrogen and estrogen in combination with 4-hydroxytamoxifen in murine mammary tissue. Levels of MIP-1α protein detected in the mammary glands of estrogen-treated mice were significantly decreased (Fig. 2A) when compared to the levels present in the mammary tissue of mice in the vehicle-treated control group. Co-treatment with 4-hydroxytamoxifen tended to partially reverse the suppressive effects of estrogen, with 39.5% less MIP-1α detected in the mammary tissue of mice treated with estrogen and 4-hydroxytamoxifen when compared to controls (Fig. 2A). Treatment with estrogen also resulted in a significant decrease in MCP-1/JE levels in murine mammary tissue when compared to the levels of MCP-1/JE detected in control mammary tissue (Fig. 2B). In contrast to MIP-1α, co-treatment of female mice with 4-hydroxytamoxifen did not result in a partial reversal of the ability of estrogen to inhibit expression of MCP-1/JE protein in mammary tissue. Although not statistically significant, treatment of mice with 4-hydroxytamoxifen alone tended to increase mammary levels of both MIP-1α and MCP-1/JE proteins in the majority of experiments conducted (Figs. 2A,B).

MIP-1α and MCP-1/JE Inhibit Estrogen-Mediated CCL-51 Mammary Tumor Formation

The observation that estrogen could decrease the detectable levels of both MIP-1α and MCP-1/JE in cultured mammary cells as well as in mammary tissue treated in vivo

Table 1
In Vivo CCL-51 Mammary Tumor Formation in Female C3H Mice[a]

Treatment groups	*n* (Total no. animals)	Incidence rate (% with tumors)	No. mammary tumors observed /animal
1. Control (vehicles)	11	72	1.8
2. Estrogen (E_2)	11	91	2.6
3. MIP-1α + E_2	12	50**	1.0*
4. MCP-1/JE + E_2	6	33**	0.2*
5. SDF-1 + E_2	4	75	2.0
6. No cells + vehicle	6	0	0
7. No cells + MIP-1α	4	0	0
8. No cells + SDF/JE	4 (2 SDF-1, 2 MCP-1)	0	0

[a]CCL-51 murine mammary carcinoma cells were injected into the mammary glands of intact female C3H mice, as described in *Materials and Methods* (groups 1–5). Mice were treated with corn oil or estradiol, alone or in combination with PBS or one of several chemokines every 72 h for 21 d, as described in *Materials and Methods*. The number of mice with tumors (incidence rate) and the number of mammary tumors present in each animal were determined at the time of sacrifice on d 21. No CCL-51 cells were administered to the mammary glands of mice in treatment groups 6–8. These animals received only injections of PBS and corn oil vehicles (group 6) or corn oil vehicle plus one chemokine (groups 7 and 8). These results summarize data from four separate experiments. *Significant difference from estrogen treatment group 2 ($p < 0.05$; Mann–Whitney *U*/Kruskal–Wallis). **Significant difference from estrogen treatment group 2 ($p < 0.001$; Pearson's chi-square).

prompted us to question whether down-regulation of these chemokines was related to the ability of estrogen to promote mammary tumor growth and, as a consequence, whether the presence of these chemokines could disrupt the ability of estrogen to promote tumor formation in the mouse mammary gland. CCL-51 murine mammary tumor cells were injected into the mammary glands of female mice and allowed to proliferate within the mammary tissue for 21 d, as described in *Materials and Methods*. For animals receiving the CCL-51 cell injections, mammary tumors formed in 72% of intact female mice in the absence of exogenous estrogen, with an average incidence of 1.8 tumors/6 injection sites/mouse (Table 1). Exposure of female mice to 5 μg E_2 every 72 h throughout the 21-d growth period resulted in increases in both the percentage of mice with tumors (91%) as well as the average number of tumors present within the mammary glands of each mouse (2.6; Table 1). No tumors were visible at d 21 in the mammary tissue of vehicle-treated or chemokine-treated female mice that had not received CCL-51 cells (Table 1). In contrast to mice treated with E_2 alone, cotreatment of mice with either 10 ng/mL MIP-1α or MCP-1/JE in the presence of estrogen lowered both the percentage of female mice with tumors ($p < 0.001$; Pearson's Chi-square) as well as the number of tumors present within the mammary glands of each mouse ($p < 0.05$; Mann-Whitney *U*), to levels below those obtained for estrogen-treated or vehicle-treated (control) mice (Table 1). In all cases, there were no signs of metastatic tumor growth to nonmammary tissues. Uterine size and degree of uterine vascularization were used as additional indicators of systemic estrogen action in all mice receiving estrogen treatment (data not shown).

Discussion

The influence of reproductive steroid hormones on various cells and factors associated with the immune system has been well documented *(14,15)*. The observation that estrogen could serve to regulate the activity of immune effector cells, especially at the local level, has prompted investigations into the interplay between estrogen action and immune cell activity within target tissues, including the mammary gland. We report here that treatment with estrogen in vivo significantly decreases the protein level of two chemokines, specifically MIP-1α and MCP-1/JE, in the mammary tissue of female mice. We believe that this is the first study that demonstrates regulation of MCP-1/JE and MIP-1α protein levels in normal murine mammary tissue treated in vivo. We obtained similar results for murine primary mammary cells treated with estrogen in culture. Our observations are consistent with those reported previously in which estrogen has been shown to decrease the expression of the mRNA for MCP-1 in murine spinal cord cells and in the human breast cancer cell line MCF-7 *(16,17)*. Estrogen has been shown to decrease MCP-1 protein levels produced by MCF-7 cells and by human uterine stromal cells, as well as the level of MCP-1 protein detected in the plasma of women receiving hormone replacement therapy *(17–19)*. Less is

known about the regulation of MIP-1α by estrogen in intact reproductive tissues, although estrogen exposure was shown to decrease MIP-1α mRNA in murine spinal cord tissue. Our observation that systemic exogenous estrogen exposure can alter chemokine protein levels within mammary tissue suggests that normal estrogen exposure during the ovarian cycle within female mice could be influential upon the microenvironment of the murine mammary gland. Our finding that 4-hydroxytamoxifen can partially reverse estrogen inhibition of MIP-1α in vivo and both MIP-1α and MCP-1/JE in culture is consistent with studies showing that tamoxifen can partially reverse estrogen suppression of MCP-1 mRNA. Although a recent study suggests that tamoxifen may induce apoptosis in some cell systems *(20)*, we were unable to detect alterations in mammary cell viability due to estrogen or tamoxifen treatment in our cell culture studies (data not shown). We are currently undertaking studies at the molecular level in order to assess whether estrogen regulation of MIP-1α and MCP-1/JE chemokine expression is occurring at the level of transcription.

Fluctuations in chemokine levels have been associated with the growth of cancer cells in different cell and tissue systems. Chemokines displaying a conserved ELR (glutamine–leucine–arginine) motif have been shown to promote the growth of some cancer cells, potentially through the stimulation of angiogenesis in solid tumors *(21,22)*. Other chemokines, primarily those that lack the ELR motif, are thought to be associated with the suppression of cancer cell or tumor growth *(23)*. Some reports have indicated that increased expression of specific chemokines, including interferon-inducible protein 10 (IP-10) and lymphotactin, are associated with tumor regression in murine models *(24,25)*. In addition, a cloned chemokine, termed BRAK, is highly expressed in normal human breast tissue, yet is detectable in only 2 out of 18 human and murine breast cancer cell lines tested *(26)*. We find it of great interest that our current studies show that the murine chemokine MIP-1α, which lacks an ELR motif, appears to decrease the frequency of CCL-51 tumor formation in the mammary tissue of female mice in response to estrogen, and that the level of MIP-1α is significantly decreased in murine mammary tissue in response to estrogen exposure.

Although we have shown that estrogen treatment can alter the level of chemokine protein in mammary tissue, we have not demonstrated that estrogen can disrupt the release of chemokines from mammary cells. While both MIP-1α and MCP-1/JE were detected in primary cultures of mammary cells and mammary tissue isolated from female mice treated in vivo, we have not reported that these levels represent the degree of chemokine secretion from mammary cells. The levels of chemokines present within whole-tissue lysate samples most likely represent a combined level of chemokines produced within cells, as well as the level of chemokines that were secreted into the interstitial fluid within the mammary gland. Our observation that estrogen exposure can decrease the levels of chemokines in murine mammary tissue could be indicative of decreased synthesis, decreased release, increased degradation, or a combination of factors. The recent observation that cathepsin-D, a protease that is upregulated in mammary tissue in response to estrogen and that is associated with poor prognosis in breast cancer patients *(27,28)*, can cleave chemokines, including MIP-1α, suggests that estrogen action could also involve the promotion of chemokine degradation in the mammary microenvironment *(28)*. Because our current understanding of chemokine bioactivity indicates that secretion of chemokines from their site of synthesis and into the surrounding interstitial environment is necessary for the attraction and activation of immune effector cells *(29)*, we will focus our attention in future studies on whether the presence of estrogen disrupts the release of, or promotes the degradation of, chemokines from murine tissue, and, if so, whether disruption of chemokine release or promotion of chemokine degradation by estrogen is related to the promotion of mammary tumor growth. It may be possible that the local effects of estrogen within mammary tissue occurs at many levels, perhaps involving the regulation of chemokine release as well as chemokine bioactivity.

Materials and Methods

Animals

Female 8–10-wk-old C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the Animal Care Unit at Canisius, and provided food and water *ad libitum*. All experimental protocols involving animals were reviewed by and approved for our use by the Canisius College Institutional Animal Care and Use Committee (IACUC), in accordance with guidelines set by the Public Health Service (PHS).

Reagents

Cell culture reagents, including fetal bovine serum (FBS), penicillin/streptomycin solution, and minimum essential media (MEM) were purchased from Invitrogen Life Technologies (Gibco; Grand Island, NY). ELISA kits for the detection of the murine chemokines macrophage inflammatory protein 1 α (MIP-1α) and monocyte chemoattractant protein 1 (MCP-1/JE) were purchased from R&D Systems (Minneapolis, MN). The murine mammary carcinoma cell line CCL-51 was purchased from ATCC. All other reagents and materials, including 17β-estradiol (estrogen; E_2) and 4-hydroxytamoxifen (tamoxifen; Tam) were purchased from Sigma Chemical Company (St. Louis, MO).

Mammary Cell Isolation and Culture

Mammary cells were isolated from murine mammary tissue, with minor modifications, as described previously *(30)*. Briefly, tissue was dissected bilaterally from the mammary pads of female C3H mice. The tissue was then minced using micro dissection scissors and placed in culture in MEM con-

taining collagenase III (18–20 units/mL of media). Mammary tissue pieces were incubated with collagenase III for 18 h at 37°C, yielding a single cell suspension with greater than 90% viability. Isolated cells were pelleted by centrifugation and resuspended in MEM containing 10% FBS. Cell count and cell viability were determined by Guava PC, and cells were placed into treatment groups on an equivalent cell number basis (2×10^6 cells/culture vessel). For chemokine studies, cultured mammary cells were treated with vehicle alone (media containing 0.1% ethanol; control), or with vehicle containing 1 n*M* estrogen (17β-estradiol; E_2) alone, or with 10 n*M* 4-hydroxytamoxifen (Tamoxifen; Tam) in combination with estrogen (E_2+Tam; E+T) for a 24 h period. The concentration of estrogen used in this study is considered to fall within the physiological effective range in female mice *(31)*. Murine mammary cells maintained in culture retain their responsiveness to estrogen, as shown by induction of progesterone receptor expression by Western immunoblotting (data not shown). Following the treatment duration, cells were pelleted by centrifugation (600*g*, 5 min at 6°C), the media supernatants were collected into separate tubes, and the sample tubes containing cell pellets were placed on ice. Ice-cold 1% NP-40 lysis buffer (100 µL/tube) was added to each cell pellet. Tubes were vortexed, centrifuged (600*g*, 5 min at 4°C), and the resulting lysates were transferred to new tubes maintained on ice. Lysates and media samples were tested immediately for chemokine levels, or quick-frozen on dry ice and stored at –80°C until assayed.

In Vivo Treatments and Analysis of Chemokine Levels

Female C3H mice received a single, 0.1 cm^3 (100 µL) subcutaneous intrascapular injection with corn oil/0.1% ethanol vehicle (for control), or with estrogen (5 µg; 0.2–0.3 µg/g body weight), 4-hydroxytamoxifen (5 µg), or a combination of estrogen and tamoxifen suspended within the corn oil/ethanol vehicle. Following the 72-h-treatment duration, mice were killed, and the mammary tissue of one mammary pad was removed and placed into a homogenization tube maintained on ice. The mammary tissue was immediately homogenized in 600 µL of ice-cold 1% NP-40 lysis buffer in order to obtain whole cell lysates. Samples were centrifuged at 900*g* for 10 min at 4–6C, and the lysate supernatants collected into separate tubes. Lysates were analyzed immediately for chemokine levels, or were quick-frozen on dry ice and stored at –80°C until assayed.

The level of the chemokines MIP-1α and MCP-1/JE present in each lysate sample, obtained from in culture or in vivo experiments, was determined using Quantikine M Murine ELISA kits from R&D Systems, as per kit instructions. Chemokine ELISA kits manufactured by R&D are specific for each murine chemokine and do not cross react with other known human and murine chemokines tested. These kits have been shown to detect MIP-1α and MCP-1/JE protein secreted from murine tissues *(32)*. We evaluated cross-reactivity of test kits with other murine and human chemokines in our laboratory. In agreement with the literature provided for the assay kit, chemokines in sample wells containing known concentrations of purified murine SDF-1a, human IP-10, murine MCP-1/JE, and murine RANTES were undetectable, whereas murine MIP-1α was detected by the murine MIP-1α ELISA assay. The concentration of MIP-1α detected in wells containing the MIP-1α positive control supplied with the kit was consistent with expected values. In a similar manner, murine MCP-1/JE was the only chemokine tested that was detected by the murine JE/MCP-1 ELISA assay kit (data not shown). Protein content of each in vivo lysate sample was determined using a Pierce BCA protein assay, as described *(33)*.

In Vivo CCL-51 Mammary Tumor Growth Studies

Murine mammary adenocarcinoma CCL-51 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in MEM supplemented with 10% FBS and antibiotics. These cells are estrogen-receptor positive and form tumors in the mammary glands of female C3H and Balb/C mice. Treatment with PBS or with any of the chemokines (10 ng/mL) MIP-1α, MCP-1/JE, SDF-1a, or IP-10 in culture did not alter CCL-51 cell viability (data not shown). For in vivo studies, CCL-51 cells were removed from culture and resuspended in phosphate-buffered saline (PBS) at a concentration of $1–2 \times 10^6$ viable cells/mL. Cells were injected, at 2×10^4 viable CCL-51 cells/50 µL/site, into the mammary glands of anesthetized female C3H mice. CCL-51 cells were injected into each mouse at each of six separate sites, with three injection sites spaced approx 1.0 cm apart along the length of the mammary gland chain on each side of the animal, 1.5 cm from ventral midline. Following the placement of tumor cells into the mammary pad, each mouse was treated every 72 h with a single 0.1 mL sc intrascapular injection of either a corn oil/0.1% ethanol vehicle (control), or with vehicle containing 5 µg of 17β-estradiol. In addition, each mouse was administered injections of PBS vehicle alone, or one of the following chemokines suspended in PBS (10 ng/mL): MIP-1α, MCP-1/JE, or SDF-1a (stromal-derived factor 1a). The PBS vehicle or each chemokine in PBS were administered every 72 h at six separate sites, approximating the ventral locations where CCL-51 tumor cells had been placed into the mammary gland previously. Each treatment group within a single experiment contained two to four animals. CCL-51 cell mammary tumors were allowed to grow within the mammary pads of experimental animals for 21 d. Mice were killed by overdose of inhalant anesthetic followed by cervical dislocation. The number, location, and relative size of all visible mammary tumors present within the mammary glands of each animal were recorded at the time of sacrifice. For experimental purposes, some animals received no CCL-51 cells and were treated with the corn oil and PBS vehicles or with corn oil and one of the chemokines every

72 h throughout the 21-d experimental period. These animals served as additional experimental controls.

Statistical Analysis

Significant differences in chemokine level or chemokine receptor level based on treatment condition was determined by analysis of variance (ANOVA) testing using SYSTAT software (SYSTAT, v10.2). Significance between two specific treatment conditions was further determined and verified using Student's paired *t*-tests on Sigma Plot Software (Sigma Plot). Significant differences in tumor incidence or tumor number for CCL-51 tumor growth studies were determined using Pearson's chi-square or Kruskal–Wallis one-way ANOVA (Mann–Whitney *U*) tests, respectively, using SYSTAT software (v10.2). Differences due to treatment condition, or differences between treatment groups, at a level of $p < 0.05$ were considered to be statistically significant.

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