Obesity Promotes Breast Cancer by CCL2-Mediated Macrophage Recruitment and Angiogenesis

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Abstract

Obesity is one of the most important preventable causes of cancer and the most significant risk factor for breast cancer in postmenopausal women. Compared with lean women, obese women are more likely to be diagnosed with a larger, higher grade tumor, an increased incidence of lymph node metastases, and elevated risk of distant recurrence. However, the mechanisms connecting obesity to the pathogenesis of breast cancer are poorly defined. Here, we show that during obesity, adipocytes within human and mouse breast tissues recruit and activate macrophages through a previously uncharacterized CCL2/IL-1β/CXCL12 signaling pathway. Activated macrophages in turn promote stromal vascularization and angiogenesis even before the formation of cancer. Recapitulating these changes using a novel humanized breast cancer model was sufficient to promote angiogenesis and prime the microenvironment prior to neoplastic transformation for accelerated breast oncogenesis. These findings provide a mechanistic role for adipocytes and macrophages before carcinogenesis that may be critical for prevention and treatment of obesity-related cancer. Cancer Res; 73(19): 6080–93. ©2013 AACR.

Introduction

Obesity is one of the leading health concerns in the United States, responsible for increased rates of diabetes, cardiovascular disease, and cancer. It is also an important preventable cause of cancer, accounting for up to 20% of cancer-related deaths in women (1). Obesity is the most significant risk factor in postmenopausal women for breast cancer (2). Compared with lean women, obese women, as defined by a body mass index (BMI) >30, are more likely to be diagnosed with larger, higher grade tumors, have an increased incidence of lymph node metastases, and an elevated risk of distant recurrence, which all contribute to an increased risk of death from breast cancer (1, 3).

The mechanisms by which obesity increases breast cancer incidence and worsens prognosis remain poorly defined. One hypothesis suggests that circulating androgens produced by the adrenal glands and climactic ovaries are converted to estrogen through aromatase activity following menopause (4–6). This higher localized estrogen in obese women could promote the progression of estrogen receptor (ER)-positive premalignant lesions, resulting in increased breast cancer incidence. However, obese pre- and postmenopausal women also have an increased likelihood of being diagnosed with ER-negative tumors compared with lean women (7, 8), suggesting that obesity may promote tumorigenesis through nonestrogenic mechanisms.

Obesity is associated with chronic adipose tissue inflammation, which has been implicated as an underlying cause of local and systemic insulin resistance, as well as increased aromatase activity (9, 10). Infiltration of the visceral adipose depots by immune cells seems to play a critical role in the development of inflammation, and a hallmark feature is the recruitment and accumulation of proinflammatory macrophages (11). In response to elevated circulating levels of insulin in obesity, adipocytes increase expression of CCL2 (also known as MCP-1), leading to an influx of macrophages into adipose tissue (12, 13). Consistent with this notion, CCL2 knockout mice, or its receptor, CCR2, show reduced macrophage infiltration and protection against obesity-induced inflammation in visceral adipose tissue (14, 15). Macrophages recruited to obese visceral adipose tissue increase CCL2 levels, as well as secrete cytokines and factors including TNF-α, inducible nitric oxide synthase (iNOS), interleukin (IL)-1, and IL-6 (16). In ovariectomized mice, subcutaneous fat depots showed elevated macrophage recruitment as well as increased expression of CCL2 compared with control mice, suggesting that CCL2 may enhance inflammation in postmenopausal breast tissue (16).
Chronic inflammation is a potent tumor promoter of many cancers, including breast, and expression of cytokines within tumors has been increasingly correlated with poor prognosis (17). CCL2 expression in tumors is correlated with higher histologic grade and is a significant indicator of early relapse (18), as well as infiltration of tumor-associated macrophages (19). Elevated CCL2 levels from adipocytes may contribute to early events in tumorigenesis by enhancing localized inflammation through adipose macrophages. Because significant differences exist between obese subcutaneous and visceral fat depots, elucidation of the inflammatory changes within the breast stroma may have important ramifications in defining the specific risks for obesity-associated breast cancer. In addition, there is still relatively little known about how signals from the stromal microenvironment in the obese state contribute to the early events in the progression to malignancy.

The understanding of obesity on human breast cancer development has been hindered due, in part, to the limited model systems to assess complex stromal events during the premalignant stages of breast cancer formation. In recent years, we developed an innovative human-in-mouse (HIM) model to study breast cancer development and progression in vivo. This model exploits the humanized mammary fat pad of humans (20, 21). This method results in the stepwise progression of normal human breast epithelium through distinct stages of premalignancy including hyperplasia and ductal carcinoma in situ (DCIS); these lesions as well as resulting invasive carcinoma are histologically and molecular similar to those found in humans (20, 21).

We have modified this in vivo model to study the role of obesity during breast cancer progression by engrafting human breast adipose stromal cells that model the inflammatory environment of the obese breast. Using this model, we provide new insights into the role of macrophage recruitment within subcutaneous fat depots before the genesis of cancer and how these changes are directly responsible for breast oncogenesis.

Materials and Methods

Cell lines and tissue culture

HL-60 and bovine retinal endothelial cells (BREC) were obtained from Drs. Richard Van Etten and Ira Herman, respectively (Tufts University, Boston, MA). Human microvascular endothelial cells (HMVEC) were obtained from Lonza; RAW 264.7 and 293T cells were obtained from American Type Culture Collection. Primary mouse heart endothelial cells were obtained from the Center for Vascular Biology Research at Beth Israel Deaconess Medical Center (Boston, MA). HL-60 cells were grown in RPMI-1640 (Invitrogen) supplemented with 10% FBS and 50 mmol/L HEPES. HMVEC were grown in Endothelial Complete Media (Lonza); BREC were grown in low-glucose Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 5% calf serum, 1% l-glutamine, and 25 mmol/L HEPES. RAW 264.7 and 293T cells were grown in high-glucose DMEM supplemented with 10% FBS. All cells were grown at 37°C and 5% CO2 and media was supplemented with 1% penicillin/streptomycin (Invitrogen). All cell lines tested negative for mycoplasma (MilliPROBE; Millipore); the identity of each cell line was not authenticated in our laboratory.

Animals and surgery

All animal procedures were conducted in accordance with a protocol approved by the Tufts University Institutional Animal Care and Use Committee. Colonies of nonobese diabetic/severe combined immunodeficient (NOD/SCID) and C57Bl/6 mice were maintained in house. SCID and DTGR EGFP-Mac mice were obtained from Jax Laboratories (strain numbers: 001913 and 006000, respectively) and mated. Genotyping was conducted by Transnetx. Mice were given food and water ad libitum. For obesity studies, 6-week-old female mice were fed either low fat maintenance chow (5% kcal from fat) or a high-fat diet (60% kcal from fat, Test Diet). Weights were measured weekly for 16 weeks. Tissue was frozen for molecular analyses or embedded, sectioned, and stained for hematoxylin and eosin at Tufts Medical Center. Details for whole mount preparation and quantification of blood vessels are provided in Supplementary Materials and Methods.

Primary tissue isolation and culture

All human breast tissues were obtained in compliance with the laws and Institutional guidelines, as approved by Institutional Review Board committees from Tufts Medical Center and Duke University. Noncancerous breast tissues were obtained from patients undergoing elective reduction mammoplasty. Breast tissues were enzymatically digested as previously described (20, 22). Cells from the lipid layer comprising the mature adipocyte fraction (MAF) and the cell pellet containing the stromal vascular fraction (SVF) were plated in DMEM supplemented with 10% calf serum. SVF cells were immortalized with the catalytic subunit of telomerase (hTERT) and transduced with lentiviruses containing either CSG empty vector (SVF/EV) or CSG-CCL2 (SVF/CCL2). Infected cells were sorted for GFP expression with a Legacy MoFlo (Beckman Coulter). For generation of SVF/CCL2 lines with shIL-1β cells were transduced with lentiviruses containing either shscrambled or shIL-1β constructs (MISSION shRNA, Sigma) and selected in 1 μg/mL of puromycin. Details for adipocyte differentiation are provided in Supplementary Materials and Methods.

For tumorigenesis studies, epithelial organoids were dissociated to single cells and infected in suspension with lentiviruses for CSG vector, pLenti-KrasG12V, and pLenti-SV40er (21). Details for lentiviral production are provided in Supplementary Materials and Methods. To humanize mice, mammary epithelium was removed from the fourth mammary glands of 3-week-old NOD/SCID females and either SVF/EV or SVF/CCL2 cells were injected into the fat pad as described (22). Two weeks posthumanization, virus-infected cells (100,000 per gland) were comixed with either SVF/EV or SVF/CCL2 cells (2.5 × 106 per gland) in a 1:1 mixture of collagen and Matrigel (BD Biosciences) and injected into...
humanized fat pads. Tumors were measured twice weekly and end stage was assessed when tumors reached 1.5 cm in diameter. For inhibitor studies, mice were treated following humanization with daily subcutaneous injections of 10 mg/kg Kineret (Amgen) or 2 mg/kg RS504393 (Tocris), and tissues were collected 2 weeks following tumor cell transplant or when tumors reached 1 cm in diameter.

**Macrophage differentiation and treatment**

Bone marrow from C57Bl/6 females was isolated and plated for 3 days in DMEM/F12 (Invitrogen) supplemented with 20% FBS. Suspended cells were differentiated into macrophages as described (23). Mouse macrophages were treated with 40% conditioned media from SVF/EV or SVF/CCL2 for 24 hours and either collected for molecular analyses or grown in DMEM supplemented with 0.5% calf serum for 24 hours for macrophage conditioned media experiments.

HL-60 cells were differentiated into adherent macrophages as described (24). Macrophages were treated with DMEM supplemented with 0.5% calf serum and vehicle, 20 ng/mL recombinant human CCL2 (rhCCL2), 10 ng/mL rhIL-1β, 20 ng/mL rhCCL2+10 ng/mL rhIL-1β (both R&D Systems), or conditioned media from SVF/EV, SVF/CCL2, SVF/CCL2 shscrambled, or SVF/CCL2 shIL-1β cells. For inhibitor studies, HL-60 macrophages were treated with SVF/CCL2 CM and vehicle (DMSO), 330 nmol/L RS504393 (Tocris), 40 ng/mL IL-1ra (R&D Systems), or 330 nmol/L RS504393 + 40 ng/mL IL-1ra. Macrophages were treated for 24 hours and either collected for molecular analyses or grown in DMEM supplemented with 0.5% calf serum for 24 hours for conditioned media experiments.

**Quantitative PCR analyses**

RNA was isolated, reverse transcribed, and analyzed as described in Supplementary Materials and Methods. Primer sequences used for quantitative PCR (qPCR) are listed in Supplementary Table S1.

**Immunohistochemistry and immunofluorescence**

F4/80 and CD31 proteins were detected as described previously (25). Details for analyses are provided in Supplementary Methods and Materials.

**Western blotting**

Proteins were isolated and concentrations measured as described (25). Antibody concentrations and detection methods are described in Supplementary Methods and Materials.

**Statistical analyses**

Results were expressed as the mean ± SEM. Statistical tests included unpaired two-tailed Student t test and one-way ANOVA, followed by Neuman–Keuls posttest (for more than 2 groups). Human qPCR data were analyzed using two-tailed Pearson correlation. BMI data were blinded until qPCR analyses were complete. P values of 0.05 or less were considered to denote significance. Statistical analyses were conducted using GraphPad Prism (GraphPad Software).

**Results**

**Stromal changes within the adipose tissue of the breast during obesity**

To examine the effects of obesity on mammary adipose tissue, nonovariectomized females were fed either a normal diet (ND) or a diet with increased calories from fat (HFD), with equivalent micronutrients and vitamins. After 16 weeks, HFD mice weighed more than twice as much as ND mice ($P = 0.006$; Fig. 1A). All fat depots expanded in obese mice compared with their lean counterparts, including the inguinal mammary glands ($1.3 ± 0.4$ g vs. $0.4 ± 0.6$ g, $P = 0.002$; Fig. 1A). The diameters of white adipocytes were significantly enlarged in mammary glands of obese mice ($P < 0.0001$; Fig. 1B). F4/80$^+$ macrophages surrounded adipocytes forming crown-like structures (CLS, Fig. 1B), which are typically present within visceral fat depots of genetically and diet-induced obese mice (11, 26). CLS were absent in the mammary glands of lean mice (Fig. 1B). Consistent with the formation of CLS, total numbers of macrophages were significantly increased in glands of HFD mice ($4.4 × 10^5 ± 0.5 × 10^5$; macrophages/gland ± SD) compared with ND mice ($2.5 × 10^5 ± 0.5 × 10^5$; $P = 0.05$).

Macrophages have at least two polarization states: M1 polarization by LPS and IFN-γ, and M2 activation by IL-4 and IL-13 (27). Macrophages that express M1-type cytokines have been proposed to have tumoricidal functions, whereas those that produce M2-type cytokines may be tumor promoting (28, 29). Mammary glands were examined for expression of IL-10 and CD206 (M2) as well as CD11c and iNOS (M1) transcripts to determine the polarization state of macrophages in the obese mammary adipose tissue. Obese glands showed significantly decreased IL-10 expression ($P = 0.02$) and significantly increased iNOS and CD11c expression ($P = 0.008$ and $P = 0.04$; Fig. 1C), consistent with an M1 polarization phenotype. However, CD206 was also significantly elevated ($P = 0.02$), suggesting that macrophages in obese mammary glands show a mixed polarization phenotype, consistent with reports from visceral adipose depots in mice and humans (30, 31), as well as peritumoral adipose tissue (32).

Adipose tissue is critically dependent on vascularization for growth (33). Therefore, we examined the effect of obesity on vascularity within the mammary glands. Inguinal mammary glands from HFD and ND females were stained with BODIPY to detect lipid and for the endothelial marker, CD31. CD31$^+$ vessels surrounding adipocytes were significantly increased in glands of HFD females compared with those fed the ND ($P = 0.0001$; Fig. 1D). Transcripts from whole glands for CD31 and pericyte marker, PDGFRβ, were significantly increased in HFD glands compared with ND glands (Fig. 1E), suggesting that both endothelial cells and pericytes were increased in the obese glands.

To determine whether obesity-induced changes in the mouse mammary gland were present in the human breast of women with increased BMI, disease-free breast tissue collected from reduction mammoplasty surgeries was examined for adipocyte size, CD11c$^+$ macrophage recruitment, and angiogenesis. Adipocyte size strongly correlated with both increasing BMI ($P < 0.0001$), as well as the formation of CD11c$^+$ CLS ($P < 0.0001$; Fig. 1F). Increased BMI was strongly correlated...
with CD11c+ CLS formation (P = 0.001) as well as increased transcripts for CD11c (P = 0.003; Fig. 1G). Elevated CD11c expression was also associated with elevated CD31 expression (P = 0.07; Fig. 1H). These results indicate that the adipose tissue of the breast during obesity exhibits increased angiogenesis and macrophage recruitment.

Generation of obesity-like humanized mammary fat pads

High-fat diet induced obesity in the C57Bl/6 genetic background is a widely used model because of the similarities to metabolic changes in obese humans. However, C56Bl/6 mice are extremely resistant to breast oncogenesis in our hands and others (34), limiting its use in the study of obesity on tumorigenesis. Therefore, to study how the obese microenvironment of the mammary gland alters breast tumorigenesis, we used the HIM model to recapitulate the paracrine stromal interactions associated with obesity. As a first step, we needed to identify factor(s) produced by the obese adipose tissue that could promote macrophage recruitment and activation. Because elevated CCL2 expression in subcutaneous adipose tissue was associated with the mild weight gain of ovariectomy (16), we examined CCL2 expression in mammary glands of obese and lean mice. Obesity induced a three-fold increase in CCL2 transcripts (P = 0.02; Fig. 2A) and protein (Fig. 2B). This association between CCL2 and obesity was further validated in human breast tissues. CCL2 and CD11c expression were correlated (P = 0.05; Fig. 2C), suggesting that CD11c+ CLS, BMI, and CCL2 are associated (Fig. 1G) in reduction mammoplasty tissues.

Next, to create a humanized adipose stromal model that mimics the inflammatory state of obesity, we isolated the adipose stromal vascular fraction (SVF) from reduction mammoplasty breast tissues; the SVF is composed of adipocyte stem cells, preadipocytes, mature adipocytes, and other stromal cells (35). The SVF was immortalized with human telomerase (hTERT) and transduced with either human CCL2 (SVF/CCL2) or empty vector (SVF/EV). SVF/CCL2 cells showed a 3-fold increase in CCL2 transcript, as well as abundant protein secretion in conditioned media compared with SVF/EV cells (P = 0.03, Fig. 2D). To characterize the adipogenic differentiation potential of the SVF cells, the immortalized and non-immortalized SVF (iSVF, pSVF, respectively) lines, SVF/CCL2, and SVF/EV cells were assayed for the expression of adipocyte markers and differentiation potential and compared with the MAF. Under adipogenic conditions, the MAF, SVF/CCL2, and SVF/EV cells all expressed similar levels of adipin (Fig. 2E), PPARγ, and FABP4 (Supplementary Fig. S1C), induced leptin expression (P < 0.05; Fig. 2E), and generated large, central lipid droplets as quantified using BODIPY or Oil Red O (Fig. 2F; Supplementary Fig. S1A and S1B). In contrast, confluent U2OS cells non-specifically developed diffuse lipid droplets, and expression of adiponectin and leptin was not detectable (Fig. 2E). Neither SVF/CCL2 nor SVF/EV cells expressed markers of epithelial cells (cytokeratins 8 and 14), endothelial cells (CD31), hematopoietic cells (CD45), or macrophages (EMR1 and CD11b; Supplementary Fig. S2A), suggesting that SVF lines were enriched in adipocytes and preadipocytes.

The murine counterpart to human CCL2 protein contains an elongated C terminus (36). As species incompatibilities between growth factor/chemokines and their receptors are well known, we evaluated whether human CCL2 (hCCL2) could enhance mouse macrophage migration to model the inflammatory state of obesity. RAW 264.7 cells migrated through Transwell chambers in response to recombinant hCCL2 (rhCCL2, P < 0.0001; Supplementary Fig. S2B), as well as further validation with SVF/CCL2 CM (Supplementary Fig. S2C). Migration was significantly attenuated in the presence of a CCL2 blocking antibody (P < 0.0001; Supplementary Fig. S2C). To evaluate the ability of SVF/CCL2 cells to enhance macrophage migration in vivo, mice were humanized as previously described (20, 22) with SVF/CCL2 or SVF/EV cells. At 2 or 4 weeks following injection, SVF/CCL2 humanized fat pads showed significantly increased macrophage recruitment compared with those humanized with SVF/EV cells (Fig. 2G), suggesting that CCL2 secreted by SVF/CCL2 cells attracts murine macrophages. Four weeks following humanization with SVF/CCL2 cells, mammary CCL2 transcripts were elevated 3.5-fold compared with those humanized with SVF/EV cells, similar to levels in obese C57Bl/6 mammary glands (Fig. 2H).

Macrophages are responsible for angiogenesis in response to the obesity-like microenvironment

Macrophages are important for angiogenesis during wound healing and tumorigenesis (37, 38), but little is known about their role during the expansion of the adipose tissue vasculature in obesity. Mammary fat pads of NOD/SCID mice were implanted with SVF/CCL2 or SVF/EV cells and examined for angiogenesis 4 weeks following humanization. SVF/CCL2 humanized fat pads showed a significant 2-fold increase in CD31+ endothelial cells compared with SVF/EV fat pads (Fig. 3A and B).

To specifically study macrophages in this process, we used DTGR EGFP-Mac transgenic mice (Mac), which express the simian diphtheria toxin (DT) receptor under the control of the CD11b promoter (39). Diphtheria treatment selectively ablates macrophages within 12 hours without altering other cell populations (39). For use with the humanized xenograft model, Mac mice were bred onto the SCID background (Mac-SCID) and treated with DT to assess macrophage ablation. One hour following DT treatment, the number of CD11b+ or F4/80+ macrophages was diminished within the bone marrow (Supplementary Fig. S2D and S2E). In mammary glands, CD11b+ and F4/80+ macrophages were also significantly decreased up to 48 hours following DT administration (Supplementary Fig. S2F and S2G). SCID littermates negative for the Mac transgene showed a significant increase in macrophages within 24 hours of DT administration, indicating that macrophage ablation was specific to Mac transgenic mice (Supplementary Fig. S2F and S2G). Although macrophages were depleted in response to DT, transcripts for leptin and adiponectin were not significantly different following DT treatment, suggesting that the adipocytes were not altered by DT treatment (Supplementary Fig. S2H).

To determine whether CCL2-associated angiogenesis was mediated by macrophage recruitment, Mac-SCID mice were
A. Weight gain (g) over 16 weeks for Normal diet (ND) and High-fat diet (HFD).

B. % CLS/HPF and average adipocyte diameter for ND and HFD.

C. Relative expression of CD11c, CD206, iNOS, IL-10 for ND and HFD.

D. Total vasc/Adipocyte area for CD31 and PDGFRβ for ND and HFD.

E. Relative expression of CD31 and PDGFRβ for ND and HFD.

F. Adipocyte diameter for ND and HFD.

G. BMI and CLS for ND and HFD.

H. Log Rel Exp CD11c and Rel Exp CD11c for ND and HFD.
implanted subcutaneously with extracellular matrix (Matrigel) plugs containing SVF/EV or SVF/CCL2 cells, and DT was administered to animals 24 and 72 hours after implantation. Plugs containing SVF/CCL2 cells significantly enhanced F4/80+ (P = 0.002) and CD31+ (P = 0.0004) cell recruitment compared with SVF/EV cells containing plugs, indicating that CCL2 expression augments macrophage recruitment and angiogenesis (Fig. 3C). DT treatment resulted in the depletion of F4/80+ and CD31+ cells recruited to Matrigel plugs (Fig. 3C), implying that macrophage recruitment is necessary for angiogenesis.

Mac-SCID mice received DT at 24 and 72 hours following humanization with SVF/CCL2 or SVF/EV cells to determine the role of macrophage recruitment on angiogenesis in mammary fat pads. After 2 weeks, humanized glands from DT-treated mice showed similar levels of GFP-expressing SVF cells (4.9 ± 3.4%; mean ± SD, n = 10) compared with those that received vehicle (5.6 ± 1.8%; n = 10), suggesting that the transplanted cells were not ablated by the DT. As SVF/CCL2 humanized gland recruit significantly more macrophages than SVF/EV humanized glands, SVF/CCL2 DT-treated mice exhibited a 2-fold reduction in the number of recruited CD11b+ cells compared with vehicle-treated cohorts, whereas this was not observed in glands humanized with SVF/EV cells (P = 0.04; Fig. 3D). DT treatment also resulted in a reduction in CD31+ cells in both SVF/EV and SVF/CCL2 humanized glands, indicating that macrophages are important for the angiogenesis induced by humanization (Fig. 3D). However, after 4 weeks when macrophage number returned to baseline levels in SVF/EV and SVF/CCL2 humanized glands following DT treatment, SVF/CCL2 humanized fat pads exhibited a greater reduction in the number of CD31+ cells (P = 0.03; Fig. 3D), suggesting that the magnitude and, thus, attenuation of the angiogenic response was greater in the presence of CCL2.

DT treatment also resulted in a long-term two-fold reduction in CD31+ vessels in SVF/CCL2 humanized fat pads compared with vehicle-treated mice, although the percentage of CD11b+ cells was similar by that time in all glands. This angiogenesis was not observed in glands humanized with SVF/EV cells (Fig. 3D). These results suggest that macrophage recruitment enhances angiogenesis, and CCL2 further augments this increase.

**Macrophage/adipose CCL2/CXCL12 crosstalk promotes angiogenesis**

To determine the mechanism by which SVF/CCL2-mediated macrophage recruitment promotes angiogenesis, we stimulated HL-60 macrophages with SVF/EV or SVF/CCL2 CM and assessed the expression of several established mediators of angiogenesis. There were no significant changes in VEGF-A, PDGF-A, and MMP-9 expression between macrophages treated with either SVF/EV or SVF/CCL2 CM (Supplementary Fig. S3A–S3C). However, CXCL12 (also known as SDF-1α) expression was significantly increased in response to SVF/CCL2 CM and was attenuated in the presence of a CCL2-blocking antibody (P = 0.0001; Fig. 4A). In addition, expression of CXCL12 in mouse bone marrow–derived macrophages was induced following stimulation with SVF/CCL2 CM (P < 0.05; Supplementary Fig. S3D). However, treatment of HL-60 macrophages with rhCCL2 did not significantly upregulate CXCL12 expression (Fig. 4B); this inability was not due to failure of rhCCL2 to induce downstream signaling, as ERK1/2 phosphorylation was increased (Supplementary Fig. S3E). These findings suggest that CCL2 is necessary but not sufficient to induce CXCL12 expression in macrophages.

It has been reported that CCL2 and IL-1β can cooperate to enhance angiogenesis (40). Consistent with this notion, abundant IL-1β protein was detected in SVF/CCL2 CM (Fig. 4C). In addition, HL-60 macrophages treated with rhIL-1β showed a significant increase in CXCL12 expression (P = 0.008; Fig. 4B), and the induction of CXCL12 by SVF/CCL2 CM was abolished in the presence of an IL-1β–blocking antibody (P = 0.0001; Fig. 4D). HL-60 macrophages treated with specific inhibitors against the CCL2 receptor, CCR2 (RS504393), or IL-1β activity (IL-1ra), significantly attenuated induction of CXCL12 following stimulation with SVF/CCL2 CM (P = 0.0001; Supplementary Fig. S3F). To further investigate the role of IL-1β in CXCL12 expression, we generated SVF/CCL2 cells with stable reduction of IL-1β protein using shRNA (Fig. 4E). Treatment of HL-60 macrophages with conditioned media from SVF/CCL2 shIL-1β cells resulted in significantly lower expression of CXCL12 compared with conditioned media from SVF/CCL2 shscrambled control cells (P = 0.008; Fig. 4F). These results indicate that IL-1β expressed by SVF/CCL2 cells cooperates with CCL2 to enhance the induction of CXCL12 in macrophages.

CXCL12 has been well characterized for its role in recruiting bone marrow–derived endothelial progenitor cells, although less is known about its effects on existing vasculature (41). Therefore, we examined the effects of macrophage-derived CXCL12 on endothelial cell migration and proliferation. HL-60 macrophages were stimulated with SVF/CCL2 or SVF/EV CM, and macrophage-conditioned media was collected and tested for its ability to promote angiogenesis. Conditioned media from macrophages treated with SVF/CCL2 CM (macCCL2)
Figure 2. Overexpression of CCL2 in human adipose stroma cells results in obesity-like conditions. A, CCL2 transcripts were significantly increased in mammary glands of C57Bl/6 mice fed HFD diet compared with those from ND mice (qPCR, n = 6 mice/group). B, CCL2 protein was elevated in glands from obese mice. Lamin A/C was used as a loading control. C, increased expression of CCL2 and CD11c were significantly correlated in mammary reduction tissue (qPCR, n = 28 samples). D, SVF/CCL2 cells expressed significantly increased levels of CCL2 mRNA and protein in conditioned media compared with SVF/EV cells (n = 3 experiments). Recombinant human CCL2 (rhCCL2) was used as a positive control. E, MAF, SVF/EV, SVF/CCL2, and U2OS cells were treated with adipogenic media (DIF) or vehicle (UN). Following differentiation, changes in expression levels of adiponectin and leptin were similar to the MAF but were not detected (nd) in U2OS cells. qPCR was conducted on RNA isolated from 3 experiments and data represented as fold change following differentiation compared with vehicle-treated cells. F, stromal cells and U2OS cells were exposed to adipogenic media or vehicle; lipid was stained with BODIPY 558/568 and counterstained with DAPI. G, mammary glands from NOD/SCID mice humanized with SVF/CCL2 cells showed increased F4/80+ cells compared with glands humanized with SVF/EV cells (n = 3 mice/group). H, after 4 weeks, CCL2 transcripts were significantly increased in glands from NOD/SCID mice humanized with SVF/CCL2 compared with glands humanized with SVF/EV cells. Original magnification, ×200 (F and G); bar, 100 μm.
significantly increased migration and proliferation of human microvascular endothelial cells (HMVEC) compared with conditioned media from macrophages treated with SVF/EV CM (macEV; Fig. 4G and H and Supplementary Fig. S3G). The migration and proliferation of HMVECs in response to macCCL2 CM was attenuated in the presence of AMD3100, an inhibitor specific for the CXCL12 receptor (Fig. 4G and H and Supplementary Fig. S3G). When HMVECs were treated, recombinant mouse CXCL12 (rmCXCL12), migration, and proliferation were significantly increased (Fig. 4G and H and Supplementary Fig. S3G).
Figure 4. Macrophage-derived CXCL12 induces angiogenesis. A, SVF/CCL2 conditioned media (CM) significantly increased CXCL12 expression in HL-60 macrophages compared with SVF/EV CM or SVF/CCL2 CM + blocking antibody for CCL2 (CCL2 Ab). B, recombinant human IL-1β (rhIL-1β) increased CXCL12 expression in HL-60 macrophages. qPCR was conducted on RNA isolated from 3 experiments. C, SVF/CCL2 CM contained increased IL-1β protein compared with SVF/EV CM. Conditioned media from HL-60 macrophages treated with LPS was used as a positive control. D, SVF/CCL2 CM + IL-1β blocking antibody significantly reduced CXCL12 expression in HL-60 macrophages compared with SVF/CCL2 CM. E, conditioned media from SVF/CCL2 shIL-1β (shIL-1β) cells showed decreased IL-1β protein compared with conditioned media from SVF/CCL2 shscrambled control cells (shscram). F, SVF/CCL2 shIL-1β CM significantly decreased CXCL12 expression in HL-60 macrophages compared with SVF/CCL2 shscramble conditioned media. (Continued on the following page.)
Supplementary Fig. S3G), suggesting that CXCL12 acts directly on endothelial cells. Similarly, conditioned media from primary mouse macrophages pretreated with SVF/CCL2 CM significantly induced migration of primary mouse vascular endothelial cells compared with conditioned media from those pretreated with SVF/EV CM, and migration was attenuated in the presence of AMD3100 (Supplementary Fig. S3i and S3j). Consistent with the role of CXCL12 on endothelial cells, conditioned media from HL-60 macrophages pretreated with conditioned media from SVF/CCL2 shiL-1β cells (mac shiL-1β) showed significantly reduced proliferation and migration compared with those treated with conditioned media from HL-60 macrophages pretreated with conditioned media from SVF/CCL2 shscrambled control cells (mac shscram; Fig. 4f and J and Supplementary Fig. S3h). The effects on migration and proliferation were specific to macrophage conditioned media, as SVF/CCL2 conditioned media could not significantly increase either migration or proliferation of either HMVECs or BRECs (Supplementary Fig. S4).

To establish whether the CCL2/IL-1β/CXCL12 crosstalk was induced during obesity, we examined mammary tissues from mice and humans. CXCL12 transcript levels were elevated in obesogenic conditions compared with those from lean mice (P = 0.03; Fig. 4k). HFD-fed DTR-GraMac mice on the C57Bl/6 genetic background treated with DT showed reduced CXCL12 expression compared with those treated with vehicle (Supplementary Fig. S3k). Similarly, fat pads humanized with SVF/CCL2 cells showed significantly elevated CXCL12 expression compared with those humanized with SVF/EV cells (P = 0.01; Fig. 4l). Likewise, IL-1β protein was more abundant in mammary glands of obese mice than those in lean mice (Fig. 4m). In human reduction mammoplasty tissues, CCL2 expression was strongly correlated with IL-1β expression (P = 0.002; Fig. 4n) and CXCL12 expression was correlated with CD31 expression (P = 0.001); CXCL12 expression was also highly correlated with CCL2 expression (P = 0.04; Fig. 4o). Taken together, these results suggest that CCL2 and IL-1β proteins are elevated in obese adipose stroma and cooperate to induce CXCL12 in macrophages that in turn promote angiogenesis.

**Stromal changes associated with obesity accelerate tumorigenesis**

We used the obesity-like humanized fat pad model to determine whether changes in the adipose microenvironment associated with obesity affects breast tumor progression.

Mammary fat pads were humanized with either SVF/CCL2 or SVF/EV cells and subsequently implanted with human breast epithelial cells that were transduced with lentiviruses encoding SV40/Kras oncogenes as previously described (21, 42). When infected cells were injected into glands humanized with SVF/CCL2 cells, palpable tumors formed more rapidly than in glands humanized with SVF/EV cells (Fig. 5a; Supplementary Table S2). In addition, obesity-like humanized stroma led to a significant increase in tumor weight compared with those humanized with SVF/EV cells (P = 0.04; Fig. 5b). Although end-stage tumors were histologically similar (Supplementary Fig. S5a), tumors that developed within SVF/CCL2 humanized fat pads exhibited significantly increased numbers of mitotic figures (P = 0.002) and Ki67+ cells (P = 0.03; Fig. 5c), suggesting that the obese-like microenvironment generates tumors of a higher grade.

To determine whether the priming of the microenvironment by macrophages and blood vessels before tumor formation accelerates tumor formation, tumorigenesis was evaluated 2 weeks following implantation of human breast epithelial cells into humanized fat pads. Within the implantation site of humanized SVF/CCL2 fat pads, premalignant lesions exhibited significantly increased recruitment of both F4/80+ macrophages (P < 0.0001) and CD31+ endothelial cells (P < 0.0001) before the formation of carcinomas (Fig. 5d). These results suggest that early macrophage recruitment and angiogenesis within the obese breast likely contribute to accelerated breast cancer formation.

**Macrophages are necessary for tumor progression associated with obesity**

To determine whether priming of the microenvironment by macrophages is necessary for accelerated tumor growth in the obese-like stroma, glands from Mac-SCID females were humanized with SVF/CCL2 cells and transplanted with reduction mammoplasty-derived epithelial cells transduced with SV40/Kras. At 24 and 72 hours following epithelial cell implantation, mice received injections of either DT or vehicle. After 2 weeks, humanized SVF/CCL2 fat pads from vehicle-treated mice showed significantly larger outgrowths compared with DT-treated mice, indicating that ablation of macrophages significantly reduced tumorigenesis (Fig. 6a). Vehicle-treated mice exhibited significantly increased number of CD31+ blood vessels, suggesting that ablation of macrophages attenuates angiogenesis (P = 0.0081; Fig. 6a). At end stage, increased tumorigenesis was observed in Supplementary Table S2). In addition, obesity-like humanized stroma led to a significant increase in tumor weight compared with those humanized with SVF/EV cells (P = 0.04; Fig. 5b). Although end-stage tumors were histologically similar (Supplementary Fig. S5a), tumors that developed within SVF/CCL2 humanized fat pads exhibited significantly increased numbers of mitotic figures (P = 0.002) and Ki67+ cells (P = 0.03; Fig. 5c), suggesting that the obese-like microenvironment generates tumors of a higher grade.

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vehicle-treated mice compared with the DT-treated cohort (6/8 vehicle vs. 1/6 DT, P = 0.03). Fat pads of the DT-treated group that did not grow expansive tumors contained small, well-circumscribed DCIS lesions that retained expression of an outer layer of cytokeratin (CK) 14+ myoepithelial cells surrounding CK8+ luminal cells (Fig. 6B). Although the pattern of CK8 and CK14 expression was different, the total staining for both markers was similar (Supplementary Fig. S5B). Epithelial cells in these DCIS lesions expressed both oncogenes, as well as Ki67, suggesting that DT treatment did not ablate the transplanted cells. In contrast, expansive tumors that developed in glands of vehicle-treated females were invasive and disorganized (Fig. 6B). These results suggest that in addition to increasing angiogenesis early during tumor formation, inflammatory macrophages are necessary to promote the progression of DCIS into aggressive, invasive tumors.

To determine whether inhibition of CCL2 and IL-1β might be a viable prophylactic strategy in treating breast cancers associated with obesity, mice humanized with SVF/CCL2 cells and implanted with SV40/Kras-transduced human breast epithelial cells were treated daily with small-molecule inhibitors for CCL2.
and of IL-1β (Kineret; IL-1ra). After 2 weeks of treatment, mice receiving inhibitors exhibited a significant reduction in recruited F4/80+ macrophages compared with fat pads of vehicle-treated mice (P = 0.02; Fig. 6C). Likewise, the CD31+ vasculature was significantly diminished within the premalignant lesions of inhibitor-treated mice compared with those receiving vehicle (P = 0.01; Fig. 6D). These results suggested that CCL2 and IL-1β inhibitors may slow the recruitment of macrophages and angiogenesis, thereby delaying tumor onset and progression. However, at end stage, there was no difference in latency among tumors in the vehicle or inhibitor treatment groups (Supplementary Fig. S5C).
tumors in the inhibitor treatment group showed significant changes in nuclear morphology including alterations in cell size, nuclear/cytoplasmic ratio, and abnormal mitoses compared with tumors from mice treated with vehicle (Supplementary Fig. S5D). The inhibitor-treated tumors no longer had diminished macrophage recruitment (Supplementary Fig. SSE) and showed a significant increase in SMAβ cancer-associated fibroblasts (Supplementary Fig. S5F). These results suggest that CCL2 and IL-1β inhibitors had direct effects on the tumor cells as well as the SMAβ cancer-associated fibroblasts. Although the DT experiments showed that ablation of macrophages limits tumor development, the method of drug delivery may need to target macrophages specifically to limit the response of other cell types within the tumor parenchyma.

Discussion

Obesity elevates the risk of postmenopausal breast cancer as well as promotes the formation of aggressive tumors (3). Our findings suggest that during obesity, the enhanced production of CCL2 and IL-1β by the breast adipose tissue increases recruitment of macrophages and the formation of CLS. The recruited macrophages are in turn stimulated by CCL2 and IL-1β to secrete CXCL12 to induce angiogenesis and support the expanding tissue (Fig. 6E). Under conditions of obesity, the microenvironment of the breast adipose tissue becomes a fertile soil for tumorigenesis such that a prelaminantigen can bypass the rate-limiting step of inducing its own vasculature during tumor progression (43). Our studies also showed that macrophages promote progression from DCIS to aggressive tumors. Given recent work implicating CXCL12 in the expansion of cancer stem cell populations as well as metastases (44, 45), we are currently investigating whether CXCL12 administration can functionally replace macrophages in the DCIS microenvironment.

The increased levels of estrogen due to the expression of aromatase in preadipocytes (4) and in the presence of inflammation (6, 46) in obese fat tissue, likely play an important role in breast cancers associated with obesity (3). In fact, increasing the levels of circulating estrogen can lead to enhanced angiogenesis and recruitment of bone marrow–derived macrophages into a growing tumor mass (25, 47). Although the tumorigenesis studies reported here were conducted in ovari-intact mice, we have not excluded a role for estrogen in CCL2-mediated macrophage recruitment or angiogenesis. Nevertheless, it is likely that in obese postmenopausal patients with breast cancer, limiting aromatase activity as well as adipose inflammation may be critical for effective prevention and/or treatment of breast cancer.

Angiogenesis plays a crucial role in the growth and progression of breast cancer, and antiangiogenesis agents that target the VEGF/VEGFR pathway have had limited effects on breast cancer (48). Our studies suggest that inflammation within the obese mammary gland contributes to angiogenesis through a novel CCL2/IL-1β/CXCL12 pathway that bypasses the VEGF/VEGFR pathway. Thus, obese women may show reduced responsiveness to VEGF-targeted therapies due to elevated CXCL12 expression.

Adipose tissue is also dependent on vascularization for its expansion, and recent reports confirm that disruption of neovascularization can prevent the onset of obesity in both genetic and diet-induced obesity models (49). However, the side-effects of these drugs may limit their usefulness in the treatment of obesity or in the prevention of cancer in obese at-risk women. Therefore, targeting inflammation in obese women in addition to angiogenesis might offer more promise in treating obesity or cancer. Consistent with this notion, treatment of mice with propargammium, a CCR2 inhibitor, suppressed inflammation in the adipose tissue and increased insulin sensitivity (50). Our findings here suggest that inhibition of inflammation can limit macrophage infiltration and angiogenesis during early tumor development. Moreover, given that tumorigenesis was almost entirely prevented in mice following macrophage ablation, development of drugs that specifically target this phagocytic population may have substantial therapeutic benefits in the treatment of obesity and/or prevention of breast cancer in obese women.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.M. Arendt, S.P. Naber, C. Kuperwasser
Writing, review, and/or revision of the manuscript: L.M. Arendt, J. McCready, S.P. Naber, C. Kuperwasser
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.M. Arendt, C. Kuperwasser
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