Cancer-Associated Adipose Tissue Promotes Breast Cancer Progression by Paracrine Oncostatin M and Jak/STAT3 Signaling

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Abstract

Increasing evidence supports the critical roles played by adipose tissue in breast cancer progression. Yet, the mediators and mechanisms are poorly understood. Here, we show that breast cancer–associated adipose tissue from freshly isolated tumors promotes F-actin remodeling, cellular scattering, invasiveness, and spheroid reorganization of cultured breast cancer cells. A combination of techniques, including transcriptomics, proteomics, and kinomics enabled us to identify paracrine secretion of oncostatin M (OSM) by cancer-associated adipose tissue. Specifically, OSM, expressed by CD45+ leukocytes in the stromal vascular fraction, induced phosphorylation of STAT3 (pSTAT3-) Y705 and S727 in breast cancer cells and transcription of several STAT3-dependent genes, including S100 family members S100A4, S100A8, and S100A9. Autocrine activation of STAT3 in MCF-7 cells ectopically expressing OSM-induced cellular scattering and peritumoral neovascularization of orthotopic xenografts. Conversely, selective inhibition of OSM by neutralizing antibody and Jak family kinases by tofacitinib inhibited STAT3 signaling, peritumoral angiogenesis, and cellular scattering. Importantly, nuclear staining of pSTAT3-Y705 identified at the tumor invasion front in ductal breast carcinomas correlates with increased lymphovascular invasion. Our work reveals the potential of novel therapeutic strategies targeting the OSM and STAT3 axis in patients with breast cancer harboring nuclear pSTAT3-Y705.

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Introduction

Cancer progression is the result of a complex interaction between the cancer cells and their microenvironment (1, 2).

Breast tumors are surrounded by type I collagen-rich tissue, including fibroblasts, blood and lymph vessels, and adipose tissue. Insights in the function of adipose tissue have shifted from a static organ for energy storage to a dynamic one, excreting growth factors, cytokines, and hormones, identifying the adipose tissue as an active player in the communication between the tumor and its microenvironment (3).

Human adipose tissue–derived adipocytes, stem cells, stromal cells, CD34+ progenitor cells, and macrophages stimulate growth, migration, and invasion of breast cancer cells by secretion of CCL5 (Rantes), IL6, IL8, and PAI-1 (4–7). A mouse mammary cancer model revealed tumor progression through secretion of adipocyte-derived type VI collagen (8). Dirat and colleagues (9) showed that mature adipocytes promote the invasiveness of estrogen receptor α (ERα)–positive and –negative breast cancer cells. IL6-mediated induction of epithelial-to-mesenchymal transition (EMT) was identified as key component of adipocyte-enhanced invasiveness of breast cancer cells (9). Zhang and colleagues (10) reported stimulation of tumor growth and angiogenesis by recruitment of adipose stromal cells and endothelial cells in a breast cancer mouse model. All together, the interaction between breast cancer cells and associated adipose tissue is a multifactorial phenomenon driving breast cancer progression.

STATs are important in cytokine receptor signaling. Several STATs contribute to normal mammary gland development...
and cellular response during pregnancy, lactation, and involution. However, recent studies revealed their seemingly contradictory participation in breast cancer progression (11). STAT3 is frequently activated in human breast cancer and correlates with poor prognosis (12).

Materials and Methods

Cell lines and transfections

MCF-7, T47D, SKBR3, and MDA MB 231 cell lines were obtained from the ATCC (http://www.lgstandards-atcc.org). Cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 2.5 μg/mL fungizone (Bristol-Myers Squibb). All cell lines have been validated in-house by short tandem repeat profiling using the Cell ID System (Promega) according to the manufacturer’s instructions. Upon receipt, cells were passaged and stored in liquid nitrogen. Every 6 months, a new aliquot of cells was resuscitated and used for experimentation. Every month cell cultures were tested for mycoplasma contamination by using the MycoAlert Plus Kit (Lonza).

MCF-7 cells secreting turbo green fluorescent protein (tGFP)–oncostatin M (OSM) fusion protein (MCF–7–OSM) were generated by Eugene Transfection (Promega). tGFP–OSM cDNA was purchased at Origene, Inc.

Antibodies and reagents

Primary and secondary antibodies are described in Supplementary Materials and Methods. Recombinant human (rh) OSM, (rh)II6, (rh)IL8, (rh)LIF, (rh)G-CSF and their respective neutralizing monoclonal antibodies were from R&D Systems. Tofacitinib citrate was purchased at Bio-Connect Diagnostics. A neutralizing (n)OSM antibody, Akt (GSK2142795) and MEK inhibitors (GSK1120212 or trametinib) were kindly provided by GlaxoSmithKline (13).

Conditioned medium of cancer-associated adipose tissue, isolation of tumor-associated adipocytes, and stromal vascular fraction

CAAT (cancer-associated adipose tissue) was obtained from patients with breast cancer undergoing a mastectomy at Ghent University Hospital (Ghent, Belgium) in accordance with the local ethics committee (Supplementary Table S1). Preparation of CMCAAT (conditioned medium of cancer-associated adipose tissue) and separation of adipose tissue in tumor-associated adipocytes (TAA) and stromal vascular fraction (SVF) and isolation of CD45+ and CD31+ fractions from SVF are described in Supplementary Materials and Methods. Supplementary Fig. S1 demonstrates the use of CMCAAT throughout the article.

Functional assays

Experimental set-up for studying morphologic changes induced by CAAT or CMCAAT is described in Supplementary Materials and Methods. Factor shape was calculated as (perimeter)²/(4 × π × area) for quantification.

MCF-7, T47D, and MDA MB 231 type I collagen gel invasion assays were performed and quantified as described in Supplementary Materials and Methods and ref. 14.

Matrigel invasion of MDA MB 231 and migration of MCF-7 and SKBR3 cells were performed using xCELLigence RTCA DP instrument (ACEA Biosciences; ref. 15).

Protein analysis

Samples for Western blot analysis were prepared, run, and immunostained as described in ref. 16.

Human phospho-kinase antibody array (R&D Systems) was used to detect relative phosphorylation levels of 44 kinases. OSM concentrations in CMCAAT were measured with a human OSM ELISA Kit (Sigma-Aldrich). Scanning densitometry was carried out with the Quantity One Program (Bio-Rad).

Microarray analysis

Total RNA was isolated using the Nucleospin RNA II Kit (Macherey-Nagel), including DNase I treatment. Quality control was performed using Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (0.5 μg) was processed and analyzed on Human GE Agilent 4 × 44 K microarrays. Four biologic samples were studied. Data can be found on GEO (GEO accession number GSE58574).

Quantitative real-time PCR

RNA was isolated using the RNeasy Plus Universal Kit (Qiagen), including DNase I treatment. cDNA synthesis and SYBR Green I RT-qPCR were carried out as described in ref. 17. Prime PCR assays for S100A7, S100A12, OSM, NTN4, LR6G1, LIFR, and GP130 were purchased at Bio-Rad. Other primer sequences are described in Supplementary Table S2. The RNA quality index (RQI > 8) was assessed using Experion software (version 3.2; Bio-Rad).

Mass spectrometry

CMCAAT samples were run on NuPAGE 4% to 20% Bis-Tris gradient gels (Invitrogen) in denaturing SDS buffer, stained with 0.5% Coomassie Brilliant Blue (Bio-Rad) in 40% methanol and 10% acetic acid for 20 minutes, and destained. Gel bands were processed and analyzed by LS/MS-MS as described in Supplementary Materials and Methods and ref. 16.

Animal studies

Animal studies were approved by the Local Ethics Committee of Ghent University Hospital (ECD 09/32). Immunodeficient mice were orthotopically injected with 1 × 10⁶ cancer cells. After 4 weeks, the mice were sacrificed and tumors were resected for IHC. Details can be found in Supplementary Materials and Methods.

Patient samples and IHC

Clinical data and paraffin-embedded primary breast carcinoma samples were collected at Ghent University Hospital (Supplementary Table S3). Written informed consent was obtained according to the recommendations of the local ethics committee. To evaluate nuclear pSTAT3-Y705, we considered an intensity score that was semiquantitative scaled as score 0, weak or absent nuclear staining; score 1, between 5% and 30% nuclear staining; score 2, more than 30% nuclear staining. Three observers quantified independently.
Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 21.0 software. Continuous data were analyzed with the Mann–Whitney test (mean ± SD) or the Student t test (difference of means and 95% confidence interval) in which appropriate. Spearman correlation was used to assess correlations. Categorical data were analyzed with the Fisher exact test. All data are representative of at least three independent experiments. Statistical tests were two-sided, P values less than 0.05 were considered statistically significant.
Results

The role of CAAT in morphologic reorganization and invasion of breast cancer cells

CAAT was confronted with MCF-7 aggregates in type I collagen, the main structural component of the mammary gland. CAAT induced aggregate reorganization resulting in infiltration of CAAT by MCF-7, engulfing single adipocytes (Fig. 1A). In contrast, MCF-7 aggregates not confronted with CAAT maintained a round shape up to 14 days of culture. The mean factor shape of CAAT-confronted MCF-7 aggregates was 4-fold that of controls (control vs. CAAT; 1.290 ± 0.095 vs. 4.017, ± 0.603, P = 0.004; Fig. 1A). We next questioned whether CM^CAAT^ could mimic the effects induced by direct coculture. Within 48 hours, 25 of 27 CM^CAAT^ (93%) induced cellular extensions and

Table 1: CAAT-secreted factors activate STAT3 signaling. A, phospho kinase array demonstrating the relative phosphorylation levels of 44 kinases on CM^CAAT^ treated MCF-7 cells (top, only the upper part of the test with 28 kinases is shown) and measurement of the fold change (bottom). B, Western blot analysis of pSTAT3-Y705 and pSTAT3-S727 in CM^CAAT^ treated MCF-7 cells. Duration of treatment is indicated: 5 (5 minutes), 10 (10 minutes), 48 hours (2 days), Rescue, 48 hours treatment with CM^CAAT^, followed by 48 hours with control medium. Total STAT3 and tubulin were loading controls. C, pSTAT3-Y705 staining of paraffin-embedded MCF-7 pellets treated as indicated (scale bar, 50 µm). D, pie chart for the distribution of at least 5-fold up- or downregulated genes in CM^CAAT^ treated MCF-7 cells in comparison with control-treated MCF-7 cells according to their functional category (DAVID database). E, relative mRNA levels of indicated genes in MCF-7 cells treated for 48 hours with control medium or CM^CAAT^. F, Western blot analysis of S100 proteins in MCF-7 cells treated for 48 hours with control medium, CM^CAAT^ or after rescue.

Figure 2. CAAT-secreted factors activate STAT3 signaling. A, phospho kinase array demonstrating the relative phosphorylation levels of 44 kinases on CM^CAAT^ treated MCF-7 cells (top, only the upper part of the test with 28 kinases is shown) and measurement of the fold change (bottom). B, Western blot analysis of pSTAT3-Y705 and pSTAT3-S727 in CM^CAAT^ treated MCF-7 cells. Duration of treatment is indicated: 5 (5 minutes), 10 (10 minutes), 48 hours (2 days), Rescue, 48 hours treatment with CM^CAAT^, followed by 48 hours with control medium. Total STAT3 and tubulin were loading controls. C, pSTAT3-Y705 staining of paraffin-embedded MCF-7 pellets treated as indicated (scale bar, 50 µm). D, pie chart for the distribution of at least 5-fold up- or downregulated genes in CM^CAAT^ treated MCF-7 cells in comparison with control-treated MCF-7 cells according to their functional category (DAVID database). E, relative mRNA levels of indicated genes in MCF-7 cells treated for 48 hours with control medium or CM^CAAT^. F, Western blot analysis of S100 proteins in MCF-7 cells treated for 48 hours with control medium, CM^CAAT^ or after rescue.
reduced cell–cell contacts in MCF-7 (Fig. 1B). Rescue experiments showed restoration of cobblestone-shaped morphology and tight cell–cell contacts. F-Actin staining of single cells revealed a rounded appearance for control cells and an elongated morphology with multiple protrusions upon CMCAAT treatment (Fig. 1C). The mean factor shape of CMCAAT-treated MCF-7 cells was 2.5-fold higher than in controls (control vs. CMCAAT; 1.221 ± 0.123 vs. 4.070 ± 1.652; \( P = 0.002 \), Fig. 1C). EMT markers showed no major expression changes in control versus CMCAAT treatment (Supplementary Fig. S2). Confocal immunocytochemistry revealed a reorganized F-actin cytoskeleton and relocation of E-cadherin and β-catenin from the plasma membrane to the cytoplasm (Supplementary Fig. S2). CMCAAT stimulated invasion of MCF-7 into type I collagen after 24 hours (control vs. CMCAAT; 9.750% ± 2.790% vs. 46.750% ± 2.408%; \( P = 0.001 \), Fig. 1D) and increased migration of MCF-7 with a 14-fold slope increment \( (P = 0.021 \), Fig. 1E).

Similar morphologic changes (Fig. 1F) and stimulation of type I collagen invasion were seen upon CMCAAT treatment of T47D (control vs. CMCAAT; 9.340% ± 3.872% vs. 33.880%, ± 5.065%; \( P = 0.008 \), Fig. 1G).

Although induction of scattering is hard to assess in MDA MB 231 given their mesenchymal phenotype (Fig. 1H), CMCAAT increased type I collagen invasion of MDA MB 231 in a 14 days assay (Fig. 1I, top). In addition, CMCAAT stimulated Matrigel invasion of MDA MB 231 with a 4.5-fold slope increment \( (P = 0.002 \), Fig. 1I, bottom).

**Paracrine activation of STAT3 by CAAT in breast cancer cells**

Phospho-kinase screening in CMCAAT-treated MCF-7 cells revealed higher phosphorylation levels of STAT3 (Y705), ERK, JNK, AKT, and CREB (Fig. 2A). Western blot analysis of pSTAT3-Y705 and pSTAT3-S727 in MCF-7 cells treated with rhOSM, IL6, IL8, and G-CSF (top). Western blot analysis of S100A7 in MCF-7 cells (Fig. 2B). A rescue experiment showed restoration of S100A8 and S100A9 levels to basal conditions, whereas S100A7 levels were reduced by 32.5%.

**Expression and secretion of OSM by CAAT**

Using a biotin label–based assay, CMCAAT from 2 patients revealed the presence of six proteins with a reported capacity to activate STAT3 signaling: OSM, IL6, IL8, G-CSF, LIF, and leptin (Supplementary Fig. S3). Addition of rhOSM dose dependently increased Y705 and S727 pSTAT3 in MCF-7 cells. In contrast, only high concentrations of rhL6 and rhG-CSF phosphorylated STAT3 Y705 but not STAT3 S727 (Fig. 3A), suggesting a reduced transcriptional activator capacity (as compared with rhOSM or CMCAAT). Moreover, only rhOSM upregulated S100A7 protein levels (Fig. 3A), rhL6, rhL8, and rhLIF had no effect on morphology and addition of their neutralizing antibodies to CMCAAT did not counter CMCAAT-induced morphologic changes (Supplementary Fig. S3). Only neutralizing (n)IL6 antibody partially inhibited CMCAAT-induced phosphorylation of STAT3 Y705 but had no effect on S727 phosphorylation (Supplementary Fig. S3). rhLeptin was not tested because it lacked the capacity to induce cellular scattering (21).

Mass spectrometry of CMCAAT revealed 10 unique OSM peptides at the expected molecular weight of 25 to 30 kDa with 55% sequence coverage (Fig. 3B). Western Blot analysis identified OSM protein in CMCAAT from 3 patients. ELISA of CMCAAT from 16 patients revealed an OSM concentration between 3.7 and 15.7 pg/mL (Fig. 3C). There was no correlation between the OSM concentration in CMCAAT and body mass index (BMI) of these patients \( (P = 0.948, \text{Spearman Rho} = -0.115) \).

To determine the source of OSM production, tumor-associated adipose tissue from 10 patients with breast cancer was separated into TAA and the SVF. RT-qPCR revealed a 20-fold
increased expression of *OSM* mRNA in the SVF compared with adipocytes (*P < 0.001*). *OSM* mRNA levels in TAA and SVF were not correlated with the BMI of the patients (*OSM* in TAA, *P = 0.650*, Spearman Rho = 0.164; *OSM* in SVF, *P = 0.235*, Spearman Rho = 0.413). *OSM* protein was found in the CD45^−^leucocyte fraction and not in the CD31^−^endothelial fraction or "rest" fraction containing CD34^+^/CD31^+^ adipocyte progenitor cells (Fig. 3D; ref. 22).

Macrophages are CD45^−^ and an increased presence of tumor-associated macrophages (TAM) has been associated with poor prognosis in human breast cancer. CD163 staining revealed an accumulation of TAMs in the adipose tissue at sites of cancer cells with high nuclear pSTAT3-Y705 infiltrating the adipose tissue (Fig. 3E).

*OSM* engages heterodimeric receptors involving gp130 and either the *OSM* receptor (*OSMR*) or the leukemia inhibitory factor receptor (LIFR). The gp130/OSMR complex is specifically activated by *OSM* and is implicated in morphologic changes (23). RT-qPCR revealed the relative expression of *OSMR* in MCF-7, T47D, and MDA MB 231 whereas SKBR3 show a 60,000-fold lower *OSM* expression (Fig. 3F). rh*OSM* treatment of these cell lines induces morphologic changes in all except SKBR3 (Fig. 3G). Moreover, migration of MCF-7 and not SKBR3 is dose dependently stimulated by rh*OSM* with a 5-fold (100 pg/mL, *P = 0.071*) and 9.9-fold (1 ng/mL, *P = 0.001*) slope increment in MCF-7 compared with 1.4-fold (100 pg/mL, *P = 0.941*) and 1.6-fold (1 ng/mL, *P = 0.422*) slope increment in SKBR3 (Fig. 3H). rh*OSM* treatment of MCF-7 and not SKBR3 is dose dependently stimulated by rh*OSM* with a 5-fold (100 pg/mL, *P = 0.071*) and 9.9-fold (1 ng/mL, *P = 0.001*) slope increment in MCF-7 compared with 1.4-fold (100 pg/mL, *P = 0.941*) and 1.6-fold (1 ng/mL, *P = 0.422*) slope increment in SKBR3 (Fig. 3I). rh*OSM* treatment of MCF-7 leads to an increase of *OSMR* (2.7-fold) and STAT3 (2.2-fold) expression (*P = 0.029* for both genes), suggesting positive feedback. The *OSM* and STAT3 mRNA response is not observed in SKBR3. In both cell lines, LIFR and GPI30 mRNA levels are not affected by rh*OSM* (Supplementary Fig. S4).

**Functional role of *OSM* and STAT3 signaling in CAAT-mediated morphologic changes**

To quantify CM^CMCAAT^ potency on STAT3 phosphorylation, MCF-7 cells were treated with rh*OSM*. pSTAT3-Y705 was significantly at 0.01 ng/mL rh*OSM*, with a 95% confidence interval (CI) equivalent to ±0.5 ng/mL rh*OSM* (Fig. 4A). rh*OSM* induced a gene signature (Con vs. rh*OSM*, *P = 0.200*, Fig. 4B) and morphologic changes (Fig. 3G) similar to CM^CMCAAT^ treatment. Preincubation of rh*OSM* with n*OSM* antibody or addition of the pan-Jak inhibitor tofacitinib abolished STAT3 phosphorylation (Fig. 4C). Preincubation of CM^CMCAAT^ with n*OSM* antibody reversed STAT3 activation (Fig. 4C, bottom left) and morphologic responses (Fig. 4D). Tofacitinib blocked CM^CMCAAT^-induced STAT3 activation and S100A7 expression (Fig. 4C), morphologic responses (Fig. 4D), and nuclear localization (Fig. 4E). The AKT pathway (inhibited by GSK2141795) but not the MEK/ERK pathway (inhibited by trametinib) is necessary for pSTAT3-S727 and transcriptional activity (Supplementary Fig. S5).

We established MCF-7 cells that ectopically secreted OSM (MCF-7–OSM) to examine the impact of constitutive OSM secretion and signaling in breast cancer progression. MCF-7–OSM cells have a decreased proliferation rate, lost the ability to form aggregates, and show an increased expression of *OSMR* compared with control MCF-7–GFP cells (Supplementary Fig. S6). OSM in the secretome coincided with constitutive pSTAT3-Y705 and increased expression of S100A7 (Fig. 5A). Tofacitinib reduced pSTAT3-Y705 and S100A7 expression (Fig. 5A).

Histology revealed that MCF-7–GFP cells organized into clusters with a compact pattern separated by Matrigel, whereas MCF-7–OSM xenografts displayed disorganized strands and single cells (Fig. 5B). Quantification of cellular organization by calculating factor shape indicated a statistically significant deviation (GFP vs. OSM, 1.4 ± 0.09 vs. 4 ± 1.4, difference, 2.6; 95% CI, 1.24–3.95; *P = 0.0016*). Pan-cytokeratin and vimentin showed no differential expression between MCF-7–GFP and MCF-7–OSM tumors. However, E-cadherin membrane expression and ERα expression was reduced in OSM-secreting tumors compared with control (Supplementary Fig. S2). Staining of pSTAT3-Y705 showed that 3.3% of MCF-7–GFP is positive compared with 69.8% of MCF-7–OSM cells (difference, 66.5%; 95% CI, 59%–73%; *P < 0.0001*; Fig. 5B).

All MCF-7–GFP xenografts had poor peritumoral vascularization (6/6) whereas MCF-7–OSM tumors developed strong peritumoral vascularization (11/12) as evidenced by macroscopic evaluation (Fig. 5B) and contrast-enhanced microcomputed tomography (μCT; Fig. 5B). Microarray data revealed the, respectively, 11- and 7-fold upregulation of angiogenic factors *NTN4* (24) and *LRG1* (25) in CM^CMCAAT^-treated MCF-7 cells. rh*OSM* and ectopic expression of *OSM* mimicked CM^CMCAAT^-induced effects on *NTN4* and *LRG1* (Con vs. CM^CMCAAT^, *P < 0.0001*; Con vs. rh*OSM* and Con vs. OSM, *P = 0.004*; Fig. 5C).

Treatment with n*OSM* antibody alleviated OSM-induced peritumoral angiogenesis as demonstrated by reduced number of mice (1/6) showing peritumoral blood vessels and blocks OSM-induced pSTAT3-Y705 (Fig. 5B), with 21.1% of MCF-7–OSM cells showing a positive nuclear signal (difference compared with untreated, 47.9%; 95% CI, 38%–57%; *P < 0.0001*). Administration of tofacitinib prevented OSM-induced peritumoral angiogenesis in 4 of 6 mice and reduced pSTAT3-Y705 (Fig. 5B) with 20% of MCF-7–OSM cells showing nuclear STAT3 (difference compared with untreated, 49%; 95% CI, 42%–56%; *P < 0.0001*). Both the n*OSM* antibody and tofacitinib restored MCF-7 cluster organization (Fig. 5B). The mean factor shape of cell clusters from MCF-7–OSM tumors treated with n*OSM* antibody and tofacitinib was, respectively, 1.78 ± 0.44 and 1.62 ± 0.26, indicating compacted organization (difference n*OSM* antibody vs. untreated, 2.2; 95% CI, 0.8–3.63; *P = 0.0058*; difference tofacitinib vs. untreated, 2.3; 95% CI, 1.00–3.75; *P = 0.0032*).

**Nuclear expression of pstat3-y705 in invasive ductal breast cancer**

We studied the expression of nuclear pSTAT3-Y705 by IHC in 50 patients with ER-positive invasive ductal carcinoma with histologically confirmed adipose tissue infiltration. Nuclear pSTAT3-Y705 staining was present at sites of adipose tissue infiltration in 18% of the samples (9/50). Interestingly, pSTAT3-Y705–positive samples showed a statistically significant
Figure 4. Tofacitinib and nOsm antibody inhibit rhOsm- and CMCAAT-induced scattering and STAT3 activation. A, Western blot analysis of pSTAT3-Y705 and pSTAT3-S727 in MCF-7 cells treated with a range of rhOsm or CMCAAT from 3 patients (P36, P40, and P44). pSTAT3-Y705 and pSTAT3-S727 by P36, P40, and P44 equals approximately 0.64, 0.86, and 0.90 ng/mL rhOsm, respectively. B, relative mRNA levels of indicated genes in MCF-7 cells treated for 48 hours with rhOsm (2 ng/mL). C, Western blot analysis of pSTAT3-Y705 and pSTAT3-S727 in MCF-7 cells treated for 48 hours with rhOsm (5 ng/mL; top) or CMCAAT (bottom), combined with a range of nOsm antibody (left) or tofacitinib (right). D, phase-contrast images of MCF-7 cells treated for 48 hours with control medium, CMCAAT, or CMCAAT combined with nOsm antibody (from R&D Systems) or tofacitinib at the indicated concentrations. E, pSTAT3-Y705 staining of paraffin-embedded MCF-7 cell pellet treated for 48 hours with CMCAAT or CMCAAT combined with tofacitinib (scale bar, 50 μm).
Figure 5. Treatment with nOSM antibody or tofacitinib inhibits OSM-induced effects in MCF-7 xenografts. A, left, validation of MCF-7 cells stably transfected with OSM cDNA (MCF-7–OSM) compared with transfection with GFP control plasmid (MCF-7–GFP). Western blot analysis of OSM, pSTAT3-Y705, and STAT3 in cell lysates (LYS) and conditioned medium (CM) of MCF-7–GFP and MCF-7–OSM cells. Total STAT3 and tubulin serve as loading control. Right, Western blot analysis of pSTAT3-Y705 and pSTAT3-S727 and S100A7 in MCF-7–OSM cells treated for 48 hours with control medium or 500 nmol/L tofacitinib. MCF-7–GFP serves as the control cell line. B, Swiss nu/nu mice injected with MCF-7–GFP or MCF-7–OSM cells in the right lower mammary fat pad and treated with neutralizing OSM antibody or tofacitinib. First row, macroscopic external view of tumors (indicated by arrows); second row, macroscopic internal view of tumors; third row, contrast-enhanced μCT images of the blood vessels surrounding the tumor (asterisk, the right hind leg bone; arrow, enhanced peritumoral angiogenesis); fourth and fifth rows, hematoxylin and eosin and pSTAT3-Y705 staining of resected tumors (scale bar, 50 μm). C, relative mRNA levels of NTN4 and LRG1 expression in MCF-7 cells treated with control medium, CMCHA or rhOSM (2 ng/mL) and in MCF-7–OSM cells.
association with lymphovascular invasion (LVI; P = 0.0032, Fig. 6). LVI is a poor prognostic factor in patients with lymph node–negative IDCA (26).

Discussion

Adipose tissue is an endocrine organ producing effectors with local and systemic actions. Tumor infiltration into the adjacent fat is a risk factor for breast cancer progression (27). We investigated the impact of CAAT in breast cancer infiltration and progression. OSM is a CAAT-secreted factor activating STAT3 and inducing proinflammatory genes, cellular scattering, and peritumoral angiogenesis (Fig. 7).

Although adipose tissue has been shown to secrete a number of cytokines potentially affecting breast cancer cells, our studies demonstrate that OSM is the most relevant factor stimulating cancer progression. First, in comparison with IL6, IL8, and G-CSF, OSM is the only inducer of cellular scattering and the most potent inducer of pSTAT3-Y705. Second, pSTAT3-S727, important for transcription, was only seen upon OSM stimulation. Third, only OSM induces S100A7 expression described as poor prognosis marker in breast cancer (28). Fourth, two distinct OSM-neutralizing antibodies (R&D Systems and GSK) abolish CMCAAT-induced morphologic changes and STAT3 activation. Fifth, besides STAT3, CMCAAT activates downstream intermediates of OSM such as AKT, JNK, ERK, and CREB (23, 29), and AKT activation is necessary for pSTAT3-S727. Sixth, nIL6, nIL8, and nLIF antibodies do not affect CMCAAT-induced scattering. Seventh, nIL6 antibody partly inhibits CMCAAT-induced pSTAT3-Y705 but not pSTAT3-S727. nIL8 and nLIF antibodies do not affect CMCAAT-induced pSTAT3-Y705. Finally, CMCAAT induces OSMR and STAT3 in a similar fashion as rhOSM. These findings are in agreement with Xiao and colleagues (30) showing a positive feedback loop for further amplification of OSM-induced signaling.

OSM and CMCAAT increase expression of S100 proteins. A rescue experiment showed a relative higher S100A7 protein level compared with S100A8/9 after rescue procedure. All studied S100 proteins have an intracellular and secreted pool, are associated with the actin cytoskeleton, and contribute to increased scattering and migration. S100A8/9 proteins are uniquely reported as promigratory. However, S100A7 has a described pro- and antimigratory function. This effect might be concentration dependent. Higher levels of S100A7 may stimulate migration; slightly lower levels support the inhibition of lamellipodia formation and restore the nonmigratory state (31). OSM and CMCAAT shift E-cadherin membrane localization to the cytoplasm but have no impact on vimentin,
fibronectin, or cytokeratin expression. Reduction of cell surface E-cadherin expression is a prerequisite for cellular scattering (32) and a marker of EMT. In contrast with Guo and colleagues and West and colleagues (33, 34), we do not observe a full EMT in our set-up. Although all studies used breast cancer cells, both West and colleagues and Guo and colleagues (33, 34) used higher OSM concentrations (100 ng/mL) and longer treatments that stimulate fibronectin, snail, and vimentin expression. In accordance with West and colleagues, withdrawal of OSM restores the epithelial phenotype, indicating that OSM does not induce permanent phenotypic changes. Concentrations of OSM provided at the adipose invasion front, a 1,000- to 10,000-fold lower compared with West and colleagues and Guo and colleagues, were capable of activating STAT3 and increasing adipose tissue infiltration by reduced cell surface E-cadherin.

Although OSM has been shown to inhibit cellular proliferation of MCF-7 and T47D (35, 36) and cell culture experiments with MCF-7 constitutively secreting OSM show a reduced growth compared with control transfected cells, our xenograft experiments revealed similar tumor sizes between control and OSM groups, most probably as a result of enhanced peritumoral angiogenesis. OSM may directly stimulate angiogenesis (37) and stimulate the secretion of VEGF by T47D cells (38). We discovered the stimulatory role of OSM on the expression of NTN4 and LRG1 genes encoding the proangiogenic factors netrin-4 and LRG1. OSM stimulates expression and secretion of S100 proteins, which promote angiogenesis by stimulating endothelial cell proliferation (31). Whether in the xenograft model, the increased presence of blood vessels is caused directly by OSM or indirectly by OSM-induced netrin-4, LRG1, S100 proteins, or a combination of them, is not known.

OSM mRNA is high in the SVF and immune-isolation of CD45+ leucocytes revealed the presence of OSM protein compared with the endothelial and preadipocyte/fibroblast fraction. A study using subcutaneous adipose tissue supports our findings showing that mature adipocytes are not the main source of OSM but derives from cells in SVF, including macrophages (39). CAAT is infiltrated by CD163+ macrophages and accumulate at sites of high pSTAT3-Y705 in cancer cells. CD163+ macrophages are defined as TAMs correlated with poor prognosis in breast cancer (40) potentially by contribution of proinflammatory cytokines stimulating invasion (9). Mature adipocytes express OSMR and OSM provided by infiltrated macrophages may contribute to dedifferentiation of mature adipocytes (41).

The underlying mechanisms linking obesity to breast cancer risk and progression are not yet fully understood. Obesity is involved in STAT3 activation by at least two mechanisms. First, high plasma levels of leptin, correlated with obesity, are mainly implicated in breast tumorigenesis and growth (42). Second, obesity is associated with a chronic state of low-grade inflammation, attracting macrophages, and lymphocytes producing STAT3-activating cytokines (43, 44). Our data show no clear contribution of obesity mediated through CMCAAT. First, CMCAAT from 9 patients showed similar STAT3 activation, regardless of their BMI (5 normal, 3 overweight, and 1 obese). Second, CMCAAT from 25 patients with a BMI ranging from 19.1 to 41 (2 underweight, 10 normal, 9 overweight, and 4 obese)
induced cellular scattering. Third, from the 9 IDCA samples with high pSTAT3-Y705 staining only one was an obese patient (6 normal, 2 overweight, and 1 obese). Fourth, OSM mRNA levels in SVF from 10 patients with breast cancer were not correlated with BMI. Fifth, we found no correlation between OSM concentration in CMCAAT from 16 patients and their respective BMI. Admittedly, the number of patient samples in our study is low. Therefore, further studies using large series of breast CAAT samples from age-matched patients, including more detailed fat deposit information and metabolic biomarkers, will yield more information whether OSM secretion by activated macrophages and pSTAT3-Y705 in infiltrating cancer cells is associated with obesity. Alternatively, recruitment of inflammatory cells is a consistent feature of the local tumor environment and an enabling characteristic of cancer progression (45). In agreement with Queen and colleagues (38), local production of chemokines by cancer cells may recruit inflammatory cells at the adipose tissue invasion front leading to increased secretion of OSM.

Nuclear pSTAT3-Y705 at sites of adipose tissue infiltration revealed an association with LVI, a poor prognosis marker in breast cancer. Although all investigated CAAT and SVF samples expressed OSM, only one fifth of patients with ER-positive breast cancer showed pSTAT3-Y705 at invasion front. This apparent contradiction may be explained by differential expression of OSMR in breast cancer cells. Conflicting data exist about activated STAT3 as a marker for prognosis. One explanation is the use of tissue microarray cores representing the bulk of the tumor, not allowing to discriminate cancer cells at the invasion front (46, 47). We used whole-tissue slides allowing examination of cancer cell–CAAT interactions. Our data are endorsed by recent findings underlining the link between pSTAT3 at the invasion front and tumor progression (48). Second, primary breast tumors displaying tyrosine phosphorylation of STAT3 and STAT5 are more differentiated and display more favorable prognostic characteristics than those with selective STAT3 activation. Indeed, both STATs mediate opposing effects on several key target genes, with STAT5 exerting a dominant role (49). In accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4. Pinnilla S, Alt E, AbdulKhalek FJ, Jotzu C, Muehlberg F, Beckmann C, et al. Tissue resident stem cells produce CCL5 under the influence of natural killer T-cell lymphoma harboring constitutive Jak mutations (51). OSM-neutralizing antibodies have been used in multiple xenograft models (52, 53). We used a monoclonal OSM-neutralizing antibody that efficiently blocks OSM-induced peritumoral angiogenesis. Further studies should indicate the potential impact of OSM and Jak targeting in breast cancer.

This is the first report demonstrating a paracrine OSM/STAT3 activation loop at the level of adipose tissue versus epithelial interactions in ER-positive breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The study sponsors have no role in the design of the study; the collection, analysis, and interpretation of the data; the writing of the article; or the decision to submit the article for publication.

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