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CEACAM-1 negatively regulates G-CSF production by breast tumor associated macrophages that mediate tumor angiogenesis

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Abstract

CEACAM1, a cell adhesion molecule expressed on epithelial cells and activated immune cells, is down-regulated in many cancers and plays a role in inhibition of inflammation in part by inhibition of G-CSF production by myeloid cells. Since macrophages are associated with a poor prognosis in breast cancer, but play important roles in normal breast, we hypothesized that CEACAM1 down-regulation would lead to tumor promotion under inflammatory conditions. Co-cultures of pro-inflammatory M1 macrophages with CEACAM1 negative MCF7 breast cells produced high levels of G-CSF (10 ng/mL) compared to CEACAM1 transfected MCF7/4S cells (1 ng/mL) or anti-inflammatory M2 macrophage co-cultures (0.5 or 0.1 ng/mL, MCF7 or MCF7/4S, respectively). The expression of CEACAM1 on M1s was much greater than for M2s and was only observed in co-cultures with either MCF7 or MCF7/4S cells. When M1 macrophages were mixed with MCF7 cells and implanted in murine mammary fat pads of NOD/SCID mice, tumor size and blood vessel density were significantly greater than MCF7 or MCF7/4S only tumors which were hardly detected after 8 weeks of growth. In contrast, M1 cells had a much reduced effect on MCF7/4S tumor growth and blood vessel density, indicating that the tumor inhibitory effect of CEACAM1 is most likely related to its anti-inflammatory action on inflammatory macrophages. These results support our previous finding that CEACAM1 inhibits both G-CSF production by myeloid cells and G-CSF stimulated tumor angiogenesis.

Keywords

CEACAM1: Carcinoembryonic antigen-related cell adhesion molecule-1; Tumor associated macrophages; angiogenesis; breast cancer; G-CSF: Granulocyte colony-stimulating factor

Introduction

Macrophages are important resident cells of all tissues, where they play a critical role in maintenance of tissue homeostasis by participating in wound healing, inflammation, and control of infections^{1, 2}. In the case of cancer, increased numbers of tumor associated macrophages (TAMs) were observed in human breast³ and ovarian cancers⁴ and in classic Hodgkin's lymphoma⁵. The presence of high numbers of TAMs in neoplastic tissues is associated with poor prognosis, increased metastatic diseases and shortened survival times^{3, 6}. In tumor models, TAMs have been shown to promote chronic inflammation, tumor

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growth, invasion, angiogenesis and suppression of anti-tumor-directed immune responses^{7–10}. However, macrophages are critically involved in mammary gland development, including gland expansion during puberty and pregnancy, and mammary gland involution at the end of lactation^{11, 12}. Thus, it is likely that their beneficial role is subverted in pre-cancerous phenomena such as inflammation. The role of TAMs in breast cancer has been studied by direct analysis of cancer tissues or in murine models of breast cancer^{13, 14}. Once recruited, TAMs produce inflammatory molecules that alter the tumor microenvironment including tumor cell growth, motility, invasion, remodeling of extracellular matrix and neoangiogenesis^{15, 16}.

The apparently contradictory role of macrophages in normal vs malignant breast has been thought to reflect a phenotypic change in macrophages between the two states. For example, macrophages have been classified as inflammatory (M1) or anti-inflammatory (M2), depending on their cytokine profiles. In the case of breast cancer, TAMs have often been classified as the M2 phenotype based on their production of anti-inflammatory cytokines such as IL-10¹⁷. Furthermore, IL-10 producing macrophages have been associated with poor T-cell mediated killing of tumor cells in animal models¹⁰. However, one would expect that if inflammation is a pre-condition to breast cancer, then pro-inflammatory M1 macrophages may also play a role, especially in the earliest stages. If that is the case, then regulation of M1 macrophages in the normal mammary gland may keep these macrophages in check and the absence of this regulation may predispose the breast to chronic inflammation and set the scene for cancer progression. In support of this idea, we have observed that CEACAM1, a major negative regulator of inflammation¹⁸ is frequently down-regulated in many cancers of epithelial cell origin^{19, 20}, including breast cancer^{19, 21}.

Carcinoembryonic antigen related cell adhesion molecule 1 (CEACAM1), a cell-cell adhesion molecule, plays a role in differentiation, apoptosis and morphogenesis of epithelial cells. CEACAM1 is highly expressed in normal breast epithelial cells, including the “normal” cell line MCF10F that forms a lumen in 3D culture, but fails to form a lumen when CEACAM1 expression is down-regulated by an anti-sense RNA²². Conversely, the breast cancer cell line MCF-7 that lacks CEACAM1 and fails to form a lumen in 3D culture regains this ability when transfected with the CEACAM1 gene²³. Furthermore, CEACAM1 induced breast cell differentiation in a murine orthotopic model of human mammary morphogenesis²⁴. These studies indicate that lack of CEACAM1 predisposes breast epithelial cells to a condition resembling the early stages of invasive breast cancer. CEACAM1 is also expressed on activated immune cells and inhibits the inflammatory response in lymphocytes, macrophages, dendritic cells and NK cells¹⁸. Recent studies revealed that CEACAM1 participates in modulating the immune response to a gram-positive infection via Stat3 mediated G-CSFR signaling²⁵ and inflammation via IL-1 from neutrophils²⁶. In addition, anti-CEACAM1 antibodies or soluble-CEACAM1 has been shown to inhibit inflammatory bowel disease²⁷. However, few studies have focused on the role of CEACAM1 in tumor-associated immune responses or how it modulates the interaction between immune and epithelial cells in terms of initiation of ECM remodeling, tumor growth and dissemination. More recently, we have shown that CEACAM1 plays an inhibitory role in tumor growth and neoangiogenesis in a murine model of melanoma and colon cancer²⁸. An inhibitory role for CEACAM1 in tumor angiogenesis is novel in that it was previously thought that CEACAM1 stimulates angiogenesis, at least in endothelial cells undergoing neoangiogenesis²⁹ or embryonic stem cells undergoing vasculogenesis³⁰. Thus, the resultant activity of CEACAM1 between epithelial tumor and myeloid cells such as macrophages on neoangiogenesis is complex and requires further investigation.

Here, we characterize the phenotypic and functional behavior of macrophages and their role in promoting tumor growth and angiogenesis upon their interaction with breast cancer

epithelial cells that do or do not express CEACAM1. The results indicate that CEACAM1 expressed on breast tumor cells directly affects the ability of TAMs to produce the critical cytokine G-CSF, which in turn, is responsible for tumor neoangiogenesis and the initial establishment of tumors. Thus, the lack of expression of CEACAM1 in breast cancer tumors may act at the critical stage of cancer cell growth, namely the initiation of tumor angiogenesis by the production of G-CSF by TAMs.

Materials and Methods

Reagents

Recombinant human GM-CSF and M-CSF were from ProSpec Tany TechnoGene Ltd. Anti-CD163-PerCP/Cy5.5, anti-CD16-PerCP-Cy5.5, anti-CD14-APC/Cy7, anti-CD45-APC/Cy7, anti-CD54- FITC (ICAM1), anti-E- cadherin-FITC, anti-IL-18R -PE, anti-CD32-APC, anti-HLA-DR-PEcy5, and anti-CD50- Alexa 647 (ICAM3) were from Biolegend; anti-CD102-PE (ICAM2), anti-CD11b-APC, anti-CD18-PE, anti-CD59- FITC, anti-IL18R -FITC and anti-CD144-FITC were from BD biosciences; anti-CEACAM1-APC was from R&D systems) and anti-GCSF-APC and anti-CD45-PE were from eBiosciences). PE-anti-CD163 was obtained from BD Biosciences. Isotype controls were from their corresponding companies. EasySep® human monocyte enrichment kit was from StemCell Technologies Inc, Vencouver, Canada; FBS and human serum from Irvine Scientific, Santa Ana, CA, and anti-mouse CD31 and anti-human CD31 antibodies from Abcam. LPS (E. coli 055:B5, Sigma) was used at 100 ng/mL.

Cell Cultures

Breast cancer cell lines, MCF7 and MCF7/4S were grown in MEM medium with L-glutamine, 10%FBS, 1% sodium bicarbonate, 1% sodium pyruvate and 1% antibacterial-anti-mycotic solution. MCF7 cells transfected with CEACAM1-4S (MCF7/4S) or vector and their selection were previously described³¹.

Isolation and differentiation of human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from discard blood (IRB number 99132) using Ficoll-Paque Plus density gradient separation. Monocytes were negatively enriched to >95% according to the manufacturer's directions (StemCell Technologies Inc). Purified monocytes resuspended in RPMI 1640 medium with 10% FBS (1×10^6 cells/mL) were treated with GM-CSF (10 ng/mL) or M-CSF (25 ng/mL) for 4 days to generate M1 or M2 phenotypes, respectively. Monocyte to macrophage differentiation was verified by macrophages markers CD163 and CD14 using flow cytometry.

Co-culture of macrophage and MCF7 or MCF7/4S cells

MCF7 or MCF7/4S cells (1 million cells) were incubated in a 6 well plates for 1 day, followed by the addition of M1 or M2 macrophages (1 million cells) for 1 day. Culture supernatants and cells were collected for further analysis.

Cytokine Multiplex Analysis

Co-culture supernatants were analyzed for inflammatory cytokines and chemokines using Human Cytokine Plex Antibody Bead Kit (Biosource International) according to the manufacturer's protocol. A Bioplex HTF Luminex reader (Bio-Rad Laboratories) was used for analysis. The human cytokine 30-Plex antibody bead kit quantitates the following: IL-1, IL-1ra, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF, IFN, IFN, G-CSF, GM-CSF, MIP-1, MIP-1, MIG, RANTES, eotaxin, MCP-1, IP-10, VEGF, HGF, FGF-basic and HGF. Bio-Plex Manager 3.0 software with an eight-parameter

algorithm was used to determine the concentration of cytokines and chemokines in the cell culture supernatants.

Flow cytometry

After macrophage and epithelial cell co-culture, cells were washed with PBS, Fc receptors were blocked with 10% human serum and stained with either isotype control IgG or specific antibodies. For G-CSF intracellular staining, co-cultured cells were permeabilized using permeabilization/fixation buffer (BD Biosciences), followed by staining with anti-G-CSF or isotype control (eBiosciences) in 0.1% saponin/PBS. Stained cells were analyzed on a FACSCanto II cytometer with Flowjo software (TreeStar Inc).

Animal studies

A modified orthotopic model of human breast cancer in NOD/SCID mice was used³¹. Briefly, MCF7 or MCF7/4S cells (0.5 million cells) plus M1 or M2 macrophages (0.5 million cells) in 50 μ L of Matrigel extracellular matrix were injected into the inguinal mammary fat pad of NOD/SCID mice. Tumor growth was measured 6–8 weeks later and xenografts collected for staining. Xenografts were fixed in 10% phosphate buffered formalin for IHC or optimal cutting temperature embedding medium (OCT) for frozen sections, and stained with antibodies to human or mouse PECAM1 (CD31).

3D Assay

Cell culture plates (100 mm $\times$ 15 mm) were coated with 1 mL of Matrigel (BD Biosciences). MCF7 or MCF7/4S cells (1×10^6) with or without M1 or M2 macrophages (1×10^6) were cultured in mammary epithelial cell growth media (MEGM, Clonetics) for 2 hours, followed by removal of media and addition of 50% Matrigel for 2 hours. Ten mL of MEGM media with BPE was added and cells incubated for 4 days at 37°C. Formation of acini was observed by confocal microscopy and scored for the presence of round, normal acini or irregular, abnormal acini. Statistical analysis was performed by Student's *t* test.

Quantitative real-time RT-PCR

Total RNA isolated from cell pellet collected by the RNeasy plus kit (Quiagen Inc) followed by preparation of cDNA using the Omniscript® reverse transcription system (Qiagen, Inc). Quantitative expression of the genes G-CSF, VEGF, TNF- α and GAPDH were measured using the Bio-Rad CX96 Real-time Detection system (Bio-Rad Laboratory) with a SYBR qPCR master mix (SA biosciences) and standard DNA primer sequences (GAPDH primers: forward, 5'-ATCCATGACAACCTTGGTATCGTG and reverse, 5'-ATGACCTTGCCCACAGCCTT-3', GCSF primers: forward, 5'-TCTGAGTTTCATTCTCCTGCCTG-3' and reverse, 5'-ATTTACCTATCTACCTCCCAGTCCAG-3', TNF- α primers: forward, 5'-CCCAGGCAGTCAGATCATCTTC-3' and reverse, 5'-AGCTGCCCTCAGCTTGA-3', and VEGF primers: forward, 5'-TCTACCTCCACCATGCCAAGT-3' and reverse, 5'-GCTGCGCTGATAGACATCCA-3'). Amplification and extension parameters for qPCR were 95 °C for 5 min, 95 °C for 30 s, 54°C for 30 s, and 72 °C for 30 s for 40 cycles, followed by 72 °C for the final extension. Expression levels of G-CSF and VEGF mRNA (triplicates) in samples were compared and normalized against GAPDH message levels.

G-CSF Cytometric bead assay

Cell culture supernatants were diluted 1/10 in PBS and analyzed with the human G-CSF flex set (BD biosciences) according manufacturers instructions.

In vivo Matrigel angiogenesis assay

MCF7 or MCF7/4S cells (5×10^5) with or without M1 macrophages (5×10^5) in 350 μ L of growth factor–reduced Matrigel (BD Biosciences) were implanted into NOD/SCID mice. The implanted mice were injected i.p. with anti-G-CSF or isotype control antibody (R&D systems) at 10 μ g/mouse for 6 days. Matrigel plugs were dissected 7 days later and stained for CD31.

RESULTS

CEACAM1 expression in MCF7 cells differentially affects cytokine production by M1 and M2 macrophages

Since previous studies have shown that tumor associated macrophages (TAMs) confer a poor prognosis in breast cancer³², including the production of inflammatory cytokines and chemokines³, we hypothesized that it was the interaction between the macrophages and breast epithelial cells that was responsible for their production. However, macrophages are resident in normal breast and responsible for mammary morphogenesis and remodeling during pregnancy, lactation and post-weaning involution¹¹. Thus, we further hypothesized that the lack of a normal anti-inflammatory molecule such as the cell-cell adhesion molecule CEACAM1 was responsible for the aberrant behavior of resident macrophages. In agreement with this idea, the loss of expression of the CEACAM1 is a common event in breast cancer epithelial cells, as well as epithelial cells of other solid tumors²¹.

Macrophages may be produced in vitro by treating monocytes with either GM-CSF to generate pro-inflammatory M1 macrophages or with M-CSF to generate anti-inflammatory M2 macrophages³³. Although M2-like TAMs predominate in advanced breast cancers where the immune response is suppressed^{10, 17}, chronic inflammation and a pro-inflammatory environment also contribute to cancer progression⁶. In order to avoid pre-activation of the monocytes using anti-CD14 antibody coated beads (positive isolation), we used a negative isolation protocol similar to that described by Lacey and coworkers³³. The resulting M1 and M2 macrophages exhibit common macrophage surface markers (Figure S1A) and produce low levels of most cytokines in the case of M2s and high levels of IL-6, MCP-1 and MIP-1 in unstimulated cultures (Tables S1 and S2). Since it has been reported that unstimulated M1 macrophages have negligible levels of CD163 compared to M2s (ref), we repeated the cell surface analysis of CD163 using the same dye conjugated antibody (same hybridoma clone) used by Rey-Giraud et al³⁴, namely PE-anti-CD163. When reanalyzed with this dye, M1s were very low compared to M2s (Figure S1B) indicating that CD163 surface analysis alone cannot be used to distinguish M1 and M2 polarization. Furthermore, it has been reported that activated M1s produce high levels of IL-12 and low levels of IL-10 compared to activated M2s³⁵. When our M1 and M2 polarized macrophages were treated with LPS, M1s produced very high levels of IL-12 compared to M2s with an IL-10 to IL-12 ratio of 0.25 for M1s and 1.24 for M2s (Figure S1C). In addition, two other inflammatory cytokines (IL-1 and IL-6) were much higher for LPS stimulated M1s compared to M2s (Figure S1C). Based on these analyses we conclude that our M1 macrophages are pro-inflammatory and M2s are anti-inflammatory.

Given that CEACAM1 plays a role in apoptosis and growth inhibition in both epithelial cells²³ and lymphocytes¹⁸, we began our studies by co-culturing MCF7 cells (no CEACAM1 expression) or MCF7 cells transfected with CEACAM1-4S (MCF7/4S) cells with M1 and M2 macrophages to determine if their interaction would lead to a change in the morphology of epithelial cells and release of inflammatory cytokines or chemokines. As expected, the morphology of MCF7 or MCF7/4S cells cultured alone had a cobblestone appearance (Figure S2A–B). However, co-culture of M1 macrophages and MCF7 cells led

to a change in morphology in which the epithelial cells became elongated, similar to mesenchymal cells (Figure S2C). The expression of CEACAM1 in MCF7/4S cells co-cultured with M1 cells was inhibitory in that the morphology of the cells was intermediate between cobblestone and elongated (Figure S2D). In contrast, co-cultures with M2 macrophages did not change the morphology of either MCF7 or MCF7/4S cells (Figure S2E–F).

Since the production of cytokines and chemokines are likely responsible for the changes in morphology of epithelial cells in the co-cultures, we collected cell pellets, and supernatants from co-culture experiments and screened for changes in inflammatory cytokine and chemokine levels with a multiplex cytokine assay. Cytokine analysis of mono vs co-culture supernatants revealed a significant increase in the production of ten cytokines and chemokines in the co-cultures (Tables S1 and S2). Among the ten cytokines that exhibited significant changes, G-CSF exhibited the most dramatic changes compared to untreated controls. Although supernatant levels of G-CSF were negligible in M1, MCF7, and MCF7/4S mono-cultures, they rose by over 10-fold in the M1-MCF7 co-culture (Figure 1A). In contrast, the levels of G-CSF fell 5-fold in the M1-MCF7/4S co-culture (p value <0.05). Similar, but less dramatic results were found for the co-cultures with M2 cells (Figure 1B). When the mRNA levels of G-CSF were measured by Q-RT-PCR, the difference between M1-MCF7 and M1-MCF7-4S co-cultures was not observed, suggesting that CEACAM1 affected protein or secreted levels rather than mRNA levels (Figure 1C). A similar trend was seen for TNF- α and VEGF, where high secreted levels were observed for M1-MCF7 co-cultures compared to M1-MCF7/4S or M2 co-cultures (Figure 1D, E and G). When mRNA levels were measured by Q-RT-PCR for VEGF and TNF- α , negligible levels were found for mono-cultures and high levels only for MCF7+M1 co-cultures (Figure 1F and H). We also performed a microarray analysis on the mono and co-cultures to screen for mRNAs not analyzed by the cytokine analysis (data not shown). Importantly, we did not see significant changes in the expression levels of angiopoietins 1 and 2 or Tie-2, angiogenic factors/receptor occasionally associated with macrophages. We conclude from these analyses that cytokine and chemokine production, especially G-CSF, is dependent on the interaction of macrophages with epithelial cells, the secreted levels of G-CSF are differentially inhibited by the expression of CEACAM1 on the epithelial cells, and the pro-inflammatory M1 macrophages exhibit a greater influence on the secretion of G-CSF than M2 macrophages.

Macrophages are responsible for the production of G-CSF in co-cultures

Since among the cytokines measured G-CSF production was highest and has been previously shown to induce tumor angiogenesis in many animal models^{28, 36}, we further investigated its source of production. Macrophages and epithelial cells were co-cultured for 1 day and analyzed for expression of G-CSF by intracellular staining of cells with anti-G-CSF antibody. We used M1 macrophage cell surface markers (CD45 and CD163) to differentiate macrophages from epithelial cells in co-culture experiments. Flow cytometric analysis revealed that CD45-/CD163-cells did not express G-CSF (Figure 2A) while CD45+CD163+ cells expressed G-CSF (Figure 2B). These results demonstrate that M1 macrophages were responsible for production of G-CSF, but only when co-cultured with the MCF7 epithelial cells. Interestingly, we did not observe a difference in expression of G-CSF by M1 macrophages that were co-cultured with MCF7/4S cells in which CEACAM1-4S was expressed, indicating that the interaction between M1 macrophages and epithelial cells that is responsible for expression of G-CSF is not dependent on CEACAM1 but the release of G-CSF into the supernatants is inhibited by CEACAM1 (see Table S1).

Since we observed increased production of G-CSF by M1 macrophages in our co-culture model system, we further investigated whether macrophages required direct contact with

tumor cells or if a soluble factor was responsible for G-CSF induction and secretion. M1 or M2 macrophages were cultured with supernatants collected from MCF7 or MCF7/4S cells and the resulting supernatants analyzed for expression of G-CSF. As shown in Figure 2C, M1 macrophages incubated in supernatants from MCF7 or MCF7-4S cells expressed a higher level of G-CSF compared to the M1 macrophages incubated in control media. However, minimal G-CSF production was observed for M2 macrophages that were incubated in tumor cell supernatants (Figure 2D). We also quantified secretion of G-CSF, TNF- and VEGF into the supernatants by macrophages using the multiflex cytokine assay (Figure 2E–G). G-CSF secretion was stimulated by M1 but not M2 macrophages in either MCF7 or MCF7/4S cell supernatants compared to media controls (Figure 2E). TNF- secretion was stimulated by M1 but not M2 macrophages in MCF7 cell supernatants but to a much lesser extent in MCF/4S supernatants (Figure 2F). VEGF secretion was equally stimulated by M1 and M2 macrophages in both MCF7 and MCF7/4S supernatants, but to a much lesser extent compared to G-CSF and TNF- (Figure 4G). Importantly, M1-MCF7 co-cultures release much higher levels (more than 4 fold) of G-CSF and TNF- (see Table S1) than when they were incubated with supernatants from MCF7 cells (see Figure 2E–F). Taken together, the results indicate that direct cell-cell contact between macrophages and MCF7 epithelial cell is more effective in production of inflammatory cytokines compared to the production of soluble factors and that M1 are more effective than M2 macrophages in cytokine release.

To determine whether direct contact between epithelial cells and M1 macrophages is responsible for the enhanced expression of G-CSF in co-cultures, we employed a trans-well migration assay, in which M1 macrophages were placed in the upper well, and the epithelial cells in the lower well, both in cell culture media with FBS (no differential chemoattractant). We collected cells from upper and lower wells separately and determined the percentage of M1 macrophages that migrated to the lower well and expressed G-CSF compared to M1 macrophages that remained in the upper well. Flow cytometric analysis revealed that 3.0% or 5.8% of M1 macrophages migrated to the lower well containing either MCF7 or MCF7-4S cells, respectively (Figure 3A–B). When analyzed for G-CSF expression, only the M1 macrophages that migrated to the lower well expressed G-CSF compared to those that remained in the upper well (Figure 3C–D). Importantly, the expression of CEACAM1 in MCF7/4S cells resulted in a lower number of G-CSF positive cells in the lower well compared to MCF7 cells (Figure 3C–D). As expected, neither MCF7 nor MCF7/4S epithelial cells in the lower well were positive for the expression of G-CSF (Figure 3E–F). These results indicate the importance of direct contact between M1 macrophages and epithelial cells to elicit a robust inflammatory response by M1 macrophages, the ability of these macrophages to migrate and associate with breast epithelial cells, and the inhibition of the induction of G-CSF secretion by CEACAM1.

Up-regulation of cell-cell adhesion molecules on MCF7 and M1 macrophages in co-culture

Since the interaction of M1 macrophages and MCF7 cells was likely mediated by cell-cell adhesion molecules, we surveyed likely candidates from the breast cancer literature. In this respect, increased expression of ICAM1 in breast cancer is associated with increased cell motility, invasion and an aggressive tumor phenotype³⁷. Since α -2-integrins such as Mac-1 (α -M β -2) are the usual receptors on macrophages that bind to ICAM1-3, we included them in our analysis. FACS analysis of MCF7 and MCF7/4S monocultures express ICAM1-3 and α -M-integrin, while M1 and M2 macrophage monocultures express ICAM1-3, and α -M-and α -2-integrins on their cell surface (data not shown). When M1 macrophages (CD45+CD163+) were co-cultured with MCF7 or MCF7/4S cells for 1 day and analyzed by FACS for changes in cell adhesion molecules, α -2-integrin (CD18) and α -M (CD11b) were down-regulated to undetectable levels compared to the M1 mono culture with no significant

changes in ICAM1-3 (Figure 4A–E). When MCF7 cells (CD45-CD163-) in co-cultures were analyzed by FACS for the same cell adhesion molecules, there was a one log increase in ICAM1 expression compared to controls (Figure 4F–J). An identical result was observed for the analysis of MCF7/4S cells in co-culture (Figure 4K–O), indicating no effect of CEACAM1 expression on the changes in these cell adhesion molecules. The up-regulation of ICAM1 on the epithelial cells in co-culture agrees with the previous observation that up-regulation of ICAM1 in breast cancer correlates with increased metastasis of breast cancer cells³⁷.

Macrophages induce abnormal acini formation in three-dimensional (3D) culture

Since TAMs have been shown to enhance the invasive behavior of malignant mammary epithelial cells in an in-vitro 3D co-culture system with primary mouse mammary organoids⁹, this enhanced invasive behavior may be responsible for metastasis. MCF7 (Figure S3A) and MCF7/4S (Figure S3B) cells form regular round smooth edged acini by day 4 in 3D culture³⁸. However, the number of abnormal acini per field (100 acini counted) increased (Figure S3C–D and quantitated in Figure S3G) for MCF7 or MCF7/4S cells with M1 macrophages in 3D co-culture for 4 days. In contrast, 3D co-cultures with M2 macrophages (Figure S3E–F and quantitated in Figure S3H) had little effect on acini formation. In addition, fewer acini per field were observed for MCF7/4S cells co-cultured with either M1 or M2 macrophages compared to MCF7 cells co-cultured with either M1 or M2 macrophages suggesting that CEACAM1-4S may induce apoptosis in 3D culture. We conclude that co-culture of epithelial cells with M1 but not M2 macrophages are responsible for changes in the morphology of acini and that the expression of CEACAM1 may induce apoptosis in the presence of either M1 or M2 macrophages.

Since we observed fewer acini per field for MCF7-4S-M1 3D co-cultures, we analyzed the extent of apoptosis with annexin V staining. As expected, MCF7/4S cells co-cultured with either M1 or M2 macrophages showed significantly higher levels of apoptosis than the MCF7 monoculture (Figure 5A–B). Thus, the expression of CEACAM1-4S on mammary epithelial cells may increase apoptosis upon exposure to activated macrophages of either the pro- or anti-inflammatory phenotype. However, breast cancer cells that lack CEACAM1 expression may be resistant to apoptosis when exposed to macrophages. This may mimic changes in the normal gland where macrophages play a role in involution of the gland, compared to breast cancer where the macrophages fail to induce apoptosis, but instead induce abnormal acini that resemble an invasive phenotype.

Since CEACAM1 is itself a cell-cell adhesion molecule and its expression is important for lumen formation, a process that involves apoptosis^{23, 38}, we asked if there was a direct CEACAM1-CEACAM1 interaction between the MCF7/4S cells and the M1 or M2 macrophages. When M1 or M2 macrophages were co-cultured with MCF7 or MCF7/4S cells for 1 day and analyzed for the expression of CEACAM1, there was a significant induction of CEACAM1 on M1 macrophages, and to a much lesser extent on M2 macrophages (Figure 5C–D). The de novo expression of CEACAM1 on M1 macrophages would allow these cells to directly interact with CEACAM1 positive (normal) breast epithelial cells in a manner consistent with the induction of apoptosis, a major function of CEACAM1 in lumen formation²³. The absence of CEACAM1 on breast cancer cells in the presence of CEACAM1 positive M1 cells is consistent with the resistance of breast cancer cells to apoptosis. Furthermore, CEACAM1 has been shown to act as an inhibitory molecule on activated immune cells especially in their interaction with other cells¹⁸. Thus, it is likely that when CEACAM1 negative M1 cells interact with normal breast epithelial cells that are CEACAM1 positive, the de novo expression of CEACAM1 on M1 cells would have a net inhibitory effect on both the M1 and epithelial cells, controlling the degree of apoptosis and/or inflammation.

M1 Macrophages enhance tumor growth via angiogenesis

As shown in previous studies, malignant human breast adenocarcinomas infiltrated with high numbers of TAMs were associated with an increased density of blood vessels³². Based on our recent studies with a murine B16 melanoma model in which CEACAM1 inhibited tumor angiogenesis via G-CSF³⁹, we hypothesized that the presence or absence of CEACAM1 in the tumor itself may directly affect TAM promoted angiogenesis. In agreement with this finding, our current study shows that co-culture of M1 macrophages with breast cancer cells results in the high production of G-CSF. To investigate this possibility *in vivo*, we utilized our previously described model of orthotopic breast cancer^{24, 40}. MCF7 or MCF7-4S cells plus or minus M1 macrophages (1:1 ratio) were implanted into the fourth inguinal mammary pad of NOD/SCID mice and tumor volume and blood vessel area and numbers was measured 8 weeks after implantation. Examples of tumors and their histochemical features are shown in Figure S4. As expected, MCF7 tumors were small ($<0.1 \text{ cm}^3$) with low blood vessel area and numbers in the absence of M1 macrophages (Figure 6A–E). However, when MCF7 cells were mixed with M1 macrophages and implanted in murine fat pads, tumor volume and blood vessel area and numbers significantly increased compared to the MCF7 only controls (Figure 6A–E). Similar to MCF7 only tumors, MCF7/4S only tumors were small ($<0.1 \text{ cm}^3$) and had low blood vessel numbers and area 8 weeks after implantation (Figure 6A–C and F). When MCF7/4S cells were mixed with M1 macrophages and implanted in murine fat pads, both tumor volume ($<0.2 \text{ cm}^3$) and blood number and vessel density significantly increased (Figure 6A–C and G), but not to the extent observed in MCF7+ M1 tumors. The same experiment performed with M2 macrophages failed to stimulate tumor growth or increases in blood vessel area (Figure S5). Staining for tumor vasculature was performed with antibodies to murine PECAM1 (CD31) which does not cross-react with human CD31. When sections were stained with antibodies to either human CD31 or human CD45 (a generic marker for human myeloid cells), the results were negative, indicating the absence of the M1 macrophages after the 8 week period (data not shown). Further staining with the mesenchymal cell marker alpha smooth muscle actin showed that only blood vessels and connective tissue were positive, indicating that the tumors did not undergo epithelial to mesenchymal transition and that blood vessel integrity was preserved (Figure S6). In addition, we performed a Matrigel plug assay in which co-mixtures of MCF7 or MCF7/4S cells and M1s were treated with anti-G-CSF over a 1 week period and blood vessel density analyzed (Figure S7). The results indicate a significant reduction in blood vessel density for treated vs untreated plugs, indicating that G-CSF is the critical cytokine for tumor angiogenesis in this system. Thus, we conclude that the effect of M1 macrophages was to promote neoangiogenesis in the early days during which the xenografts became established, after which the M1 macrophages disappeared from established tumors. In the absence of M1 macrophages the MCF7 or MCF7/4S cells were unable to promote neoangiogenesis and thus failed to form substantial tumors even after 8 weeks in the murine fat pads. These results, taken together with the *in vitro* co-culture studies, suggest that the production of pro-angiogenic factors by the M1 macrophages was responsible for the observed increase in blood vessel density and tumor growth. Since G-CSF was by far the major factor produced in the *in vitro* co-culture studies and is known to promote VEGF-independent angiogenesis^{41, 42}, we conclude that G-CSF is the critical factor. This conclusion is supported by our previous studies that show CEACAM1 can inhibit G-CSF promoted tumor angiogenesis⁶ and the Matrigel plug assay (Figure S7). Similarly, the presence of CEACAM1 in the mammary fat pad tumor model system dramatically inhibited both tumor blood vessel density and tumor growth. Thus, the role of CEACAM1 in tumor inhibition is largely due to its inhibition of G-CSF mediated angiogenesis.

Discussion

Although inflammation and cancer have been invariably linked, the mechanism of how inflammation may initiate cancer remains debatable. Since macrophages play a role in inflammation, and TAMs in breast cancer correlate with a poor prognosis, TAMs have received increasing scrutiny for a role in this process. Yet macrophages are present in normal breast and play beneficial, if not crucial roles in breast function. Thus, it is likely that some change in either macrophages themselves or their interacting partners, the epithelial cells, must predispose the breast to cancer. The goal of this study was to determine if breast epithelial-macrophage interactions were influenced by the absence of the homotypic cell adhesion receptor CEACAM1 that is present in normal but down-regulated in malignant breast. CEACAM1 is a prime suspect for such a role because it has been repeatedly shown to regulate inflammation and is often down-regulated in cancers that originate from the epithelium. To access the role of CEACAM1, the two major classes of macrophages (pro-inflammatory M1 and anti-inflammatory M2 macrophages) were co-cultured with MCF7 cells that differed in their expression of CEACAM1 and which were previously shown to behave like normal glandular epithelial cells (+CEACAM1) or malignant breast cancer cells (-CEACAM1). The co-cultures revealed that M1 macrophages secreted high levels of G-CSF, TNF and VEGF when stimulated by MCF7 cells lacking CEACAM1 and that M2 macrophages secreted significantly less of each, indicating that the pro-inflammatory activity of M1 macrophages was stimulated compared to M2 macrophages, and that CEACAM1 played its expected inhibitory (anti-inflammatory) role. Notably, the cytokine profiles observed do not correspond to the profiles reported for LPS or IFN (classic) or IL-4/IL-13 (alternative) stimulated Ms. This is not unexpected, since tumor stimulated inflammation is considered a sterile inflammation. It should also be noted that we isolated monocytes by a negative isolation protocol, a protocol less likely to cause activation during the isolation step of the procedure. Strikingly, M1 macrophages up-regulated CEACAM1 on their cell surface when co-cultured with MCF7/4S cells expressing CEACAM1 implying that there was a direct cell-cell interaction involving CEACAM1. When other cell-adhesion molecules were analyzed in the co-cultures, CD18/CD11b on M1 macrophages was down-regulated and its ligand ICAM1 was up-regulated on both MCF7 and MCF7/4S cells indicating that CEACAM1 expression did not affect this classical interaction involving a α 2-integrin and its ligand. Thus, CEACAM1 was able to inhibit the activity of pro-inflammatory M1 cytokines.

In agreement with our recent studies on the role of CEACAM1 in the inhibition of G-CSF action on Gr1⁺ cells³⁹, we show that CEACAM1 inhibits G-CSF secretion by M1s in co-culture with MCF7/4S cells. Since G-CSF directly stimulated tumor angiogenesis in an all murine melanoma or colon cancer models³⁹, we hypothesized that increased production of G-CSF by M1s mixed with MCF7 cells would also stimulate tumor angiogenesis in a orthotopic model of human breast cancer. Indeed, this was the case, including the observation that this only occurred in the absence of CEACAM1 expression. Thus, we have been able to better define the role of CEACAM1 in an animal model in which human M1 cells are comixed with human breast epithelial cells. Furthermore, increased tumor angiogenesis is observed even though the M1 cells are no longer present at the end of the 8 week period when tumor size and blood vessel density was measured. This indicates that the effect of M1 cells and their G-CSF production acts at the earliest stages of tumorigenesis when the tumor requirement for blood vessel formation is most critical. Neither MCF7 nor MCF7/4S cells can grow to significant sizes unless M1 cells are present and MCF7/4S cells expressing CEACAM1 significantly inhibit tumor growth even in the presence of M1 cells. Thus, in normal breast where both macrophages and CEACAM1 positive epithelial cells are present, tumor growth would be minimal unless CEACAM1 is down-regulated. During inflammation when M1 cells would be likely present, the pro-angiogenic activity of the M1

cells would be inhibited in normal breast epithelia where CEACAM1 is expressed, but promoted in those cells lacking CEACAM1. In this way, we can explain why the down-regulation of CEACAM1 is so strongly correlated with tumor progression. Importantly, CEACAM1 is also down-regulated in prostate epithelial tumors, resulting in increased angiogenesis and the release of VEGF^{43, 44}.

Perhaps a surprising result of this study is the lesser role for anti-inflammatory M2 macrophages in tumor promotion. This result seems to contradict the current idea that TAMs in advanced breast cancers resemble the M2 phenotype. However, this can be reconciled with the fact that M1 macrophages were not recovered from advanced tumors in our tumor model, similar to the lack of M1 macrophages in advanced human breast cancer. It appears that the M1 cells perform their pro-angiogenic function early in tumorigenesis and once the tumors are established, they no longer play a role. The finding of M2 cells in advanced human tumors may reflect further steps in tumorigenesis not observable in our model because there was no source for human M2 or anti-tumor T-cells. In fact, our study suggests the possibility that M2 cells may be beneficial at the early stages of tumorigenesis and only become tumor promoting at later stages when adaptive immune response are critically important to reduce tumor growth. Since the adaptive immune response is known to take place over an extended time period, this idea is consistent with our current knowledge of both the innate and adaptive immune responses and their interactions. Furthermore, both M1 and M2 macrophages promoted more apoptosis in MCF7 cells that express CEACAM1 molecule, but not in wild type MCF7 cells. Thus, macrophage induced apoptosis may well play a role inhibiting tumor growth when CEACAM1 is present.

In this study, we have not fully analyzed the importance of both the VEGF and TNF responses of M1s in the co-cultures, both of which may contribute to tumor progression. The potential role of VEGF in angiogenesis, though obvious, is partially discounted by the finding that many tumors are VEGF independent⁴⁵, thus leading many to search for other pro-angiogenic factors such as Bv8 which is specifically stimulated by G-CSF^{41, 46}. Bv8, also known as prokineticin-2, is mainly produced by granulocytic myeloid cells and is also regulated by CEACAM1⁶. The role of TNF is also hard to assess, although it may promote inflammation, it is also a potent tumor inhibitory factor²⁶. Thus, at this stage of our analysis we favor the more critical role of G-CSF since it was secreted at especially high levels compared to the other factors and was totally dependent on the interaction of M1 cells with breast epithelial cells. The potent effect of CEACAM1 on inhibition of G-CSF secretion is in good agreement with our other studies^{25, 39}, connecting the probable sequence of events that protect normal breast from tumor formation, while promoting tumors in CEACAM1 negative breasts undergoing M1 associated inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and impact statement

This manuscript describes a novel mechanism by which tumor associated macrophages (TAMs) interact with MCF7 breast epithelial cells (MCF7 cells are Er+,Pr+, Her2 normal) to promote tumor angiogenesis via production of G-CSF. MCF7 cells were chosen for their lack of expression of the anti-inflammatory, cell adhesion molecule CEACAM1 that is frequently down-regulated in breast cancer. These findings relate to the current studies in human breast cancer that correlate TAMs with poor outcomes in breast cancer. They provide a novel insight to the earliest stages of invasive breast cancer, suggesting that pro-inflammatory TAMs play a significant role.

Fig.1. G-CSF, TNF-α and VEGF expression in co-culture supernatants

G-CSF, TNF-α and VEGF levels in the supernatants and mRNA levels in cell pellets from macrophage and epithelial cell co-culture experiments were analyzed using a multiplex cytokine assay and real time RT-PCR. The concentration of G-CSF (A and B), TNF-α (D and E) and VEGF (G) were measured in supernatants collected from M1 (A and D) and M2 (B and E) macrophages co-cultured with MCF7 or MCF7/4S cells. G-CSF (C), TNF-α (F) and VEGF (H) mRNA expression was measured in the co-culture experiments by real time RT-PCR. The experiments were repeated more than 3 times, * $p < 0.05$.

Fig.2. Identification of G-CSF producing cells and the effect of epithelial cell supernatants on macrophages

A. Analysis of G-CSF expression by MCF7/4S cells co-cultured with M1s (orange), MCF7 co-cultured with M1s (green), or by mono-cultures of MCF7/4S (blue) or MCF7 cells (red). Intracellular anti-G-CSF staining was performed on these cells which are CD45-CD163- to differentiate them from macrophages which are CD45+CD163+. **B.** Analysis of G-CSF expression by M1s co-cultured with MCF7/4S (yellow) or MCF7 (blue), or in mono-cultures (red). **C.** To determine if epithelial cells release factors into supernatants that can activate macrophages to produce G-CSF, M1s were incubated in supernatants collected from MCF7/4S (yellow) or MCF7 (blue) cells for 1 day, and G-CSF expression analyzed by

intracellular staining). **D**. Similar analysis for M2s incubated with supernatants from MCF7/4S (yellow) or MCF7 (blue) epithelial cells or mono-cultures (red). Secreted levels of G-CSF (**E**), TNF- (**F**) and VEGF (**G**) in supernatants from **C** and **D** were measured by multiplex cytokine analysis. Experiments were repeated twice, * $p < 0.05$.

Fig.3. G-CSF production by M1 macrophages in a transwell assay

M1s were placed in upper wells, MCF7 or MCF7/4S cells in lower wells of transwell plates with no chemoattractant and incubated for 1 day. Percent of M1 macrophages (CD163+CD45+) that migrated to the lower wells containing MCF7 (A) or MCF7/4S (B) cells. Migrated (blue) or non-migrated (green) M1s were analyzed for G-CSF expression in plates with MCF7 (C) or MCF7/4S (D) or no (red) cells in the lower wells. Controls included analysis of G-CSF in CD163-CD45- MCF7 cells (E) with M1s (blue) or in mono-culture (red), and CD163-CD45- MCF7/4S cells (F) with M1s (blue) or in mono-culture (red). The experiments were repeated twice.

Fig.4. Effect of co-culture on expression of cell-cell adhesion molecules on macrophages and breast cancer epithelial cells

M1 macrophages and MCF7 or MCF7/4S epithelial cells were co-cultured for 1 day and analyzed for expression of ICAM1-3, CD18 and CD11b molecules by flow cytometry. Expression of ICAM1-3, CD18 and CD11b on M1 macrophages (A to E), MCF7 cells (F to J) and MCF7-4S cells (K to O). First column: analysis of M1 macrophages (CD45+CD163+) that were co-cultured with MCF7/4S (orange) or MCF7 (green) cells M1 (4S) or in monoculture (blue). Second column: analysis of MCF7 cells (CD45-CD163-) that were co-cultured with M1s (green) or in mono-culture (blue). Third column: analysis of

MCF7/4S cells that were co-cultured with M1s (green), or in mono-culture (blue). Isotype controls (red). The experiments were repeated three times.

Fig.5. Induction of apoptosis and CEACAM1 expression on macrophages co-cultured with MCF7 or MCF7/4S cells

A. M1 (blue) or M2 (green) macrophages were co-cultured with MCF7 cells for 1 day and the CD45-CD163- MCF7 cells analyzed for Annexin V expression. **B.** M1 (blue) or M2 (green) macrophages were co-cultured with MCF7/4S cells for 1 day and the CD45-CD163- MCF7/4S cells analyzed for Annexin V expression. **C.** CD45+CD163+ M1 macrophages co-cultured with MCF7 (green) or MCF7/4S (orange) or in mono-culture (blue) were analyzed for CEACAM1 expression. **D.** CD45+CD163+ M2 macrophages co-cultured with MCF7 (green) or MCF7/4S (orange) or in mono-culture (blue) were analyzed for CEACAM1 expression. The experiments were repeated twice.

Fig.6. Effect of M1 macrophages on tumor volume and blood vessel number and area in an orthotopic NOD/SCID mouse model system

MCF7 or MCF7/4S cells alone or mixed with M1 macrophages (1:1 ratio) in Matrigel were implanted into the inguinal mammary fat pads of NOD/SCID mice, and tumor volumes (**A**), blood vessel area (**B**) and blood vessel number (**C**) measured after 8 weeks of tumor growth. Values were analyzed by student t-test, * $p < 0.05$. Examples of immunostaining for murine anti-CD31 in MCF7 only tumors (**D**), MCF7 plus M1 tumors (**E**), or MCF7/4S only tumors (**F**) and MCF7/4S plus M1 tumors (**G**). The experiments were repeated twice (n=8 mice per group).