

Research Article

Breast Cancer Cells Stimulate Neutrophils to Produce Oncostatin M: Potential Implications for Tumor Progression

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Abstract

Tumor-associated and tumor-infiltrating neutrophils (TAN) and macrophages (TAM) can account for as much as 50% of the total tumor mass in invasive breast carcinomas. It is thought that tumors secrete factors that elicit a wound-repair response from TAMs and TANs and that this response inadvertently stimulates tumor progression. Oncostatin M is a pleiotropic cytokine belonging to the interleukin-6 family that is expressed by several cell types including activated human T lymphocytes, macrophages, and neutrophils. Whereas oncostatin M can inhibit the proliferation of breast cancer cells *in vitro*, recent studies suggest that oncostatin M may promote tumor progression by enhancing angiogenesis and metastasis. In addition, neutrophils can be stimulated to synthesize and rapidly release large quantities of oncostatin M. In this article, we show that human neutrophils secrete oncostatin M when cocultured with MDA-MB-231 and T47D human breast cancer cells. Neutrophils isolated from whole blood or breast cancer cells alone express little oncostatin M by immunocytochemistry and ELISA, but neutrophils express and release high levels of oncostatin M when they are cocultured with breast cancer cells. In addition, we show that granulocyte-macrophage colony-stimulating factor produced by breast cancer cells and cell-cell contact are both necessary for the release of oncostatin M from neutrophils. Importantly, neutrophil-derived oncostatin M induces vascular endothelial growth factor from breast cancer cells in coculture and increases breast cancer cell detachment and invasive capacity, suggesting that neutrophils and oncostatin M may promote tumor progression *in vivo*. (Cancer Res 2005; 65(19): 8896-904)

Introduction

Neutrophils, also known as polymorphonuclear leukocytes, represent 50% to 60% of the total circulating leukocytes and constitute the first line of defense against infectious agents or “nonself” substances that penetrate the body’s physical barriers. Neutrophils form a primary defense against bacteria, fungi, protozoa, viruses, and virally infected cells and also target tumor cells (1). In the blood stream, inactivated neutrophils are short-lived (8 hours), terminally differentiated cells, that under

conditions of inflammation are able to survive for longer periods of time and can up-regulate a variety of cytokines (2) including interleukin-1 β (IL-1 β), IL-6, IL-8, tumor necrosis factor- α , granulocyte macrophage colony-stimulating factor (GM-CSF; ref. 1), and oncostatin M (3). Oncostatin M is not secreted in significant amounts by resting neutrophils but is produced in response to inflammatory mediators (4–6), and the tumor-infiltrating neutrophil population was originally identified as a primary source of oncostatin M (7). Human neutrophils have the capacity to direct the progression of an inflammatory reaction by influencing the activity of other immune cells and tissues (5).

Tumor-associated neutrophils (TAN) have been shown to promote tumor progression via a variety of mechanisms, including the stimulation of angiogenesis and invasion (8). TANs can also stimulate tumor growth by releasing growth factors such as epidermal growth factor, transforming growth factor- β (TGF- β), and platelet-derived growth factor (PDGF; ref. 9). In fact, it is thought that tumors secrete factors that elicit a wound-repair response from TANs and tumor-associated macrophages and that this response inadvertently stimulates tumor progression (10). TANs have been detected in adenocarcinoma of the bronchioloalveolar carcinoma (BAC) subtype (11) and in colon (12) and ovarian cancer⁴ by immunohistochemistry. Wislez et al. (11) have shown that tumor cells drive local neutrophil recruitment and persistence in the lung via cytokine release, and this increase in TANs are linked to poorer outcome of patients with BAC subtype.

Oncostatin M is a 28-kDa pleiotropic cytokine of the IL-6 family (13) that is a product of activated T lymphocytes (14), monocytes (15), neutrophils (4), and some tumor cells including breast cancer epithelial cells (16). Oncostatin M has been shown to exert proinflammatory effects by inducing adhesion and chemotaxis of neutrophils and chemokine production by endothelial cells (17, 18). Although oncostatin M was originally identified as an inhibitor of melanoma tumor cell growth *in vitro* (19) and has been shown to inhibit breast cancer cell proliferation *in vitro* (14), it is increasingly apparent that this cytokine plays a role in breast cancer cell detachment (20) and angiogenesis.⁵ These results suggest that oncostatin M may enhance tumor progression and metastasis *in vivo*.

Oncostatin M-specific receptors are expressed in a wide variety of cell types, including endothelial, hepatic, lung, bone marrow cells, and many tumor cell lines (21–23). Oncostatin M elicits its biological effects through two types of transmembrane receptors:

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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⁴ R.E. Ryan et al. The expression of oncostatin M in tumor-associated macrophages and polymorphonuclear leukocytes.

⁵ A.E. Ide et al. Oncostatin M induction of vascular endothelial growth factor in human breast cancer cells promotes angiogenesis.

the type I oncostatin M receptor is identical to the high-affinity leukemia inhibitory factor (LIF) receptor, composed of gp130 and LIFR β , and the type II oncostatin M receptor, composed of gp130 and the oncostatin M-specific receptor β subunit (OSMR β which is specific for oncostatin M; refs. 21, 22). Oncostatin M can signal through the Janus-activated kinase/signal transducer and activator of transcription (JAK-STAT) pathway (7), the mitogen-activated protein kinase pathway (14, 24), and the phosphatidylinositol 3-kinase pathway (25).

Angiogenesis, the growth of new blood vessels from a preexisting microvascular bed (26), is crucial for the growth, maintenance, and metastasis of solid tumors (26, 27). When tumor cells and/or tumor-associated stromal and inflammatory cells express proangiogenic molecules, the normal, neutral balance of proangiogenic and antiangiogenic factors is disrupted, such that angiogenesis is initiated (28). The association of neutrophils with certain tumors and the ability of neutrophils to secrete a variety of proinflammatory cytokines make these cells ideally situated to influence angiogenesis and tumor progression.

Depending on the cytokine milieu in which they act, TANs may promote tumor progression by secreting growth factors (TGF- β , PDGF, and oncostatin M; ref. 7), angiogenic factors (vascular endothelial growth factor or VEGF, hepatocyte growth factor, and IL-8; ref. 9), and matrix-degrading enzymes (elastase and cathepsin G; ref. 9). However, which factors secreted by neutrophils play a role in breast tumor progression remains unclear. Here we have focused on the cytokine oncostatin M and invasive breast cancer. Using an *in vitro* model, we have examined the expression of oncostatin M by neutrophils in the presence and absence of breast cancer cells and asked whether oncostatin M can influence the invasive capacity of breast cancer cells. Our results show that freshly isolated human neutrophils express little oncostatin M until "primed" with human breast cancer conditioned medium. Oncostatin M production by neutrophils can be inhibited by the addition of anti-GM-CSF-neutralizing antibody to the conditioned medium before priming the neutrophils. Cell-to-cell contact is required for oncostatin M release from neutrophils. This neutrophil-derived oncostatin M signals human breast cancer cells to secrete VEGF and increases breast cancer cell detachment and invasive capacity. Our findings could provide grounds for the development of cancer therapies designed to inhibit oncostatin M release from leukocytes.

Materials and Methods

Breast cancer cell conditioned medium. MDA-MB-231 and T47D human breast cancer cells were plated in RPMI (Life Technologies, Inc., Grand Island, NY) + 10% fetal bovine serum (FBS, Life Technologies) at 37°C, 5% CO₂. Once cells were 40% to 60% confluent, the medium was removed, cells were washed with PBS, and RPMI + 1% FBS was added for 48 hours. After 48 hours, the medium was collected, centrifuged, and conditioned medium was transferred to a new tube. Conditioned medium was then stored at -20°C and used in all experiments to prime neutrophils unless described otherwise.

Purification of neutrophils. Blood was collected from healthy human volunteers by venipuncture into heparin-coated vacutainers. Ten to 20 mL of fresh blood were carefully layered over a histopaque column (5 mL 1119 and 5 mL 1077; Sigma-Aldrich, St. Louis, MO) and then centrifuged. After centrifugation at 700 \times g for 30 minutes (18-22°C), bands for plasma, platelets, granulocytes, and erythrocytes were visible. The lower band consisted primarily of neutrophils and was transferred to a new tube and diluted by addition of one volume of 1 \times PBS (Sigma-Aldrich) solution. Collected cells were centrifuged at 300 \times g for 10 minutes (18-22°C) and washed in 9 mL of distilled water for 20 seconds to lyse remaining

RBC. One milliliter of 10 \times PBS (Sigma) without calcium was added, and cells were centrifuged again, followed by resuspension in conditioned medium.

Neutrophil priming. Freshly isolated neutrophils were placed in the appropriate breast cancer cell conditioned medium (either MDA-MB-231 or T47D) for 18 to 24 hours, for priming, before coculturing with either cell lines. Neutrophil priming increased cell survival by allowing neutrophils to adjust to their environment before being plated with breast cancer cells (29).

Breast cancer cell and neutrophil coculture. Primed neutrophils were counted with a hemacytometer (American Optical, Buffalo, NY) and plated at a concentration of 2.5 \times 10⁵ cells/mL along with breast cancer cells in RPMI + 1% FBS.

Immunocytochemistry for oncostatin M. Human breast cancer cells were plated in a 24-well dish with or without fresh neutrophils in RPMI + 1% FBS for 24 hours. After incubation, breast cancer cells were scraped off the bottom of the 24-well dish with the side of a pipette tip, and the medium containing both the neutrophils and breast cancer cells was collected. The medium was centrifuged at 12,000 rpm for 2 minutes and the cells were resuspended in 300 μ L serum-free RPMI. Thirty-five percent bovine serum albumin (80 μ L; Life Technologies) was added to the cell suspension before cytospinning. Samples were placed in a cytospin chamber (Shannon Southern Instruments, Sewickley, PA) and spun onto slides for 10 minutes at 800 rpm. Slides were allowed to air-dry and were immunostained with anti-oncostatin M antibody (Chemicon, Temecula, CA) at 1:100 dilution for 24 hours at 4°C using 3,3'-diaminobenzidine as a substrate (Zymed Laboratories, Inc., San Francisco, CA), as per protocol. Images were taken with an Olympus IX70 inverted microscope (Olympus America, Inc., Melville, NY; 400 \times ; light).

Oncostatin M ELISA. Oncostatin M ELISAs (R&D Systems, Minneapolis, MN) were done according to the manufacturer's protocol. The absorbance was recorded using a microplate reader (Bio-Rad, Hercules, CA) at 450-nm wavelength.

To access a role for GM-CSF, neutrophils were primed in conditioned medium with or without 10 μ g/mL anti-GM-CSF-neutralizing antibody (R&D Systems) for 18 to 24 hours and primed neutrophils cocultured with human breast cancer cells \pm anti-GM-CSF-neutralizing antibody (10 μ g/mL). Sample medium was collected at 15-minute, 1-, 12-, and 24-hour time points and analyzed by oncostatin M ELISA.

To determine neutrophil granule release, primed neutrophils were cocultured with breast cancer cells \pm formylated methionine-leucine-phenylalanine (FMLP, 5 \times 10⁻⁷ mol/L). As a control, neutrophils alone were plated \pm FMLP (5 \times 10⁻⁷ mol/L). Medium was collected at 15-minute, 1-, 12-, and 24-hour time points and analyzed by oncostatin M ELISA.

Cell-to-cell contact was analyzed by plating primed neutrophils in the lower chamber of a 24-well transwell dish (BD Biosciences, Bedford, MA) in RPMI + 1% FBS. A transwell insert (3 μ m) containing human breast cancer cells in RPMI + 1% FBS was added and incubated for 24 hours. Medium from the upper and the lower chamber of the transwell insert was collected, combined, and analyzed using an oncostatin M ELISA.

Three independent experiments were done for each experiment with each data point representing six replicates. SDs were calculated and error bars represent \pm SD.

Vascular endothelial growth factor ELISA. VEGF ELISAs (Cytimmune Science, Inc., Rockville, MD) were done according to the manufacturer's protocol. The absorbance was recorded using a microplate reader (Bio-Rad) at 490-nm wavelength.

Breast cancer cells (MDA-MB-231 or T47D) were plated with 0.1 μ g/mL anti-OSMR β -neutralizing antibody (Amgen, Inc., Thousand Oaks, CA), 0.1 μ g/mL anti-LIFR β -neutralizing antibody (R&D Systems), 0.1 μ g/mL anti-gp130-neutralizing antibody (R&D Systems), or 0.1 μ g/mL of both anti-OSMR β - and anti-LIFR β -neutralizing antibodies, 1 hour before the addition of primed neutrophils and plated in RPMI + 1% FBS, incubated for 72 hours, and collected medium was analyzed using the VEGF ELISA.

Three independent experiments were done with each data point representing six replicates. SDs were calculated and error bars represent \pm SD.

Breast cancer cell detachment. Human MDA-MB-231 and T47D breast carcinoma cells were plated on uncoated six-well plates in RPMI + 10% FBS. After the cells had grown to ~60% to 70% confluency, the medium was removed and 2.5×10^5 cells/mL primed neutrophils were added in 1 mL serum-free medium. The following treatments were done: breast cancer cells alone, breast cancer cells plated with primed neutrophils, or breast cancer cells plated with primed neutrophils pretreated with oncostatin M-neutralizing antibody (50 μ g/mL) 1 hour before plating. Cultures were incubated for 2, 4, and 6 days, and images were collected using a Zeiss Axiovert25 Microscope (400 \times , phase contrast). In addition, detached and adherent cells were collected at each time point and counted with a hemacytometer (American Optical, Buffalo, NY). Detachment was expressed as a percentage of total number of cells and calculated using the following equation: % detachment = detached cells / [total cells (detached cells + adherent cells)]. Viability of detached cells was assessed by trypan blue exclusion. Four independent experiments were done with each data point representing eight replicates. SDs were calculated and error bars represent \pm SD.

Invasion assay. Matrigel-coated invasion chambers (BD Biosciences, Bedford, MA) were thawed and allowed to rehydrate for 2 hours with serum-free RPMI at 37°C. After rehydration, medium was removed and RPMI + 15% FBS (as a chemoattractant) was added to the lower chamber. Breast cancer cells were plated with or without neutrophils in RPMI + 1% FBS in the upper chamber. Neutrophils cocultured with breast cancer cells were also plated with and without 50 μ g/mL (single dose at time 0) of anti-oncostatin M-neutralizing antibody. Cells were then incubated for 22 hours and then a cotton swab was used to remove the noninvading cells from the upper chamber. The Matrigel-coated inserts were fixed in 100% methanol for 2 minutes and stained in 1% toluidine blue, 1% borax in PBS for 2 minutes. Stained inserts were rinsed in distilled water and allowed to air-dry. Invading cells were counted using a Zeiss Axiovert25 Microscope (200 \times , light) and percent invasion was calculated. Percent invasion was calculated by the following equation: % invasion = [mean no. of cells invading through Matrigel insert

membrane / mean no. of cells migrating through control insert membrane (inserts without Matrigel coating)] \times 100. Five independent experiments were done with each data point representing 10 replicates. SDs were calculated and error bars represent \pm SD.

Results

Neutrophils cocultured with human breast cancer cells express oncostatin M. Freshly isolated (nonprimed) neutrophils express no oncostatin M versus neutrophils that had been primed with conditioned medium for 18 to 24 hours (Fig. 1A), as detected by immunocytochemistry. Little or no oncostatin M expression was detected in MDA-MB-231 (highly metastatic) or T47D (weakly metastatic) human breast cancer cells cultured alone at 24 hours (Fig. 1A). Coculture of freshly isolated neutrophils with MDA-MB-231 or T47D cells for 24 hours induced expression of oncostatin M, as indicated by brown staining within neutrophils surrounding each particular cancer cell (Fig. 1A). Cocultured T47Ds have a slight brown staining that may be due to the fact that both T47D and MDA-MB-231 human breast cancer cells make and secrete small amounts of oncostatin M. This staining was not seen in the absence of anti-oncostatin M antibody (secondary antibody alone; Fig. 1A). Neutrophils showed multi-lobed nuclei by hematoxylin staining (Fig. 1A).

Freshly isolated neutrophils primed overnight with conditioned medium from MDA-MB-231 or T47D human breast cancer cells showed low levels of oncostatin M secretion by ELISA, as did breast cancer cells cultured alone (Fig. 1B-C). A time course of cocultured cells indicated a relatively rapid release of oncostatin M from neutrophils by 15 minutes, which was sustained over time with MDA-MB-231 cells (Fig. 1B) and decreased over time with T47D cells (Fig. 1C). Our findings indicate that neutrophils cocultured

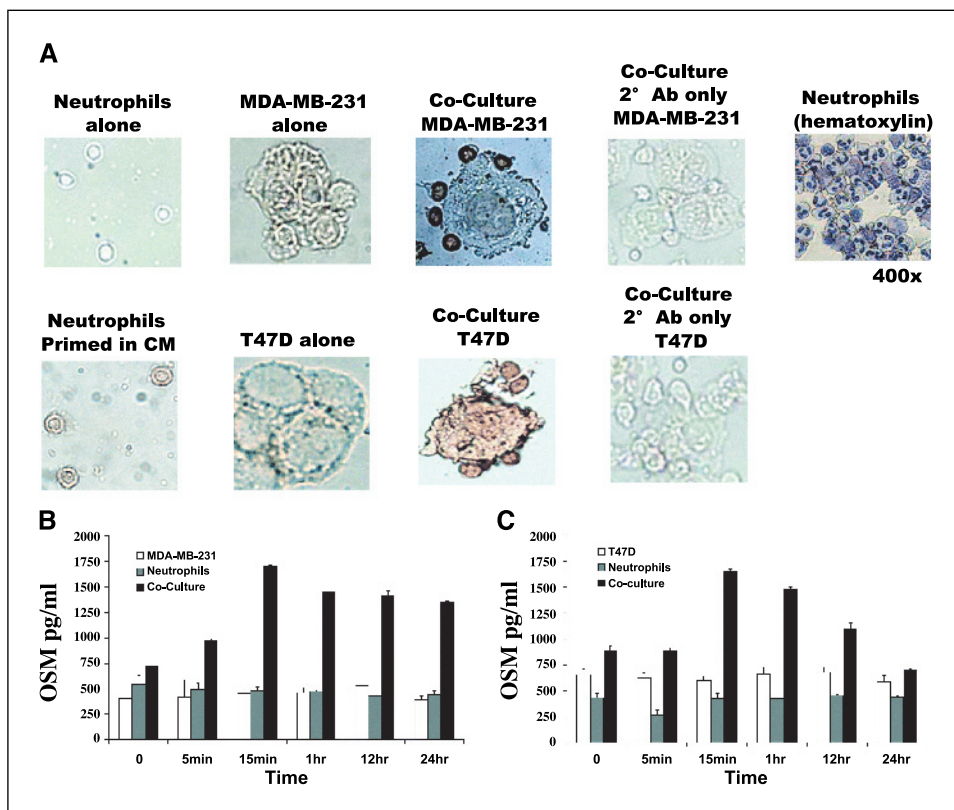


Figure 1. Neutrophils cocultured with human breast cancer cells express oncostatin M (OSM). A, immunocytochemistry of freshly isolated nonprimed neutrophils stained with anti-oncostatin M antibody shows no oncostatin M expression, whereas neutrophils primed in conditioned medium (CM) show some oncostatin M expression (brown staining). MDA-MB-231 and T47D human breast cancer cells cultured alone express little oncostatin M. Freshly isolated neutrophils cocultured 24 hours with MDA-MB-231 and T47D cells express increased levels of oncostatin M. During coculture, MDA-MB-231 cells show little oncostatin M expression and T47D cells show slightly more oncostatin M expression. Neutrophils cocultured with MDA-MB-231 or T47D human breast cancer cells show no staining with secondary (2°) antibody alone. Hematoxylin-stained neutrophils show multilobed nuclei. MDA-MB-231 (B) and T47D (C) human breast cancer cells cocultured with neutrophils secrete increased levels of oncostatin M compared with breast cancer cells or neutrophils cultured alone, as measured by ELISA. Time course analysis shows highest amounts of secreted oncostatin M by 15 minutes, suggesting a granular release of presynthesized oncostatin M.

with human breast cancer cells secrete increased levels of oncostatin M compared with neutrophils alone.

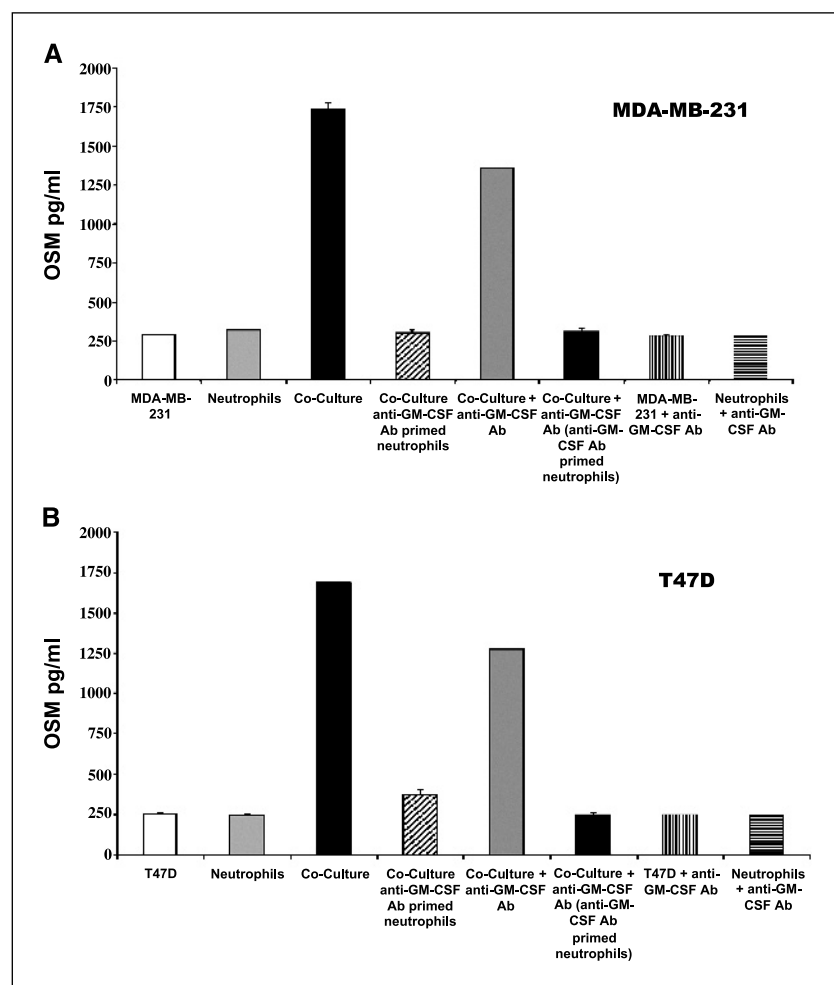
Granulocyte-macrophage colony-stimulating factor signals accumulation of granule-stored oncostatin M. We have found by immunocytochemistry that freshly isolated human neutrophils express little oncostatin M, yet upon "priming" 18 to 24 hours in conditioned medium from human breast cancer cells, they acquire the ability to secrete oncostatin M. We investigated what factor in the conditioned medium was responsible for oncostatin M accumulation. Wu et al. (29) found that MDA-MB-231 breast cancer cells produced high levels of GM-CSF, and we sought to determine whether GM-CSF secreted from human breast cancer cells was the signal for neutrophils to produce and secrete oncostatin M. To block GM-CSF in breast cancer conditioned medium, anti-GM-CSF-neutralizing antibody (50 $\mu\text{g}/\text{mL}$) was added 1 hour before neutrophil priming relative to normally primed neutrophils. Neutrophils primed in conditioned medium plus anti-GM-CSF antibody and cocultured with MDA-MB-231 human breast cancer cells secreted significantly less oncostatin M (5.8-fold decrease) at 15 minutes by ELISA (Fig. 2A), similar to the amount of oncostatin M secreted by MDA-MB-231 cells or neutrophils cultured alone. When normally primed neutrophils were cocultured with MDA-MB-231 cells, and anti-GM-CSF-neutralizing antibody was added only at the time of plating, the levels of oncostatin M secretion decreased slightly (1.3-fold;

Fig. 2A), suggesting that GM-CSF is important in signaling production of oncostatin M during neutrophil priming as opposed to during coculture. Similar results were seen with the T47D human breast cancer cell line.

Cell-to-cell contact is necessary for release of oncostatin M by neutrophils cocultured with human breast cancer cells. To assess the amount of oncostatin M stored in neutrophil granules, cultures were incubated with the peptide FMLP, a peptide known to stimulate total granular release of neutrophils (30). FMLP (2×10^{-7} mol/L) was added to primed neutrophils, and oncostatin M levels in the medium were measured over time (15 minutes, 1, 12, and 24 hours) by ELISA and compared with the amount of oncostatin M released by neutrophils cocultured with MDA-MB-231 (Fig. 3A) or T47D (Fig. 3B) human breast cancer cells. At 15 minutes, roughly 93% of total granule-stored oncostatin M was released by neutrophils cocultured with MDA-MB-231 cells, and 89% by neutrophils cocultured with T47D cells. The percentage of oncostatin M released was calculated as % oncostatin M released = [(coculture - MDA-MB-231 or T47D alone) / neutrophils + FMLP] = [amount released by cocultured neutrophils into medium / total oncostatin M in neutrophil granules (released by FMLP)]. Over time, the levels of oncostatin M released into the coculture medium never dropped below 73% of total granule-oncostatin M (Fig. 3A-B).

To determine if cell-to-cell contact was required for neutrophils to release oncostatin M, a transwell assay was done

Figure 2. GM-CSF signals accumulation of oncostatin M (OSM) in neutrophils. A, MDA-MB-231 human breast cancer cells and neutrophils plated with or without anti-GM-CSF-neutralizing antibody. When neutrophils were primed for 18 to 24 hours with an anti-GM-CSF-neutralizing antibody before coculture with MDA-MB-231 human breast cancer cells, the level of secreted oncostatin M at 15 minutes were equal to control levels, indicating that GM-CSF is needed for oncostatin M accumulation in neutrophils. When neutrophils were primed with normal conditioned medium before coculture and then anti-GM-CSF-neutralizing antibody was added at the time of plating, there was only a small decrease in oncostatin M level as seen by ELISA. Similar results were obtained when T47D breast cancer cells were plated with neutrophils (B).



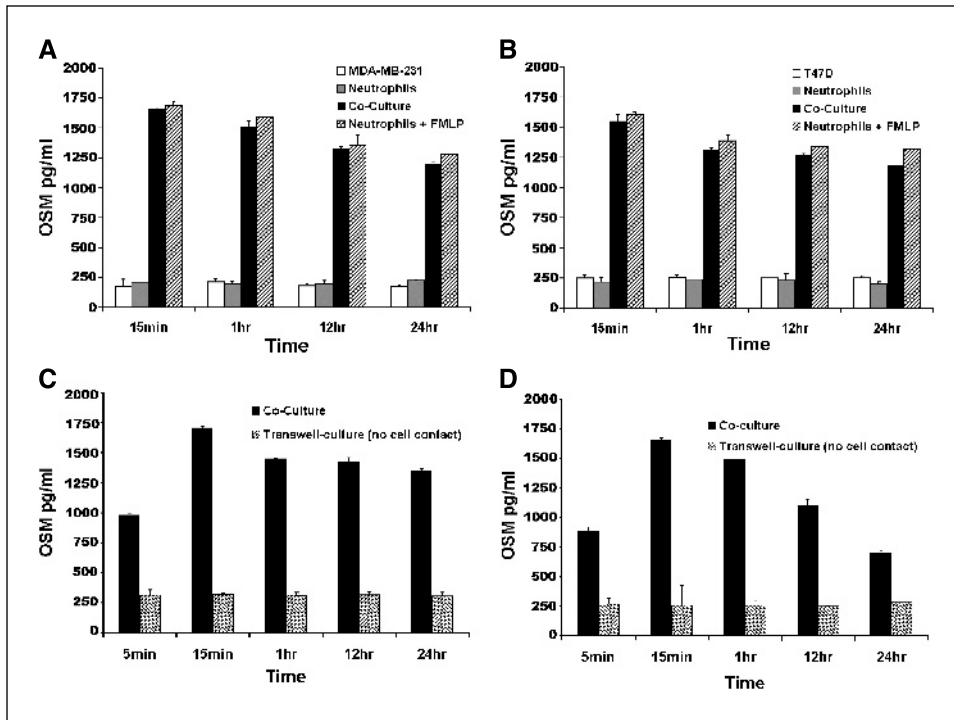


Figure 3. Cell-to-cell contact is needed for granule release of oncostatin M (OSM) by neutrophils cocultured with human breast cancer cells. Total granular release in neutrophils was induced by FMLP (2×10^{-7} mol/L). The level of oncostatin M released at 15 minutes from primed neutrophils cocultured with MDA-MB-231 (A) or T47D (B) cells was ~90% of the total oncostatin M stored in the granules of neutrophils. Coculture of primed neutrophils plated in the lower chamber and MDA-MB-231 (C) or T47D cells (D) plated in the upper chamber and separated by a 3- μ m membrane. Separation of the breast cancer cells from the neutrophils lead to a decrease in oncostatin M levels (transwell culture) compared with normal coculture (coculture), indicating cell-to-cell contact was needed for release of oncostatin M by 15 minutes.

separating neutrophils from breast cancer cells. The transwells contained MDA-MB-231 or T47D human breast cancer cells in the upper chamber. The breast cancer cells could not come into contact with the primed neutrophils in the bottom chamber, although the medium could flow freely between the two chambers. In the transwell culture, no increase of oncostatin M was detected throughout the time course (Fig. 3C-D) compared with primed neutrophils cocultured with breast cancer cells.

Neutrophil-derived oncostatin M induces vascular endothelial growth factor expression in human breast cancer cells. VEGF is a potent proangiogenic factor and has been implicated in invasion and metastasis. Coculture of primed neutrophils with MDA-MB-231 human breast cancer cells showed a modest increase in secreted VEGF by ELISA (Fig. 4A) compared with either MDA-MB-231 cells or neutrophils cultured alone. Greatest levels of secreted VEGF were seen at 3 days (Fig. 4A). T47D human breast cancer cells cocultured with neutrophils showed a 3.5-fold

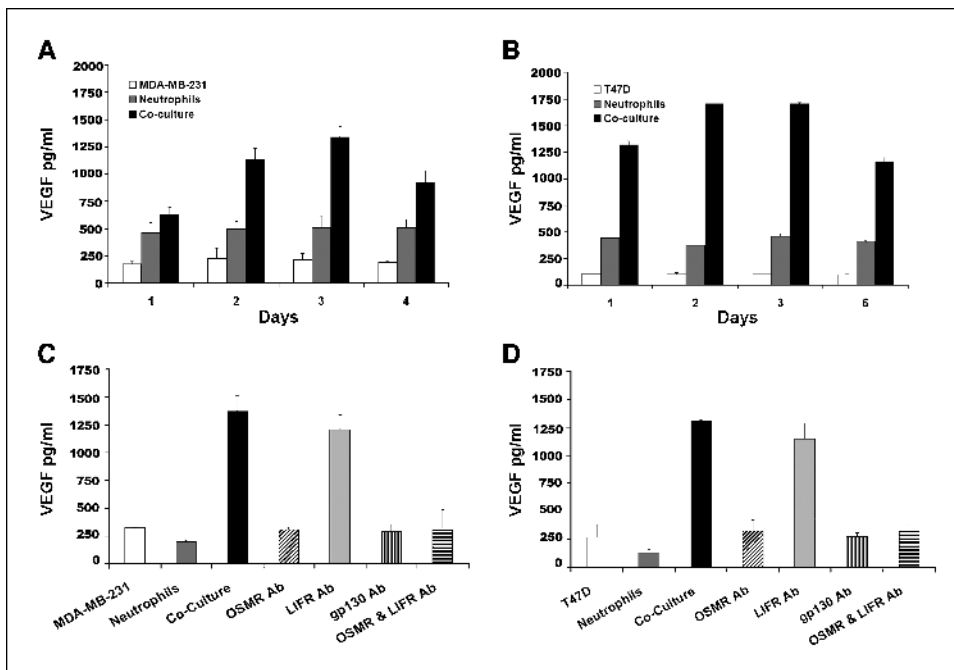


Figure 4. Neutrophil-derived oncostatin M (OSM) induces VEGF expression. VEGF ELISA analysis demonstrates an increase in secreted VEGF by coculture of neutrophils with MDA-MB-231 (A) or T47D (B) human breast cancer cells versus neutrophils or human breast cancer cells alone. VEGF secretion peaked at three days. C-D, neutrophil-derived oncostatin M signals through the oncostatin M specific receptor. Neutrophils were cocultured with MDA-MB-231 (C) or T47D (D) human breast cancer cells for 3 days. Anti-oncostatin M receptor-neutralizing antibody (0.1 μ g/mL) added to the breast cancer cells 1 hour before coculture showed a decrease in VEGF levels, whereas little decrease in VEGF levels was seen with the anti-LIFR β -neutralizing antibody. With the addition of anti-gp130-neutralizing antibody, VEGF levels decreased to levels similar to those with anti-OSMR β . VEGF levels did not decrease further even when anti-OSMR β - and anti-LIFR β -neutralizing antibodies were both added to the coculture.

increase in VEGF levels at days 2 and 3 compared with T47D cells or neutrophils cultured alone (Fig. 4B). A dose response experiment was done to determine the anti-oncostatin M–neutralizing antibody concentration (50 $\mu\text{g}/\text{mL}$) needed for maximal VEGF inhibition that did not effect cell proliferation (data not shown). Anti-oncostatin M–neutralizing antibody added to neutrophils cocultured with MDA-MB-231 cells resulted in a 52% inhibition of total VEGF secretion (Supplementary Fig. S1A) and a 57% inhibition of VEGF secretion when added to T47D cell coculture (Supplementary Fig. S1B).

To determine whether oncostatin M signals VEGF induction through the oncostatin M receptor, LIF receptor, or both receptors, we cultured breast cancer cells in the presence of neutralizing antibodies to either OSMR β , LIFR β , or gp130 (a shared component of both receptors). A 5-fold decrease in VEGF secretion was observed by ELISA when anti-OSMR β -neutralizing antibody was added to the MDA-MB-231 coculture system (Fig. 4C), whereas when the LIFR β -neutralizing antibody was added, there was little change in VEGF secretion. The addition of gp130-neutralizing antibody decreased VEGF secretion to levels seen with anti-OSMR β -neutralizing antibody. When both neutralizing antibodies (anti-OSMR β and anti-LIFR β) were added to the breast cancer cells, VEGF levels decreased to that of anti-gp130 and anti-OSMR β alone. Neutralizing antibody concentrations had previously been optimized at 0.1 $\mu\text{g}/\text{mL}$ (data not shown). Similar results were seen with T47D coculture system (Fig. 4D).

Neutrophil-derived oncostatin M induces cell detachment and increases invasive potential of human breast cancer cells. A decrease in cell-cell and cell-substratum adhesiveness *in vitro* has been correlated with increased metastatic potential. We assessed the effects of neutrophil-derived oncostatin M on the detachment of MDA-MB-231 and T47D human breast cancer

cells from tissue culture plates. MDA-MB-231 cells cocultured with primed neutrophils showed a 3-fold increase in viable detached cell compared with MDA-MB-231 cells cultured alone (Supplementary Fig. S2A-B). This detachment was largely inhibited by the addition of anti-oncostatin M–neutralizing antibody (Supplementary Fig. S2B). Similar results were seen with cocultured T47D cells (Supplementary Fig. S2C-D).

Oncostatin M released from neutrophils increased the invasive capacity of human breast cancer cells during coculture as measured by the Matrigel invasion assay. MDA-MB-231 human breast cancer cells cocultured with primed neutrophils showed a 2.9-fold increase in invasive capacity (Fig. 5A-B) relative to MDA-MB-231 cells alone. Similar results were seen with T47D cells (Fig. 5C-D), which showed a 3-fold increase in invasion when cocultured with neutrophils. Importantly, oncostatin M–neutralizing antibody was able to decrease neutrophil-promoted invasiveness in cocultures (Fig. 5B-D), which suggests that a large portion of the increased invasive capacity of cocultured breast cancer cells results from neutrophil-derived oncostatin M.

Discussion

In this article, we show that (i) resting neutrophils produce oncostatin M when they are “primed” by breast cancer conditioned medium; (ii) the signal for oncostatin M synthesis by neutrophils is breast cancer cell–derived GM-CSF and the release of oncostatin M is dependent on cell-cell contact; and (iii) neutrophil-derived oncostatin M induces VEGF expression, breast cancer cell detachment, and invasive capacity. Oncostatin M is a potent modulator of inflammation (18, 31, 32). Although oncostatin M was originally shown to inhibit breast cancer cell (14) and melanoma cell proliferation *in vitro* (19), it has more recently been implicated

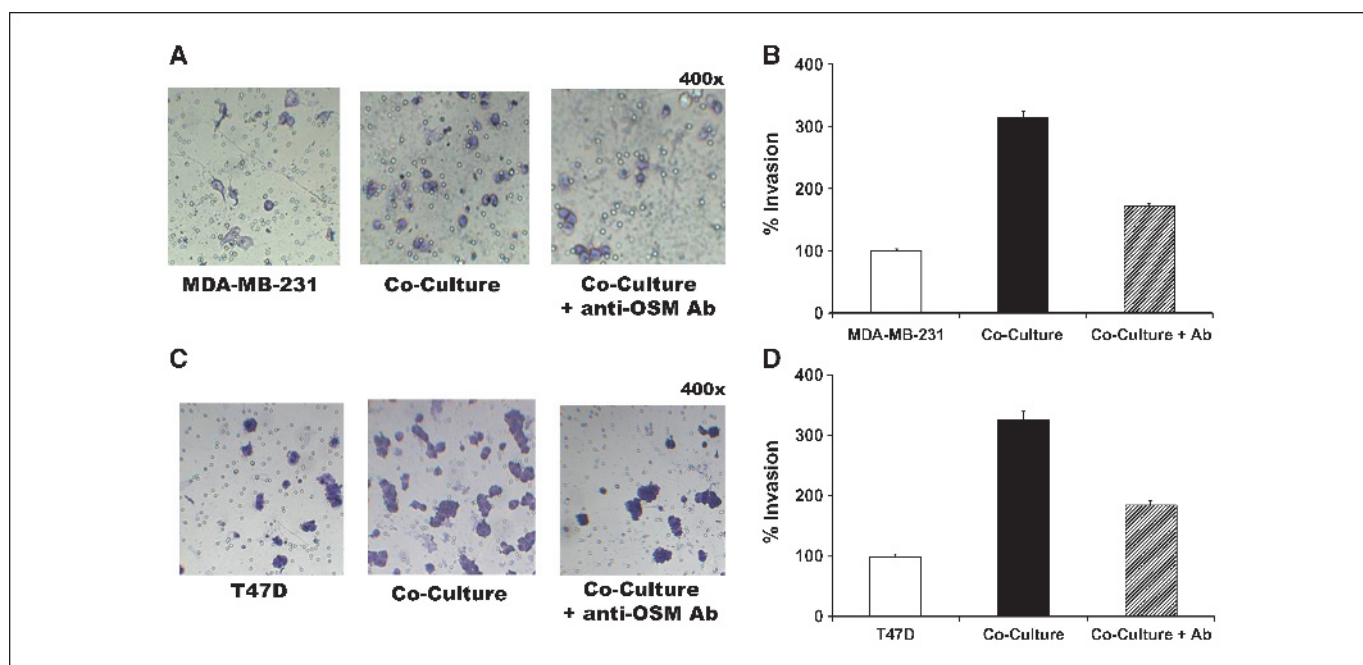


Figure 5. Neutrophil-derived oncostatin M (OSM) induces human breast cancer cell invasiveness. A, MDA-MB-231 human breast cancer cells plated alone show some invasion in the Matrigel invasion assay (cells stained blue have invaded through Matrigel), whereas MDA-MB-231 cells show increased invasiveness when cocultured with primed and anti-oncostatin M–neutralizing antibody decreased invasiveness of MDA-MB-231 cells cocultured with primed neutrophils. B, quantification of results showed a 3-fold increase in invasive capacity of MDA-MB-231 cells cocultured with primed neutrophils, which was partially abrogated by anti-oncostatin M–neutralizing antibody. Similar results were obtained when T47D human breast cancer cells were plated with neutrophils (C-D).

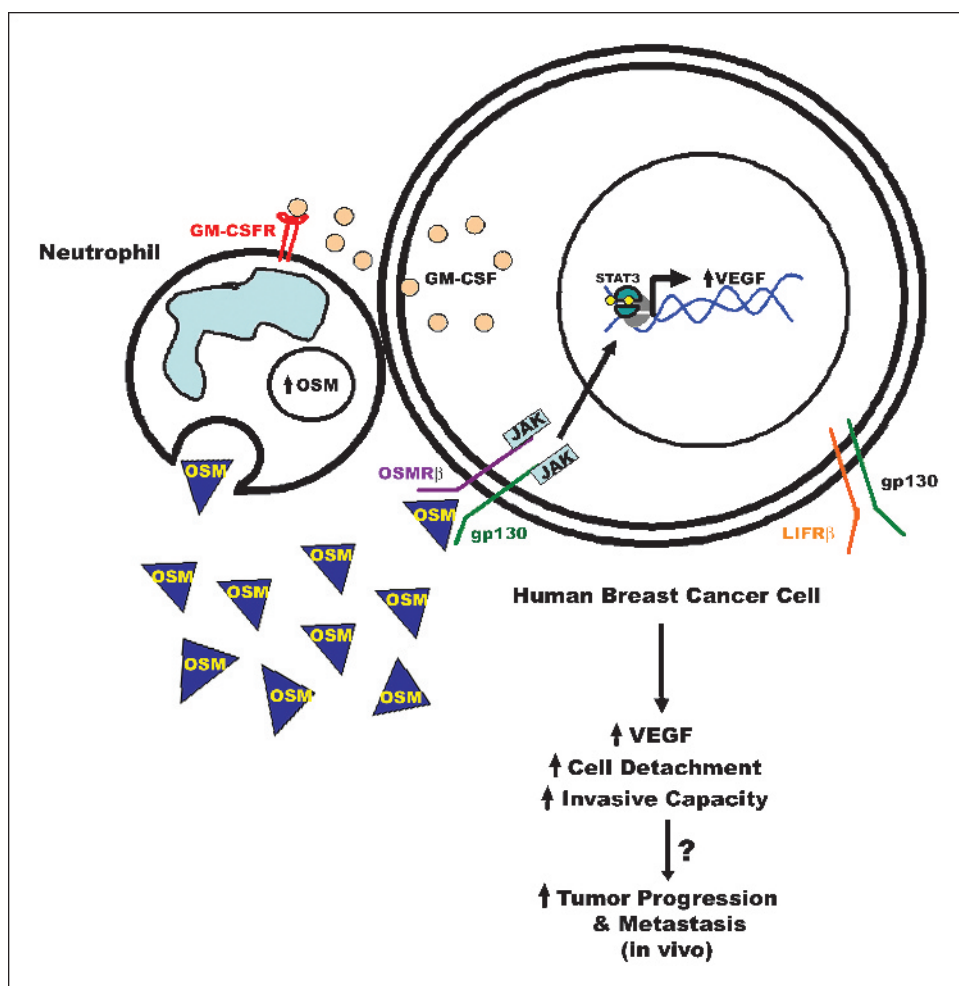


Figure 6. Proposed pathway by which neutrophil-derived oncostatin M (OSM) leads to increased invasive and metastatic capacity of human breast cancer cells. Breast cancer cells supply a signal, GM-CSF, to neutrophils, which then, produce oncostatin M and store it in granules. Cell-to-cell contact between neutrophil and breast cancer cells causes neutrophils to release oncostatin M. Oncostatin M binds to oncostatin M receptors on the breast cancer cells signaling increased cell detachment, VEGF secretion, and invasiveness. Our results suggest that neutrophils may provide a source of oncostatin M for cancer cells, resulting in oncostatin M-induced tumor progression and metastasis.

in angiogenesis and invasion (33). These data, together with the fact that neutrophils are an important infiltrating cell type in many types of cancer (34), suggest that neutrophil-derived oncostatin M could be a promoter of tumor progression and metastasis.

Our immunocytochemistry analyses allowed visualization of oncostatin M expression in resting (nonstimulated) neutrophils, primed (activated) neutrophils, and primed neutrophils that had been cocultured with human breast cancer cells. Oncostatin M expression was absent or very low in resting neutrophils, somewhat higher in primed neutrophils, and highest in primed neutrophils cocultured with human breast cancer cells. It has been previously shown that oncostatin M is not secreted by resting (nonstimulated) blood neutrophils, but its expression and secretion are rapidly activated by proinflammatory signals such as GM-CSF (4).

Oncostatin M secretion occurs through a two-step mechanism in neutrophils, consisting of early release of preformed stock oncostatin M in granules followed by *de novo* protein synthesis (4). The intracellular stock of preformed oncostatin M in activated neutrophils is rapidly mobilized by degranulating agents such as phorbol myristate acetate and GM-CSF (4). In our studies, we used FMLP in our coculture system to initiate granular release and found that almost all oncostatin M stored in the neutrophil granules was released.

GM-CSF is a cytokine that promotes the growth and differentiation of cells of monocyte and granulocyte lineages (35). GM-CSF is produced by many cancer cells types including, colon

(36), prostate (37), and breast (38). GM-CSF has been implicated in the delay of apoptosis of both neutrophils and lymphocytes and thus potentially functions to increase the number of neutrophils at sites of inflammation (39). GM-CSF released by activated endothelium can act to increase neutrophil survival and function in the peripheral blood allowing the neutrophil to retain their functional capabilities (39). High levels of exogenous GM-CSF have been shown to trigger the release of oncostatin M by neutrophils (5) and GM-CSF produced by tumor cells, including the metastatic human breast cancer cell line MDA-MB-231, can alter normal neutrophil function (29). Lastly, it has been shown that GM-CSF produced by MDA-MB-231 cells can alter normal neutrophil function, and these neutrophils can facilitate tumor cell transmigration through the endothelial barrier potentiating the process of tumor metastasis (38). Grenier et al. (4) did a kinetic study that showed GM-CSF as a physiologic inducer of not only oncostatin M synthesis but neutrophil degranulation as well.

We tested whether breast cancer cell-derived GM-CSF was involved in the activation of resting neutrophils to produce oncostatin M in our coculture system. We showed an almost complete abrogation of oncostatin M secretion by cultured neutrophils when an anti-GM-CSF-neutralizing antibody blocked GM-CSF during the time of activation (priming). Yet inhibition of GM-CSF at the time of coculture resulting in only a small decrease in neutrophil-secreted oncostatin M compared with normal coculture. Our results concur with Grenier et al. (4) in

that GM-CSF can activate/prime neutrophils and act as an important inducer of oncostatin M synthesis.

In this article, we show that inhibition of direct neutrophil-breast cancer cell contact prevented neutrophil release of stored oncostatin M from neutrophil granules. A previous study showed that contact between glioma cells and neutrophils was required to influence the infiltration of the neutrophils into the tumor (40). Hor et al. (40) also showed that improved survival of neutrophils in the presence of glioma cells required cell-to-cell contact. In addition, our immunocytochemistry results show that those neutrophils that are in direct contact with breast cancer cells produce high levels of oncostatin M, whereas little oncostatin M staining was observed in the ~50% of neutrophils that did not directly associate with breast cancer cells (data not shown).

As neutrophils and mononuclear leukocytes show no expression of OSMR β or LIFR β (7), and human breast cancer cells (MDA-MB-231 and T47D) express both OSMR β and LIFR β by Western blot analysis (data not shown), we believe that neutrophil-derived oncostatin M primarily acts on breast cancer cells and that neutrophil-derived oncostatin M is unlikely to work in an autocrine manner. Therefore, we propose that GM-CSF secreted by breast cancer cells signals neutrophils to produce and store oncostatin M, which is then released by neutrophils in response to cell-to-cell contact with breast cancer cells (Fig. 6). We have yet to identify the signal needed for oncostatin M release by the neutrophil-breast cancer cell interaction, although future studies will address this issue.

Invasive and metastatic capabilities positively correlate with a decrease in cell-cell and cell-substratum adhesiveness. We showed a 3-fold increase in MDA-MB-231 and T47D human breast cancer cell detachment during coculture with neutrophils. We believe this is largely due to neutrophil-secreted oncostatin M, as anti-oncostatin M-neutralizing antibodies inhibited >50% of breast cancer cell detachment. Other neutrophil-secreted factors, such as VEGF and matrix metalloproteinases (MMP), may also play a role in cell detachment (2). In addition, we have previously shown that exogenous oncostatin M is able to induce significant cell detachment of T47D cells⁶ and mammary carcinoma cells (20) from tissue culture plastic, as well as from Matrigel-coated dishes.⁷

VEGF, a potent proangiogenic factor, plays a crucial role in tumor angiogenesis, progression, and metastasis. The proangiogenic effects of VEGF are mostly due to its ability to promote the survival, proliferation, and motility of endothelial cells (41). VEGF is produced by endothelial cells (41), vascular smooth muscle cells (42), monocytes (43), neutrophils (44–46), and breast cancer cells (47). In this article, we show that coculture of neutrophils with human breast cancer cells leads to an increase in VEGF secretion, above the combined amounts released by neutrophils or breast cancer cells alone. This VEGF induction is inhibited by anti-oncostatin M-neutralizing antibody indicating that neutrophil-derived oncostatin M induces VEGF. In addition to neutrophil-derived oncostatin M (endogenous oncostatin M), our lab has studied the addition of exogenous recombinant oncostatin M to human breast cancer cells and observed a similar increase in oncostatin M-induced VEGF.⁵ Here we show an increase in VEGF expression by 2 to 3 days, whereas neutrophil-released oncostatin

M was highest at 15 minutes. These results are in agreement with the time course of exogenous oncostatin M treatment previously done in our lab.⁵ Increased levels of VEGF in a tumor environment disrupt the neutral balance of proangiogenic and antiangiogenic factors, initiating angiogenesis and promoting growth, maintenance, and metastasis of solid tumors.

We have shown that the increase in VEGF secretion by breast cancer cells is the result of oncostatin M binding to the OSMR on breast cancer cells and that the LIFR is not important. Similar results have also been shown using astrogloma cells (33). We predict that neutrophil-derived oncostatin M will signal VEGF induction through the JAK-STAT pathway, as we have previously shown using exogenous oncostatin M treatment of human breast cancer cell lines⁵ and others have shown with astrogloma cell lines (33).

Invasive capabilities of breast cancer cells are positively correlated with an increase in protease expression and VEGF expression and a decrease in cell-cell and cell-substratum attachment (20). Because we observed both increased cell detachment and increased VEGF expression by breast cancer cells in our coculture system, we investigated breast cancer cell invasive potential. We showed an increase in invasive capacity for both MDA-MB-231 cells (324%) and T47D cells (420%) when cocultured with primed neutrophils. Anti-oncostatin M-neutralizing antibody inhibited invasiveness of cocultured breast cancer cells, suggesting a role for neutrophil-derived oncostatin M. The addition of anti-oncostatin M-neutralizing antibody did not completely inhibit breast cancer cell invasive capacity, suggesting that neutrophil-derived oncostatin M may not be solely responsible for the increased invasiveness seen in our cocultures. Other neutrophil-derived factors may contribute to the increased invasion of cocultured breast cancer cells, including MMPs (48) and cathepsins (9).

In other systems, oncostatin M has been shown to induce transmigration of human neutrophils through monolayers of endothelial cells by stimulating endothelial cells to express adhesion molecules and chemokines (18). Wu et al. (29) have shown that tumor-conditioned medium isolated from the human breast cancer cell line MDA-MB-231 alters normal human neutrophil function by suppressing the cytotoxic capacity of neutrophils and at the same time increasing expression of neutrophil adhesion receptors.

In conclusion, our studies show that neutrophil-derived oncostatin M is involved in VEGF induction, breast cancer cell detachment, and increased invasiveness (Fig. 6). Based on our data, neutrophils could be a possible target for the inhibition of tumor progression and metastasis. Ultimately, developing leukocyte inhibitory therapies could be a useful approach for treatment of invasive cancers with infiltrating neutrophils. Future work will be directed towards investigating the mechanism of cell-to-cell contact required for the release of neutrophil-derived oncostatin M, as well as assessing the significance of neutrophil-derived oncostatin M in human breast cancer progression and metastasis *in vivo*.

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⁶ C.L. Jorcyk et al. Oncostatin M induces cell detachment and enhances the metastatic capacity of T47D human breast carcinoma cells.

⁷ Holzer, unpublished.

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