Oncostatin M Induces Growth Arrest of Mammary Epithelium via a CCAAT/enhancer-binding Protein δ-dependent Pathway[1]

Julie A. Hutt and James W. DeWille[2]

Department of Veterinary Biosciences [J. A. H., J. W. D.] and Division of Molecular Biology and Cancer Genetics, Ohio State Comprehensive Cancer Center [J. W. D.], The Ohio State University, Columbus, Ohio 43210

Abstract

Oncostatin M (OSM), an interleukin 6-type cytokine, induces sustained up-regulation of CCAAT/enhancer-binding protein (C/EBP) δ mRNA and protein in nonneoplastic HC11 mouse mammary epithelial cells. This up-regulation is dependent on signaling by phospho-Stat3 (signal transducers and activators of transcription). The same signaling pathway is activated in two human breast cancer cell lines, a neoplastic mouse mammary epithelial cell line and a second nonneoplastic mouse mammary epithelial cell line. [^{3}H]Thymidine incorporation and flow cytometry demonstrate that OSM inhibits the growth of HC11 cells by reducing the number of S-phase cells. These phenotypic changes are accompanied by reduced expression of S-phase genes with a corresponding increased expression of G_0 genes in HC11 cells. Reduction of C/EBPδ protein in HC11 cells by expression of a C/EBPδ antisense construct inhibits OSM-mediated growth arrest. These data demonstrate that OSM induces up-regulation of C/EBPδ via a Stat3-dependent pathway in mammary epithelial cells and that the growth inhibition induced by OSM depends on the presence of C/EBPδ.

Introduction

OSM[3] has recently been proposed for use as a potential cancer therapeutic agent, due to its ability to induce growth arrest of normal and neoplastic human breast epithelial cells (1). OSM is a member of the IL-6 family of cytokines, which also includes IL-6, IL-11, LIF, and ciliary neurotrophic factor. These cytokines signal by binding to cell surface receptors, which consist of cytokine-specific binding subunits and a common signaling subunit, glycoprotein 130 (2, 3). OSM is produced by a variety of cell types, including neoplastic breast epithelial cells (4). Other IL-6-type cytokines are also produced by normal and neoplastic breast epithelium and breast adipose cells (4). Furthermore, the IL-6 expression level in some types of mammary carcinoma is inversely correlated with the histological grade of malignancy (5, 6).

OSM stimulates proliferation of some cell types, such as myeloma cells and Kaposi's sarcoma cells, and inhibits proliferation of others, such as melanoma cells and normal and neoplastic mammary epithelial cells (7–12). In normal and neoplastic human mammary epithelial cells, OSM inhibits cell cycle progression, with a reduction in the proportion of S-phase cells and an adoption of morphological phenotype characteristic of differentiated breast epithelial cells (11–13). However, little is known about the signaling pathways involved in determining whether OSM induces cell proliferation or growth inhibition. To understand the biological role of OSM in the mammary gland and enhance the effectiveness of OSM as a therapeutic agent, an understanding of the OSM-activated signaling pathways leading to growth arrest of normal and neoplastic cells is of crucial importance.

Recent studies have demonstrated that OSM and IL-6 simultaneously induce growth inhibition and increased migration of T47D breast cancer cells via different, simultaneously activated signaling pathways (14). The growth inhibition induced by both OSM and IL-6 depends on Stat3 activation, but no downstream mediators of growth arrest have been identified. The increased cell migration induced by IL-6 is independent of Stat3 and depends on activation of the MAPK/phosphatidylinositol 3′-kinase pathway. This suggests that the ultimate cell fate depends on a balance between the different cytokine-activated signaling pathways.

Stats are latent cytoplasmic transcription factors, which, upon activation by tyrosine phosphorylation, form homo- or heterodimers, translocate to the nucleus, and bind consensus DNA sequences to activate transcription. Stat3 has previously been shown to be involved in regulation of both proliferation and growth arrest, depending on the cell type (15–19). Several human breast cancer cell lines exhibit dysregulation of Stat3 signaling characterized by constitutively activated Stat3 (20, 21). In contrast, Stat3 activation in the involuting mouse mammary gland has been proposed as the "driving force" for mammary epithelial apoptosis (22–24). Conditional Stat3 knockout in the involuting mouse mammary gland confers delayed involution (25). Previous work from our laboratory demonstrates that mammary gland involution *in vivo* and serum and growth factor withdrawal *in vitro* result in Stat3 activation, with subsequent Stat3 binding

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[2] To whom requests for reprints should be addressed, at Department of Veterinary Biosciences, The Ohio State University, 1900 Coffey Road, Columbus, OH 43210. Phone: (614) 292-4261; Fax: (614) 292-6473; E-mail: dewille.1@osu.edu.
[3] The abbreviations used are: OSM, oncostatin M; IL, interleukin; LIF, leukemia inhibitory factor; C/EBP, CCAAT/enhancer-binding protein; JAK, Janus kinase; EGF, epidermal growth factor; TRPM2, testosterone repressed specific message 2; CP, cyclophilin; Stat, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; FBS, fetal bovine serum; ATCC, American Type Culture Collection; TK, thymidine kinase; ERK, extracellular signal-regulated kinase.

to the acute phase response element of the C/EBPδ promoter, increased Stat3-dependent C/EBPδ transcription, and, ultimately, growth arrest and apoptosis of mammary epithelium (26–28). Thus, these contrasting roles for Stat3 in mammary epithelium demonstrate that there are other factors (presumably other signaling pathways) that interact with the Stat3 pathway to influence the ultimate fate of the cell.

C/EBPδ is a member of the C/EBP family of leucine zipper DNA-binding proteins, which have been implicated in the control of growth and differentiation in a variety of tissue and cell types (29–34). The six C/EBP family members (α, β, δ, γ, ε, and C/EBP homologous protein-10) form homo- or heterodimers with C/EBPs and other leucine zipper proteins and bind to DNA to activate or repress transcription (35, 36). C/EBPδ has an important role in the induction of G_0 growth arrest and apoptosis in cultured mouse mammary epithelium in response to serum and growth factor withdrawal (37, 38).

To investigate the signaling pathways involved in the OSM-mediated growth arrest response of mammary epithelial cells, nonneoplastic HC11 mouse mammary epithelial cells were treated with OSM. The work described herein demonstrates that the OSM-induced growth arrest response in HC11 cells and other mouse mammary epithelial cells is similar to what occurs in human mammary epithelium. OSM treatment of HC11 cells decreases [^{3}H]thymidine incorporation and reduces the number of S-phase cells, with corresponding increases in expression of G_0 genes and decreases in expression of S-phase genes. In addition, in both mouse and human mammary epithelium, OSM induces sustained Stat3 phosphorylation and phospho-Stat3-dependent C/EBPδ up-regulation. Furthermore, we show that the growth arrest response induced by OSM is markedly diminished in HC11 cells expressing C/EBPδ antisense mRNA. These data support a direct role for C/EBPδ in the OSM-mediated growth arrest of mammary epithelial cells and suggest that the integrity of the Stat3-C/EBPδ pathway is important for the growth arrest response of mammary epithelial cells to OSM.

Materials and Methods

Cell Culture. HC11 cells (a generous gift of Dr. Wolfgang Doppler; Universitat Innsbruck, Innsbruck, Austria), a nontransformed line of mouse mammary epithelial cells, were cultured in RPMI 1640 supplemented with 10% FBS, 10 ng/ml EGF, and 10 μg/ml bovine insulin (Invitrogen Life Technologies, Inc., Carlsbad, CA). NMuMG mouse mammary epithelial cells (ATCC, Manassas, VA) were cultured in DMEM (4.5 g/ml glucose) supplemented with 10% FBS and 10 μg/ml insulin. The transformed mouse mammary epithelial cells, MM5MT (ATCC), were cultured in DMEM (4.5 g/ml glucose) supplemented with 10% FBS. The human mammary carcinoma cells, SKBR3 and MCF-7 (ATCC), were cultured in DMEM (4.5 g/ml glucose) supplemented with 10% FBS. For the cytokine growth assays (thymidine incorporation, cell counting, and flow cytometry), all cells were cultured in RPMI 1640 containing 2% heat-inactivated FBS (12, 39). The following cytokines were added for the designated times at the designated concentrations: IFN-γ, LIF, IL-6 (Peprotech, Rocky Hill, NJ), and OSM (mouse and human; R&D Systems, Minneapolis, MN).

Transfections. To make the bulk-transfected C/EBPδ antisense cell lines, HC11 cells were transfected with the pcDNA3-C/EBPδ antisense plasmid or the empty pcDNA3 plasmid using Transfectam reagent (Promega, Madison, WI; Ref. 38). Selection was carried out in the presence of 700 μg/ml Geneticin (Invitrogen Life Technologies, Inc.) for 2 weeks. Clonal cell lines were established from individual colonies, and Western blotting identified the δ_1 clone as having maximal inhibition of C/EBPδ protein expression. Other colonies were pooled to establish bulk transfected cell lines to dilute the influence of clonal variation. The δ_1 antisense plasmid has been described previously (38).

Northern Blot Analysis. Total RNA was isolated from cultured cells using RNazol B (Tel-Test, Friendswood, TX). Northern blots were carried out using standard procedures (40). Blots were probed with the following random primer-labeled ([α-^{32}P]dCTP) cDNAs: C/EBPδ, C/EBPβ (a generous gift of Dr. Steven McKnight; University of Texas Southwestern Medical Center), GAS1 (growth arrest-specific gene 1; a generous gift of Dr. Claudio Schneider; Cosorzio Interuniversitario Biotecnologie), TRPM 2, TK, and histone 2B (Oncor, Gaithersburg, MD). Cyclophilin receptor protein partial cDNA was used as a constitutive probe. Fold induction was calculated from densitometric measurements taken using an AlphaImager 2000 (Alpha Innotech, Staffordshire, United Kingdom). Figures depicting multiple mRNAs were either coprobed or stripped and sequentially reprobed. Results are representative of experiments performed two to four times.

Western Blot Analysis. Whole cell protein lysates were prepared from tissue culture cells. Cells were washed with cold PBS, scraped into microfuge tubes, and centrifuged for 30 s at 14,000 × *g* to pellet cells. The supernatant was removed, and cells were suspended in whole cell lysis buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, and 2 mM EDTA]. Protease, kinase, and phosphatase activities were inhibited by the addition of Complete tablets (Roche Molecular Biochemicals, Indianapolis, IN), 1 mM NaF, 1 mM $NaVO_3$, 1 mM Na_2MoO_4, and 10 nM okadaic acid to the protein isolation solution. Proteins were quantified using the BCA microprotein assay kit (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Evenness of loading was verified by examination of gels stained with Coomassie Blue. Western blots were probed with primary antibodies to Stat3, phospho-Stat3 (Y705), Stat1, phospho-Stat1 (Y701), p42/p44, phospho-p42/p44 (T202 and Y204; Cell Signaling Technology, Beverly, MA), p21, C/EBPδ (Santa Cruz Biotechnology, Santa Cruz, CA), p27, and cyclin D1 (Transduction Laboratories, Lexington, KY). Horseradish peroxidase-conjugated antimouse or antirabbit antibodies (Cell Signaling Technology) were used to detect primary antibodies, and the signal was developed using the ECL system (Amersham Biosciences, Piscataway, NJ). Fold induction was calculated from the densitometric measurements taken using an AlphaImager 2000 (Alpha Innotech). Figures depicting multiple proteins were either stripped and sequentially reprobed or

prepared by running multiple gels using the same lysates. Results are representative of experiments performed two to four times.

[^{3}H]Thymidine Incorporation Studies. Cells were plated in 24-well tissue culture dishes at a density of 1.2×10^4 cells/well in 400 μl of medium containing 2% heat-inactivated FBS (12, 13). After 5 h, OSM was added to the desired final concentration. At the designated time points, cells were pulsed for 2 h with [^{3}H]thymidine (1 μCi/ml), harvested by precipitation with cold 10% trichloroacetic acid, solubilized in 0.3 N NaOH, and counted by liquid scintillation counting. Results represent the average of two to six independent experiments with 3–6 wells/time point/condition.

Flow Cytometric Cell Cycle Analysis. Cells were plated in 6-cm tissue culture dishes at a density of 1.3×10^5 cells/plate, in 3.5 ml of medium containing 2% heat-inactivated FBS. After 5 h, OSM was added to the desired final concentration. At the designated time points, cells were harvested from the various treatments, fixed in 70% ethanol overnight, and stained with propidium iodide. The percentage of cells in each cell cycle phase was assessed by fluorescence-activated cell sorter analysis. Results represent the average of three independent experiments with 2–3 samples/time point/condition.

Statistical Analysis. Statistical analyses were performed using SAS JMP software (SAS Institute, Cary, NC). Student's *t* test or a one-way analysis of variance and Tukey-Kramer comparison of all pairs were used to compare averages for different treatment groups.

Results

The Growth Rate of Mouse Mammary Epithelial Cells Is Reduced in the Presence of OSM. We investigated the effect of OSM on mouse mammary epithelial cells. The growth of nonneoplastic HC11 mouse mammary epithelial cells was decreased after 4 days in the presence of increasing concentrations of OSM, as measured by [^{3}H]thymidine incorporation (Fig. 1*A*). As the concentration of OSM approached 100 ng/ml, the dose-response curve reached a plateau. HC11 cells treated with 50 ng/ml OSM showed 60% and 81% reductions in [^{3}H]thymidine incorporation after 1 day and 4 days, respectively (Fig. 1, *B* and *C*). Simultaneous cell counting experiments at 4 days demonstrated a corresponding 5-fold reduction in cell number in the OSM-treated cells (data not shown). The influence of OSM on the growth of the neoplastic human (SKBR3) and mouse (MM5MT) mammary cell lines was also tested. OSM treatment decreased [^{3}H]thymidine incorporation in SKBR3 and MM5MT cells by 37% and 90%, respectively (Fig. 1*D*).

OSM Decreases the Number of HC11 Cells in S-Phase. Flow cytometry (propidium iodide-stained cells) was used to confirm the [^{3}H]thymidine incorporation data and to directly assess the effect of OSM on the cell cycle status of HC11 cells after 4 days. A representative histogram is shown in Fig. 2, and the average number of cells in each phase of the cell cycle is reported above each histogram. OSM treatment resulted in a 48% reduction in the number of S-phase cells, with a corresponding increase in the number of G_0-G_1-phase cells. OSM treatment also reduced the number of SKBR3 cells in S phase (data not shown).

OSM Alters the Expression of a Number of Growth-related Genes in HC11 Cells. To investigate the molecular mechanisms of OSM-mediated growth arrest, we evaluated OSM-induced changes in the levels of cell cycle-related genes. Proteins and RNA were harvested from HC11 cells after being grown for 4 days with or without OSM, and Western and Northern blots were performed (Fig. 3). The levels of phospho-Stat3 (Y705) and C/EBPδ both increased almost 6-fold (Fig. 3*A*). The mRNA levels of the S-phase markers histone and TK were reduced 2–3-fold (Fig. 3*B*), and the mRNA levels of the G_0 markers GAS1, TRPM2, and C/EBPδ were increased 3–5-fold (Fig. 3*C*). These results are consistent with the flow cytometry results (Fig. 2) and suggest that OSM induces cell cycle exit into G_0. OSM also induces changes in the levels of other growth-regulated proteins (Fig. 3*D*). OSM induced 3-fold decreases in the protein levels of cyclin D1 and $p21^{WAF1/CIP1}$, whereas $p27^{KIP1}$ remained unchanged. The levels of phospho-p44/p42 (ERK1 and ERK2) increased 3-fold.

The Induction of C/EBPδ mRNA by Cytokines Is Unique to OSM. To determine whether the induction of C/EBPδ by cytokines was a general characteristic of IL-6-type cytokines or a unique feature of OSM, HC11 cells were treated with IL-6, LIF, and OSM. In addition, cells were treated with IFN-γ. IL-6, LIF, OSM, and IFN-γ are known to inhibit proliferation of mammary epithelial cells (5, 11–13, 41–46). Cells were treated with cytokines at the indicated concentrations, and RNA was harvested after 1, 8, and 24 h. C/EBPδ mRNA was induced 7-fold after 1 h in cells treated with IFN-γ but disappeared by 8 h (Fig. 4). In contrast, C/EBPδ mRNA was induced 8-fold after 1 h in cells treated with OSM and remained elevated at 8 and 24 h. Neither IL-6 nor LIF had any effect on C/EBPδ mRNA levels. Similar induction patterns and levels were observed at the 15 and 30 min time points (data not shown). The C/EBPβ mRNA level remained relatively unchanged for all cytokines at all time points.

The Induction of C/EBPδ mRNA and Protein by OSM Corresponds to Sustained Stat3 Phosphorylation. IL-6-type cytokines signal through a common gp130 receptor component and typically transduce signals to the nucleus via activation of the Stat family of proteins. Although it signals through binding to a different receptor superfamily, IFN-γ also transduces signals via activation of Stat proteins. HC11 cells were treated with the IL-6-type cytokines IL-6, LIF, and OSM, along with IFN-γ, and proteins were harvested after 1, 8, and 24 h. Western blots probed with antibodies to phospho-Stat3 (Y705), Stat3, phospho-Stat1 (Y701), and Stat1 are shown in Fig. 5. Stat3 phosphorylation was increased at 1 h (3-fold), 8 h (6-fold), and 24 h (6-fold) in the OSM-treated cells. Stat1 was phosphorylated at 1, 8, and 24 h (3–4-fold) in the IFN-γ-treated cells. Similar induction patterns and levels were observed at the 15 and 30 min time points (data not shown). IFN-γ treatment increased Stat3 phosphorylation (2-fold) only before and at the 1 h time point. Interestingly, IFN-γ induced C/EBPδ mRNA only at time points that corresponded to Stat3 activation, and not Stat1 activation. Stat3 and Stat1 protein levels were unchanged by cytokine

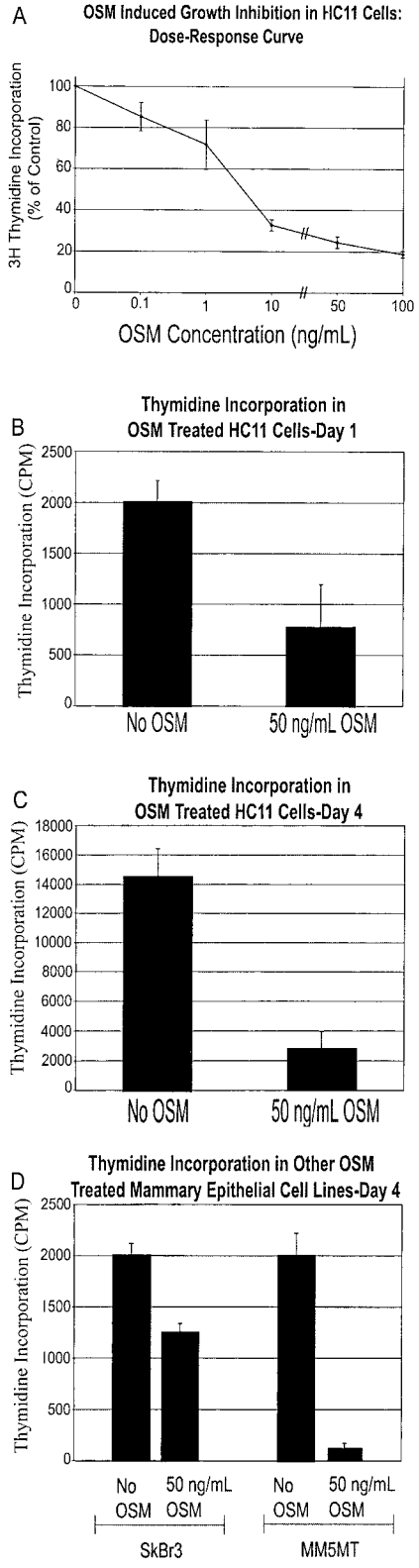

Fig. 1. OSM reduces the growth rate of mammary epithelial cells. *A,* HC11 cells were grown in the presence of varying concentrations of OSM. [^{3}H]Thymidine incorporation was measured after 4 days, and the results are presented relative to untreated controls (100% incorporation). *B,* HC11 cells were grown in the presence or absence of 50 ng/ml OSM for 1 day. Data represent the average from three experiments with 6 replicates/condition/experiment ($P < 0.0001$, Student's *t* test). *C,* HC11 cells were grown in the presence or absence of 50 ng/ml OSM for 4 days. Data represent the average of six experiments with 4–6 replicates/condition/experiment ($P < 0.0001$, Student's *t* test). *D,* SKBR3 and MM5MT cells were grown for 4 days in the presence or absence of 50 ng/ml OSM (human and mouse, respectively). Data represent the average of two experiments with 3–6 replicates/condition/experiment ($P < 0.0001$, Student's *t* test).

treatment. Stat5 activation was not detected at any of the time points examined (data not shown). Neither IL-6 nor LIF had any effect on phosphorylation of Stat1 or Stat3 in HC11 cells.

OSM Induction of Stat3 Phosphorylation and C/EBPδ Protein Is Not Unique to HC11 Cells. To determine whether OSM activates Stat3 and induces C/EBPδ in mammary epithelial cells other than HC11 cells, the levels of phospho-Stat3 and C/EBPδ protein in response to OSM were examined in several other mammary epithelial cell lines. HC11, NMuMg, and MM5MT cells, which are nontransformed (HC11 and NMuMg) and transformed (MM5MT) mouse mammary epithelial cell lines, were treated with OSM for 24 h. Western blots probed with phospho-Stat3, Stat3, and C/EBPδ are shown in Fig. 6*A*. In all cell lines at all time points, there were sustained increases in both Stat3 phosphorylation (3–6-fold) and C/EBPδ protein levels (3–8-fold) in response to OSM treatment. Fig. 6*B* shows Western blots of two neoplastic human mammary epithelial cell lines, MCF-7 and SKBR3, treated with OSM for 24 h. Increased Stat3 phosphorylation (3–4-fold) and increased C/EBPδ protein levels (3–4-fold) were also present in these human cell lines in response to OSM. These data demonstrate that the activation of Stat3 and induction of C/EBPδ are a general response of mammary epithelial cells to OSM treatment.

Inhibition of JAK2 Decreases the OSM-induced Activation of C/EBPδ in HC11 Cells. OSM and other IL-6-type cytokines signal via activation of the JAK family of nonreceptor tyrosine kinases, with subsequent activation of Stats. To verify that the increase in C/EBPδ protein depends on Stat3 activation and not a Stat3-independent mechanism, HC11 cells were pretreated with 50 μM AG490 (a specific inhibitor of JAK2) or a DMSO control for 24 h, followed by treatment with OSM for 1 h. Western blots comparing the OSM induction of phospho-Stat3 (Y705) and C/EBPδ, with and without AG490 pretreatment, are shown in Fig. 7. Pretreatment with AG490 at the 50% inhibitory concentration caused a 2–3-fold reduction in both the amount of phospho-Stat3 and C/EBPδ protein induced in response to OSM. This supports a role for the JAK family members in the OSM-mediated activation of Stat3 and demonstrates the dependence of C/EBPδ up-regulation on Stat3 phosphorylation.

The OSM-induced Decrease in Growth of HC11 Cells Is Dependent on C/EBPδ. To examine the role of C/EBPδ in the OSM induction of growth arrest of HC11 cells, [^{3}H]thymidine incorporation studies and flow cytometric analysis of cell cycle status were examined in HC11 cells with reduced expression of C/EBPδ protein. HC11 cells were transfected with a pcDNA3 control plasmid (pc_{con}) or a pcDNA3-C/EBPδ antisense plasmid and selected for 2 weeks in G418. In subsequent experiments, both the δ_1 antisense (clonal) cell

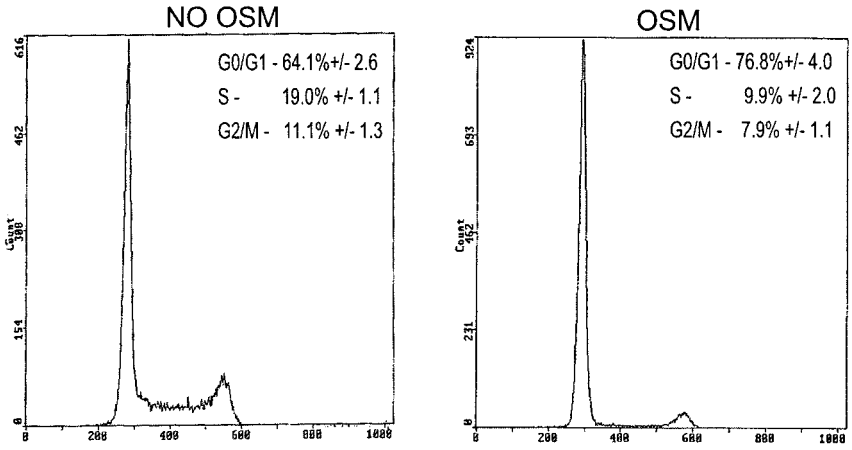

Fig. 2. OSM reduces the percentage of HC11 cells in S phase and increases the percentage of HC11 cells in G_0-G_1. HC11 cells were grown in the presence or absence of 50 ng/ml OSM. After 4 days, cells were harvested, stained with propidium iodide, and analyzed for DNA content by fluorescence-activated cell sorting. Results represent the average of three independent experiments with 2–3 replicates/condition/experiment ($P < 0.0001$, Student's *t* test).

line and the bulk transfected C/EBPδ antisense cell line (δ_{bulk}) were used to reduce the contribution of clonal variation. The level of C/EBPδ protein induction in response to OSM treatment was compared for these three cell lines (Fig. 8*A*). The OSM-treated δ_{bulk} cells and δ_1 cells expressed approximately 80% and 30%, respectively, of the C/EBPδ protein expressed by the pc_{con} cells, demonstrating the efficacy of the antisense construct at reducing C/EBPδ protein expression.

The average [^{3}H]thymidine incorporation in these three cell lines in response to OSM treatment is shown in Fig. 8*B*. After 4 days of OSM treatment, the pc_{con} cells exhibited a 76% reduction in [^{3}H]thymidine incorporation in response to OSM, similar to that seen in the parental HC11 cell line (81% reduction). In contrast, the C/EBPδ antisense cells exhibited significantly less of a reduction in [^{3}H]thymidine incorporation when treated with OSM (42% reduction for δ_{bulk} cells and 13% reduction for δ_1 cells).

To confirm the results of the [^{3}H]thymidine incorporation data, flow cytometry was used to assess the cell cycle status of the OSM-treated antisense cells. The results are summarized in Table 1. The pc_{bulk} cells showed a 49% decrease in the number of S-phase cells after 4 days treatment with OSM, similar to the parental HC11 cell line (48% decrease; Fig. 2). In contrast, the δ_{bulk} and δ_1 cells showed only a 16% decrease and a 10% increase, respectively, in the number of S-phase cells. These results suggest that C/EBPδ is an important mediator of OSM-induced growth arrest.

Discussion

Several reports have demonstrated that OSM induces growth arrest of human mammary epithelial cells (12, 13, 47); however, only recently have the OSM-activated signaling pathways responsible for inducing growth arrest been investigated. Badache and Hynes (14) show that mammary epithelial growth arrest induced by IL-6 and OSM depends on activated Stat3. Our data suggest that C/EBPδ is a downstream mediator of the Stat3-dependent growth arrest response in OSM-treated mammary epithelium.

This is the first report to examine the effects of OSM on mouse mammary epithelium. Nonneoplastic and neoplastic mouse mammary epithelial cells undergo growth arrest in response to OSM in a manner similar to human mammary epithelial cells (12, 13). In addition, C/EBPδ is increased in all OSM-treated mammary epithelial cell lines examined (Fig. 6). The similarities between mouse and human mammary epithelial cells in the signaling pathways initiated and the growth arrest responses induced after treatment with OSM indicate that the mouse is a suitable model for investigating the therapeutic response of mammary cancers to OSM.

Previous results show that C/EBPδ is up-regulated in mammary epithelium only during G_0, and not during any other stage of the cell cycle (37). The reductions in the S-phase markers histone and TK and the increases in the mammary epithelial G_0 markers C/EBPδ, GAS1, and TRPM2 (Fig. 3) suggest that OSM causes mammary epithelial cells to withdraw from the cell cycle. The reduction in cyclin D1 protein is also consistent with G_0 growth arrest (13). The persistent increase in C/EBPδ for the 4-day duration of the growth assays (Fig. 3*A*) suggests that C/EBPδ has a significant role in maintaining the OSM-induced growth arrest. A similar, persistent up-regulation of C/EBPδ has previously been shown to be responsible for cell cycle exit and maintenance of G_0 growth arrest in mammary epithelium after serum and growth factor deprivation (37, 38).

The reduction in $p21^{CIP1}$ induced by OSM is consistent with other reports that activated Stat3 represses $p21^{CIP1}$ expression in mammary epithelial cells. During mammary gland involution, when Stat3 is normally activated, conditional Stat3 knockout mice demonstrate a marked increase in $p21^{CIP1}$ expression compared with wild-type controls (25). The lack of change in $p27^{KIP1}$ level during this time is expected because these cells were not confluent and hence were not contact inhibited (48–50).

HC11 cells are commonly used as a model to study mammary epithelial growth and differentiation. Lactogenic hormone treatment of HC11 cells induces differentiation via two different signaling pathways, MAPK-dependent synthesis of enzymes involved in cytoplasmic lipid droplet formation and Stat5-dependent synthesis of β-casein (51, 52). Treatment of human mammary carcinoma cells with OSM causes activation of the MAPK pathway, with MAPK-dependent formation

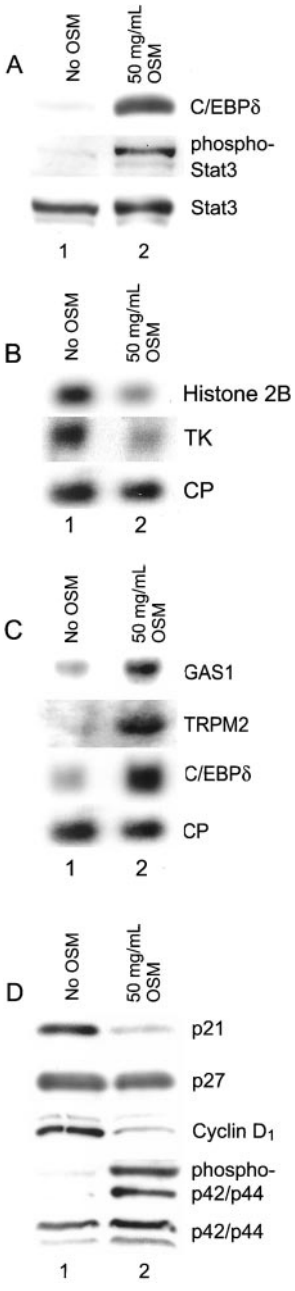

Fig. 3. OSM alters the expression of a number of growth-regulated genes in HC11 cells. Subconfluent HC11 cells were treated with 50 ng/ml OSM for 4 days, and proteins and RNA were harvested. *A,* Western blot probed with antibodies to Stat3, phospho-Stat3 (Y705), and C/EBPδ. *B,* Northern blot probed with the S-phase markers histone and TK. CP was used as a loading control. *C,* Northern blot probed with the G_0 markers GAS1, TRPM2, and C/EBPδ. CP was used as a loading control. *D,* Western blot probed with $p21^{WAF1/CIP1}$, $p27^{KIP1}$, cyclin D1, phospho-p44/p42 (ERK1 and ERK2), and p44/p42.

of cytoplasmic lipid droplets (53). OSM-treated HC11 cells exhibit similar MAPK activation (Fig. 3) and cytoplasmic lipid droplet formation (data not shown). However, neither Stat5 activation nor β-casein synthesis is observed in OSM-treated HC11 cells (data not shown). Thus, the differentiation pathway induced by OSM is not completed.

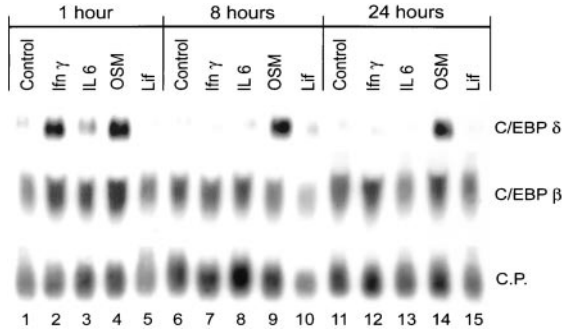

Fig. 4. OSM, but not IFN-γ, IL-6, or LIF, induces a sustained increase in C/EBPδ mRNA in HC11 cells. HC11 cells were treated with IFN-γ (500 units/ml), IL-6 (50 ng/ml), OSM (50 ng/ml), and LIF (20 ng/ml) for 1, 8, and 24 h. RNA was harvested at the designated time points, and Northern blots were performed. CP was used as a loading control.

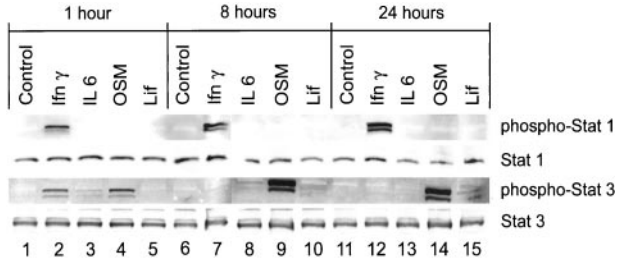

Fig. 5. OSM, but not IFN-γ, IL-6, or LIF, induces sustained activation of Stat3, but not Stat1, in HC11 cells. HC11 cells were treated with IFN-γ (500 units/ml), IL-6 (50 ng/ml), OSM (50 ng/ml), and LIF (20 ng/ml) and for 1, 8, and 24 h. Proteins were harvested at the designated time points, and Western blots were performed.

The growth status of mammalian cells at a given point in time is the result of a balance between mitogenic and growth arrest signals. The growth arrest of OSM-treated HC11 cells is blocked in the presence of high levels of EGF and insulin, even though Stat3 is activated, and C/EBPδ expression is increased (data not shown). A similar phenomenon occurs in HC11 cells transfected with a CMV-C/EBPδ plasmid that constitutively overexpresses C/EBPδ protein. In growth medium containing the mitogens insulin and EGF, the C/EBPδ-overexpressing cells grow at the same rate as controls. However, under serum and growth factor withdrawal conditions, the C/EBPδ-overexpressing cells undergo growth arrest more rapidly than controls (38). These data suggest that the expression of C/EBPδ alone is not sufficient to inhibit growth. Alternatively, posttranslational modifications of C/EBPδ, which may not occur in the presence of mitogens, may be required for C/EBPδ growth-inhibitory activity. Liu *et al.* (12) have shown that OSM antagonizes the mitogenic response of human mammary epithelial cells to EGF in a manner that is dependent on the dose of both EGF and OSM. The difference between our observation (that OSM cannot block mitogenic signals) and that of Liu *et al.* (12) may be due to the amount of EGF added, the addition of insulin, or the effects of autocrine growth factors produced in the different cell lines.

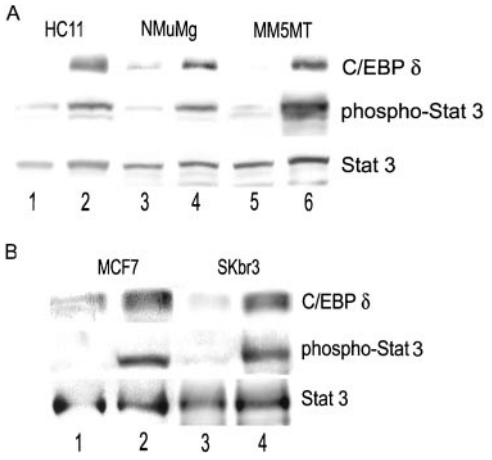

Fig. 6. The activation of Stat3 and up-regulation of C/EBPδ by OSM is not unique to HC11 cells. Other nontransformed and transformed mammary epithelial cell lines were examined for their ability to activate Stat3 and up-regulate C/EBPδ in response to OSM. Cells were treated with OSM for 24 h, and then proteins were harvested, and Western blots were performed. *A,* HC11 (nontransformed), NMuMg (nontransformed), and MM5MT (transformed) mouse mammary epithelial cells. Mouse OSM (50 ng/ml). *B,* MCF-7 and SKBR3 human mammary carcinoma cells. Human OSM (50 ng/ml).

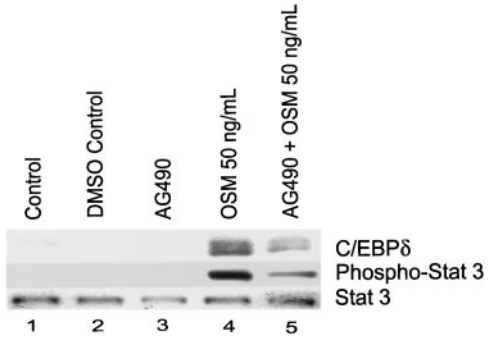

Fig. 7. The OSM induction of C/EBPδ protein is dependent on Stat3 activation by the JAK pathway. HC11 cells were pretreated with AG490, a JAK2 inhibitor, or with DMSO control for 24 h, followed by treatment with OSM. Proteins were harvested, and Western blots were performed.

The reduction in thymidine incorporation induced by OSM varies for the different cell lines examined (Fig. 1). This may be due to variations in the endogenous growth factors produced in the different cell types or to variations in the growth-regulating signaling pathways altered in the different cell types during the process of immortalization and/or transformation. In addition, because human-derived cell lines generally double at a slower rate than mouse-derived cell lines, the relative level of growth arrest induced by OSM in SKBR3 cells may have been greater with treatment times longer than 4 days.

In human mammary epithelial cells, the signaling pathway responsible for inducing growth arrest after treatment with OSM is cell line specific. In T47D human mammary carcinoma cells, OSM induces growth arrest by a Stat3-dependent pathway and cell migration by MAPK- and phosphatidylinositol 3′-kinase-dependent pathways (14). Li *et al.* (47)

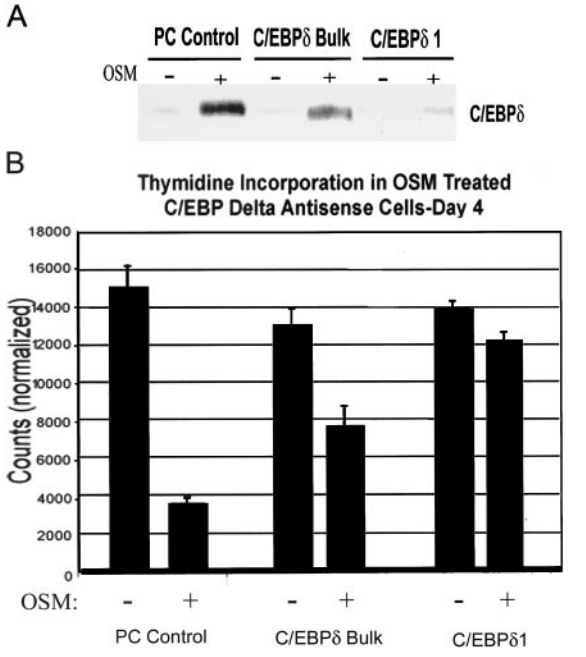

Fig. 8. C/EBPδ antisense cells have a smaller reduction in [^{3}H]thymidine incorporation in response to OSM. C/EBPδ antisense cells and pc_{con} cells (all HC11-derived) were grown in the presence of 50 ng/ml OSM for 4 days. *A,* proteins were harvested, and Western blots were probed with antibodies to C/EBPδ. *B,* [^{3}H]thymidine incorporation was measured in the pc_{con}, δ_{bulk}, and δ_1 antisense cell lines after 4 days with and without OSM. Data represent the average of three experiments with 3 replicates/condition/experiment. The [^{3}H]thymidine incorporation for both antisense cell lines after treatment with OSM was significantly different from the [^{3}H]thymidine incorporation for the control cells. The [^{3}H]thymidine incorporation for the OSM-treated δ_1 cells, which have the greatest reduction in C/EBPδ, was significantly greater than that for the δ_{bulk} cells ($P < 0.001$, ANOVA, Tukey-Kramer).

demonstrate that in MCF-7 human mammary carcinoma cells, the MAPK pathway is not involved in the growth arrest induced by OSM. In contrast, they show that the OSM-induced growth arrest of MDA-MB231 human mammary carcinoma cells does depend on MAPK. In HC11 cells, inhibition of the MAPK pathway using the specific MAPK inhibitor PD90859 does not affect the induction of C/EBPδ and does not interfere with the growth arrest response induced by OSM (data not shown).

We treated HC11 cells with other cytokines that have been reported to induce growth arrest of mammary epithelial cells. OSM and IFN-γ are the only two cytokines examined that induce Stat3 phosphorylation and increase C/EBPδ mRNA and protein in HC11 cells (Figs. 4 and 5). Subsequent evaluation showed that HC11 cells do not express the cytokine-specific components of the IL-6 and LIF receptors (data not shown), which explains their lack of response to these cytokines. Preliminary data from our laboratory demonstrate that IL-6 does induce Stat3 phosphorylation and C/EBPδ protein up-regulation in human breast cancer cell lines.[4] IFN-γ in-

[4] Sivko. Growth arrest and oncostatin M treatment activate Stat3 and increase C/EBPδ expression in mammary epithelial cells, manuscript in preparation.

Table 1 Cell cycle changes in C/EBPδ antisense cells treated with OSM for 4 days

Control (pc_{con}) and C/EBPδ antisense cells (δ_{bulk} and δ_1) were grown in the presence or absence of OSM and analyzed by flow cytometric analysis of propidium iodide-stained cells. Results represent the average of three independent experiments with 2–3 replicates/condition/experiment. The S-phase reduction for both antisense cell lines in response to OSM treatment was significantly less than that for the control cell line. The δ_{bulk} (pooled transfected antisense) cells had a greater reduction in S-phase cells than the δ_1 (clonal transfected antisense) cells. There was no statistically significant change in the number of S-phase δ_1 cells ($P = 0.01$, ANOVA, Tukey-Kramer).

Cell cycle phase	pc_{con}		δ_{bulk}		δ_1	
	No OSM	With OSM	No OSM	With OSM	No OSM	With OSM
G_0-G_1	59.7[a] (5.5)	72.5 (4.1)	62.6 (7.0)	64.4 (7.5)	59.5 (5.4)	57.0 (6.4)
S	26.2 (3.5)	13.3 (2.7)	22.3 (4.0)	18.8 (3.9)	21.5 (3.0)	23.7 (4.0)
G_2	11.0 (1.6)	8.2 (1.9)	10.6 (3.0)	10.6 (1.3)	11.2 (3.1)	13.5 (3.0)

[a] Values are percentages, with [±] SD shown in parentheses.

duces only a transient increase in phospho-Stat3 and C/EBPδ. IFN-γ also induces sustained Stat1 phosphorylation; however, this does not correspond to any changes in C/EBPδ mRNA levels. This observation is consistent with the report of Cantwell *et al.* (54), which demonstrates that phospho-Stat3, but not phospho-Stat1, binds to the acute phase response element of the mouse C/EBPδ promoter. C/EBPβ, which also regulates growth in the mouse mammary gland, is not influenced at the mRNA level by the addition of any of these cytokines (32, 33).

The specific JAK2 inhibitor AG490 reduces both Stat3 activation and C/EBPδ up-regulation in HC11 cells treated with OSM (Fig. 7), which is consistent with previous reports that demonstrate that OSM signals through the JAK family of non-receptor tyrosine kinases (55). These data also demonstrate that the increase in C/EBPδ occurs specifically in response to Stat3 phosphorylation and not other OSM-activated signaling pathways.

The [^{3}H]thymidine incorporation data in Fig. 8 and cell cycle analysis data in Table 1 demonstrate a role for C/EBPδ in the induction of growth arrest in response to OSM. The greater the reduction in the levels of C/EBPδ protein by the antisense construct, the less able the cells are to initiate a full growth arrest response to OSM. The dependence of the OSM-induced mammary epithelial cell growth arrest on the presence of C/EBPδ, reported here, is consistent with a recent report by Badache *et al.* (14), which demonstrates that IL-6-type cytokines induce growth arrest of T47D human mammary carcinoma cells by a Stat3-dependent mechanism. We propose that this dependence is due to Stat3 induction of C/EBPδ in these human cells.

C/EBPs inhibit cell proliferation via mechanisms that depend on their transcription factor activities and via protein-protein interactions with cell cycle-regulatory proteins. In IL-6-treated M1 leukemia cells, C/EBPs, including C/EBPδ, have a role in the transcriptional regulation of the growth arrest-associated gene *GADD45γ* (growth arrest and DNA damage; Ref. 56). In contrast, in hepatocytes, C/EBPα inhibits cell proliferation through direct binding to cyclin-dependent kinases 2 and 4 and blocking their association with cyclins (57). In adipocytes and granulocytes, C/EBPα binds to E2F and directly inhibits E2F-dependent transcription (58). Furthermore, dominant negative mutations in human C/EBPα are associated with acute myeloid leukemia, establishing a tumor suppressor function for C/EBPα (59). Interactions between C/EBPδ and cell cycle-regulatory proteins are currently under investigation in our laboratory.

In summary, the data presented here are significant on two levels. First, C/EBPδ has an important role in the induction of G_0 growth arrest in response to both OSM and serum and growth factor withdrawal of near confluent, mouse mammary epithelium (37, 38). Thus, C/EBPδ appears to be an important, common, downstream factor regulating the entry of mammary epithelial cells into G_0 in response to a variety of growth-inhibitory signals. This growth-regulatory role for C/EBPδ is supported by recent reports of dysregulation of C/EBPδ levels in mouse mammary carcinomas and in androgen-independent human prostate carcinomas (60, 61).

Second, the potential usefulness of OSM as a therapeutic agent for treating breast cancer is enhanced by knowledge of the signaling pathways responsible for growth arrest induction. Understanding the signaling pathways involved in determining whether OSM-treated cells proliferate or undergo growth arrest is important for evaluating when OSM may be therapeutically useful. Specifically, it may be important to evaluate the integrity of the Stat3-C/EBPδ pathway before using OSM to treat breast cancer.

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Molecular Cancer Therapeutics

Oncostatin M Induces Growth Arrest of Mammary Epithelium via a CCAAT/enhancer-binding Protein δ-dependent Pathway 1 Supported in part by NIH Grant RR00136CA (to J. A. H.) and NIH Grants CA 57607 and P30CA16058 from the National Cancer Institute (to J. W. D.). 1

Julie A. Hutt and James W. DeWille

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