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13. ABSTRACT *(Maximum 200*

Oncostatin M (OM), a T cell, and macrophages derived cytokine, has profound effects on malignant and normal mammary epithelial cells. In this study, we have investigated the growth inhibitory mechanism of OM on breast cancer cells and the receptor subtype involved in OM action. We show that OM inhibits the growth of a variety of breast cancer cell lines. Our data show that the growth inhibitory activity of OM is partially due to its functional antagonism of breast cancer mitogens including EGF, EGF-like growth factor, and bFGF. The proliferative activities of these growth factors were totally abolished by cotreatment of breast cancer cells with OM. Examination of proto oncogene expression demonstrated that OM down regulates the *c-myc* gene in growth-inhibited breast cancer cells. Interestingly, we also found that the transcription of p53 gene was inhibited by OM in breast cancer cells that contain the mutated p53 gene, but OM had no effect on p53 gene in cells contain the normal p53 gene. The biological effects reported herein are not shared by the OM-related cytokines IL-6, IL-11, or LIF. Our data suggest that the biological functions of OM in breast cancer is mediated predominantly through the OM-specific receptor, and activation of this receptor abrogates growth factors stimulation and down regulates the *c-myc* protooncogene.

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Jingwen Cui 9/8/98

PI - Signature Date

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A: INTRODUCTION

Human oncostatin M (OM), a product of activated T cells and macrophages, is a 28 kDa glycoprotein that regulates cell growth and differentiation. OM stimulates the growth of normal fibroblasts, normal vascular smooth muscle cells, and myeloma cells. OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including melanoma and lung carcinoma (1-7). The growth inhibitory or growth stimulatory activity exhibited by OM seems to depend on target cell type.

OM signal transduction occurs through two types of heterodimeric receptors (8-10). A shared receptor mediates both OM, leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1) signals and is composed of the LIF receptor α subunit and gp130 (9,11). Many overlapping functions of OM and LIF are mediated through this common receptor. However, we and others have demonstrated that a specific OM receptor exists in certain cell types including breast cancer cells. (9,10,12). Therefore, In the grant, we originally proposed to isolate and characterize this OM specific receptor and we predicted that the OM-specific receptor transduces exclusively OM signals and would be consist of gp130 as a binding subunit and a second subunit as an affinity conversion subunit. However, during our initial work to construct a cDNA library and to clone this second subunit, an abstract reporting the isolation of signaling subunit of OM receptor was published in the full of 1994 at the International Cytokine Society Meeting. Bruce Mosley at Immunex reported the cloning of this subunit (13). Therefore, we decided not to waste our resource to continue to clone this subunit, instead to start a collaboration with Immunex to characterize the expression of this newly identified molecule in breast cancer and in normal mammary epithelial cells, and to investigate the functional roles of OM-specific receptor versus the OM/LIF shared receptor in OM mediated growth inhibition, since currently, little is known about how the cellular growth response to OM is controlled at the receptor level, and the molecular mechanism(s) by which OM regulates cell growth remains largely uncharacterized. Unfortunately, although Immunex had agreed to provide the cDNA of OSMRβ for our transfection experiment in the beginning of 1995, their manuscript that describes the cDNA sequence and cloning procedure was delayed for publication until December 1996 (14). Therefore, we were unable to start the experiments as planed in our grant. Finally, we received the OSMRβ expression vector from Immunex in August 1997. Since then, we have spent a lot of time trying to establish an OSMRβ expression breast cancer cell line that does not express endogenous OSMRβ.

In the mean time, we have focused our investigations on understanding the biological functions of OM and its receptors in human breast malignancy from 5 different aspects. 1. To examine the growth inhibitory effects of OM on breast cancer cells and on normal mammary epithelial (HME) cells. 2. To examine the differential expression of OM specific receptors in malignant and normal breast epithelial cells. 3. To examine the effects of OM on proto oncogene, tumor suppressor gene, and breast cancer specific gene expressions. 4. To examine the signaling transduction pathways that lead to inhibition of tumor cell growth, and 5. To examine the role of endogenous OM, produced by the host defense system (T cells and macrophages) in breast cancer development by immunohistochemistry.

Although we have not directly examine the activity of OM specific receptor by transfection experiments, our studies are very fruitful and yield important information with regard

to understand the fundamental role of cytokine OM and its specific receptors in breast cancer cells.

B. MATERIALS AND METHODS

Cells and Reagents. The human breast cancer cell lines H3396, H3477, H3630, H3680B, H3730, H3914, H3922, and HBT3464 were isolated at Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle WA. Cell lines H3477, H3630, and H3680B were derived from primary solid tumors. H3396 was derived from malignant pleural effusion and H3914 and H3922 were derived from infiltrating ductal carcinoma. The MCF-7 and ZR-75-1 breast cancer cell lines were obtained from American Type Culture Collection (ATCC), Rockville, MD. The cells were cultured in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human recombinant OM was expressed by Chinese hamster ovary cells and purified as previously described (22). The other growth factors and cytokines were obtained from R&D Systems, Minneapolis, MN. The plasmid containing the cDNA probe for *c-Myc* was obtained from ATCC. A human p53 cDNA probe was provided by Dr. Linda Boxer at Palo Alto VA Medical Center, Palo Alto, CA. A human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) cDNA probe was generously provided by Dr. Jeff L. Ellsworth at CV Therapeutics, Palo Alto, CA.

Northern Blot Analysis

Total cellular RNA was isolated by the method of Peppel and Baglioni. Approximately 20 μg of each total RNA sample was separated on a 1.0% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane before crosslinking to the membrane. Prehybridization and hybridization steps were performed under the conditions previously described. The blot was hybridized at 60° C to ^{32}P-labeled DNA probe. The probe was labeled using 50 μCi [α-^{32}P] dCTP with random primed DNA labeling kit (Boehringer Mannheim Corp, Indianapolis, IN). The membrane was then washed 3 times at ambient temperature with 2X SSC, 0.1% SDS and twice at 37° C with 0.1X SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) for 1-3 days at -80° C. The autoradiographs were scanned by a laser densitometer (Personal Densitometer^{TM} SI, Molecular Dynamics, Sunnyvale, CA) and the integrated intensity of each band was analyzed with the program ImageQuaNT^{TM}, version 4.1. Densitometric analysis of autoradiographs in these studies as well as those discussed below included various exposure times to ensure linearity of signals.

Nuclear Run-on Analysis. These analyses were conducted using a procedure adapted from one that had already been described. Briefly, 1.8 x 10^7 adherent H3922 cells were harvested with cell scrapers into a minimal volume of cold phosphate buffered saline (PBS). The cells were pelleted by low-speed centrifugation and lysed with lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation and the lysis procedure was repeated once. The nuclei were recovered by centrifugation a second time and resuspended at 10^8 nuclei/ml in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The nuclei samples were immediately frozen under liquid nitrogen and stored at -80°C.

The frozen nuclei were subsequently thawed and 100 μl of each sample received 100 μl 2X reaction buffer (70% glycerol, 0.02 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.16 M KCl, 2 mM DTT, 0.2 mM EDTA, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 2.6 μCi/μl [^{32}P] rUTP). The

reactions were incubated, with shaking at 30°C for 30 minutes. Labeled nuclei were pelleted and resuspended with 100 µl DNase buffer (50% glycerol, 20 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 10 mg/ml RNase-free DNase I). The reactions were incubated with shaking at 30°C for 15 minutes. Samples were brought up to 125 µl with 7.5 µl 13.6 mg/ml proteinase K, 5 µl 10 mg/ml yeast tRNA, and 12.5 µl 10X SET buffer (5% SDS, 0.05 M EDTA, 0.01 M Tris-HCl, pH 7.4) and incubated at 42°C for 30 minutes.

Labeled RNA transcripts were extracted by adding the following: 275 µl GCSM solution [4 M guanidinium isothiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M b-mercaptoethanol], 45 µl 2.0 M sodium acetate, 450 µl water-saturated phenol, and 90 µl chloroform:isoamyl alcohol (49:1). The samples were vortexed and incubated on ice for 15 minutes. Nuclear run-on transcripts were precipitated with isopropanol and pelleted by high speed centrifugation. Extractions and isopropanol precipitations were repeated and the samples were dissolved with 102 µl TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). Assays for radioactivity were conducted by liquid scintillation. Approximately 2.0 x 10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham Life Sciences, Arlington Heights, IL) slot blot. Each blot received the following three plasmids: 5 µg plasmid with the human *GAPDH* cDNA insert, 3 µg of the 0.3 kb fragment of the *BCSG1* cDNA which is the 3' end of the cDNA, or 3 ug of p53 cDNA.

Actinomycin D/mRNA Stability Analysis. H3922 cells in 100 mm tissue culture plates were incubated with or without OM for 6 h. Actinomycin D (5 µg/ml) was added to cells for different lengths of time. At the end of each time point, total RNA was harvested as described above under "Northern Blot Analysis". Electrophoresis of total RNA samples, blotting, and hybridization to radiolabeled probes were also carried out as described above.

Cell Proliferation Assay

Cells were seeded in 96-well tissue culture plates (Costar, Cambridge, MA) in IMDM medium containing 2% FBS at a density of 3000 cells/well in 100 µl of medium. Three to 5 hours after seeding, 50 µl of the same culture media containing various factors was added to each well. Three days later [^{3}H]thymidine (0.5 µCi/50 µl/well) in medium was added to the culture plates 4 hours prior to harvest. The amount of [^{3}H]thymidine incorporated into cells was measured using a liquid scintillation counter (Pharmacia, Piscataway, NJ). The differences in counts/min incorporated between experimental and control cultures were used as an index for DNA synthesis. Each data point represents the average of triplicate cultures and each experiment was performed at least 3 times.

Immunoprecipitation and Immunoblotting. H3922 cells were cultured in 60 mm culture plates for 2 days in 2% FBS IMDM with or without OM (100 ng/ml). The cells were then stimulated with 10 ng/ml of EGF for 10 minutes, or 100 ng/ml of OM for 15 minutes. Cells were rinsed with cold PBS and lysed with 0.5 ml of lysis buffer (50 mM Tris [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF, 5 µg/ml of aprotinin, 1 µg/ml of leupeptin, and 1.25 µg/ml of pepstatin). The cell lysate (250 µl, approximately 1µg/µl of protein) was precleared with 50 µl of protein A-sepharose slurry and then incubated overnight with 30 µl of sepharose-conjugated

anti-phosphotyrosine monoclonal antibody 4G10. The sepharose beads were collected by microcentrifugation and resuspended in 50 μl of 2X SDS sample buffer containing 5% β-mercaptoethanol. Immunoprecipitates were analyzed by western blot with anti-phosphotyrosine monoclonal antibody 4G10 using an enhanced chemiluminescence (ECL) detection system.

C. RESULTS AND DISSCUSSION

1. OM INHIBITS CELLULAR PROLIFERATION OF A HIGH PROPORTION OF BREAST CANCER CELLS

Although OM was shown to inhibit the growth of a variety of tumor cells such as melanoma and lung carcinoma cells, its antitumor activity in malignant human breast carcinoma has not been extensively examined. To explore the potential clinical application of cytokines in the treatment of breast cancer, we examined systematically the activity of OM on the cellular proliferation of 9 different breast cancer cell lines. As summarized in Figure 1, among the 9 cell lines, DNA synthesis of four cell lines (H3922, H3680B, H3396, and H3630) was inhibited by OM to 70 to 95 % of that of control cells in culture for three days. The growth of cell lines (MCF-7, ZR-75-1, and H3730) was moderately inhibited to 40 to 60% of untreated cells. In comparison, OM had no effect on the DNA synthesis of two breast tumor cell lines (HBT3464 and H3914).

Breast cancer cells have different dependence on steroids for their growth. Among the cell lines tested, the growth of H3922, H3680B, MCF-7, and ZR-75-1 in phenol red free medium containing steroid depleted serum was decreased to more than 50% compared with cells cultured in regular medium, whereas the cellular proliferation of H3630 and H3730 cells was not affected significantly by the absence of steroids. However, the growth inhibitory effect of OM on all of these cells was observed in the presence or absence of steroid in the culture medium. This suggests that the estrogen receptor may not be involved in the inhibitory activity of OM in breast cancer cells.

2. THE RESPONSES OF BREAST CANCER CELLS TO OM TREATMENT ARE CORRELATED WITH THE EXPRESSION STATUS OF OM HIGH-AFFINITY RECEPTORS.

In a attempt to understand why OM affects the growth of some breast cancer cells but not the others, radioligand receptor binding assays were conducted to detect the presence of high-affinity OM receptors in different breast cancer cell lines. As shown in Table 1, high-affinity binding sites for OM were detected in all the 7 cell lines whose cellular proliferation was inhibited by OM. Scatchard analysis of the binding data revealed a single class of high-affinity receptors present in these cells. The affinity of these receptors for OM range from 300 to 500 pM in these cells. Apparently, H3922 cells have more receptors than other cell lines. In contrast, specific binding of ^{125}I-OM to H3477 and H3914 cells was not detected. This correlated with the lack of response of these two cell lines to OM treatment. These data suggest that the growth inhibitory activity of OM is mediated by the OM high-affinity receptor. The presence of this high-affinity receptor on breast cancer cells is necessary for OM to exert its biological function.

3. OM ANTAGONIZES EGF MITOGENIC ACTIVITY. To study potential mechanisms of growth inhibition, we investigated whether OM inhibited the mitogenic activity of a variety of epithelial cell mitogens. Initial studies focused on EGF, as EGF has been shown to stimulate the growth of normal and malignant mammary epithelial cells, and reports from several groups have suggested that the EGF ligand/receptor system may play an important role in the proliferation of breast cancer cells and in tumor development. Treatment of H3922 for 3 days with EGF stimulated increased DNA synthesis with an EC_{50} of approximately 80 pg/ml (Figure

2A). Maximal increases of 2 to 4-fold in DNA synthesis were routinely obtained at concentrations of 1 ng/ml and higher. To examine whether OM can antagonize the proliferative activity of EGF H3922 cells were stimulated with 1 ng/ml of EGF in the presence or absence of OM. As shown in Figure 2B, OM blocked EGF induced DNA synthesis in a concentration-dependent manner. The increased proliferative activity with the addition of EGF was antagonized completely by OM at a concentration of 20 ng/ml and higher.

4. SUPPRESSION OF C-MYC GENE EXPRESSION. We next examined the regulation of *c-myc* gene expression by OM, as this gene product plays a central role in the regulation of cell growth and differentiation. Much evidence links *c-myc* gene expression with cell proliferation, and reduction of its expression correlates with cellular differentiation. Further, over expression of the *c-myc* gene has been found in 15-40% of the breast tumors tested. H3922 cells were cultured in the presence or absence of OM for 3 days prior to stimulation with EGF for 4 hours. The mRNAs isolated from these cells were analyzed by northern blot for the presence of the *c-myc* gene transcript as well as another cell growth-related gene, *c-jun*. As shown in Figure 3, the basal expression of *c-myc* in OM treated cells was reduced to approximately 15 to 20% of that observed in control cells. EGF stimulation increased *c-myc* mRNA level to approximately 180% of controls. This increased *c-myc* expression was completely abolished in EGF stimulated cells that had been pretreated with OM. In contrast to the changes seen in *c-myc* mRNA expression, the basal expression and the EGF-stimulated expression of *c-jun* was not suppressed at all in OM treated cells. In fact, the mRNA level of *c-jun* was increased in OM treated cells to approximately 166% of control. These data suggest that the *c-myc* gene transcription is regulated selectively by OM. The results clearly showed that OM not only suppresses the constitutive expression but also antagonizes mitogen stimulated expression of the *c-myc* gene.

5. COMPARISON OF OM ACTIVITY WITH RELATED CYTOKINES. The biological activities of OM can be mediated by two related receptors that bind OM. One receptor as been found to be OM-specific. A second, more promiscuous receptor has been shown also to bind LIF and more recently CT-1. In addition to OM sharing a receptor with related cytokines, many biological responses of OM are common to LIF, IL-6, and IL-11. These effects can be exerted by either the same receptor or related receptors each containing a common signal transduction unit, gp130. To determine which receptor complex is responsible for transmission of the OM anti-growth signal in breast cancer cells, we performed competition binding assays in H3922 cells. The result showed that [^{125}I]OM binding to H3922 cells was displaced by unlabeled OM, but not by unlabeled LIF (Figure 4). This suggested a direct binding to the OM-specific receptor. We therefore tested the growth regulatory activity of LIF and IL-6. LIF and IL-6 did not inhibit DNA synthesis of the cells in the presence or the absence of exogenous EGF, suggesting that these cytokines do not repress proliferation of the malignant epithelial cells driven either by serum or by EGF. In fact, cell growth was slightly stimulated by these factors. Consistent with the different effect of these cytokines on cell growth, northern blot analysis showed that LIF, IL-6, and IL-11 did not significantly decrease the level of *c-myc* mRNA in H3922 cells (Figure 5). These results suggest that the OM growth inhibitory activity in breast cancer cells is a function of this cytokine that is not shared with related cytokines, and that the activity is mediated predominantly through the OM-specific receptor, not the shared LIF/OM/CT-1 receptor.

6. EFFECTS OF OM ON NORMAL HUMAN MAMMARY EPITHELIAL CELLS.

To investigate whether OM has a similar effect in normal human mammary epithelium (HME), growth assays were conducted in normal primary HME cells derived from four donors. OM produced a dose-dependent inhibition of DNA synthesis in these cells (cultured in mammary epithelium growth medium) (Figure 6). In contrast, LIF had no effect on HME cell proliferation. Flow cytometry analysis using antigp130mAb and antiOMRβmAbs (the monoclonal antibodies against the second subunit of OM specific receptor, provided by Immunex) demonstrated that these cells express both receptor subunits, but the OMRβ is expressed at higher level than found in breast cancer cells. We then examined the mRNA levels of each OM receptor subunit including gp130, OMRβ and LIFR in different breast cancer cell lines and in HME cells. The quantitative PCR analysis demonstrates that the mRNA level of OMRβ is much higher in HME cells compared with breast cancer cells. The LIF receptor messenger is expressed at a lower level in both normal and malignant mammary epithelial cells compared with the levels of OMRβ mRNA (Table II).

7. TRANSCRIPTIONAL REGULATION OF BREAST CANCER SPECIFIC GENE (BCSG1) BY ONCOSTATIN M

Northern blot analysis of BCSG1 expression in human breast cancer cell lines.

By utilizing a high-throughput direct-differential cDNA sequencing approach, a novel breast cancer specific gene, designated *BCSG1* was recently isolated from a breast tumor cDNA library (15). *BCSG1* gene is transcripted into a 1 kb mRNA, and the open reading frame of the full length gene is predicted to encode a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with genetic data base reveals that *BCSG1* is highly homologous to the non-Aβ fragment of human AD amyloid protein with 54% sequence identity (16).

BCSG1 mRNA expression was found exclusively in neoplastic epithelial cells. In situ hybridization analysis has demonstrated a stage-specific expression pattern of *BCSG1* mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade in situ breast carcinoma, to high expression in advanced infiltrating carcinomas. This implies that *BCSG1* may play a role in breast cancer malignant progression. The high level expression of BCSG1 in the neoplastic breast epithelial cells suggest that the expression of BCSG1 may be up-regulated in the mammary gland during breast cancer onset and progression. Therefore, we initiate a study to examine BCSG1 expression in breast cancer cell lines. As shown in Figure 7, among the 8 different cell lines, BCSG1 mRNA was detected in 2/4 human breast cancer cell lines derived from pleural effusion and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas (ZR-75-1, MDA-MB-231, H3914, and H3922). In contrast, no hybridization signals were detected from mRNAs isolated from 4 normal breast tissues.

Dose- and time-dependence of OM-induced suppression of *BCSG1* mRNA expression in breast cancer cells.

Among the cell lines that were examined, H3922 expressed the highest level of *BCSG1* mRNA. Since OM has an inhibitory and differentiative effect on H3922 cell growth, we examined the effect of OM on *BCSG1* mRNA expression. The results in Figure 8 demonstrated a dramatic time-dependent suppression of *BCSG1* mRNA level by OM. Treatment of H3922 cells with OM

initiated an immediate decrease of *BCSG1* mRNA as early as 30 minutes. By 4 h treatment, the level of *BCSG1* mRNA was decreased to 70% of that in control, and by 24 h, the mRNA was completely undetectable.

We next investigated the dose-dependent effect of OM on *BCSG1* gene expression. H3922 cells were treated with OM for 6 h at different concentrations before the total RNA was harvested for northern blot analysis of *BCSG1* mRNA. Densitometry analysis of *BCSG1* hybridization signal with normalization to *GADPH* signal showed that cells treated with 0.2, 1.0, 5.0 , 25, and 125 ng/ml OM expressed 58%, 46%, 47%, 45%, and 41% as much *BCSG1* mRNA, respectively, as that observed in control cells treated with OM dilution buffer (1 mg/ml BSA in PBS). This data suggested that OM produced a maximum suppressive effect on *BCSG1* expression at a concentration of 1-5 ng/ml with an Ec50 of 0.08 ng/ml.

Transcriptional regulation of *BCSG1* expression by oncostatin M.

To determine whether the down regulation of *BCSG1* expression by OM occurs at transcriptional or post-transcriptional levels, we conducted nuclear run-on assays to measure the relative transcription rate of *BCSG1* in control cells and in the cells treated with OM. As shown in Figure 9, upon the pretreatment with OM for 16 h, H3922 cells contained only 28.5% as many active *BCSG1* mRNA transcripts as that observed in control cells. Data were normalized by the signals observed in the *GADPH* slots. The level of reduction of BCSG1 transcripts is consistent with the results obtained from northern blot. These results suggest that transcriptional regulation is the major component of the observed OM-mediated suppression of *BCSG1* gene expression.

8. OM REGULATES P53 GENE TRANSCRIPTION

H3922, a breast cancer cell line developed from a human infiltrating ductal carcinoma, is most sensitive to OM treatment. In an attempt to understand the growth inhibitory mechanism, we examined the tumor suppressor gene p53 protein expression, as p53 protein plays a critical role in cell proliferation by controlling cell cycle progression, and the p53 gene is frequently mutated in human cancers (17). The expression of p53 protein in H3922 cells was detected by immunohistochemistry. Interestingly, western blot analysis showed that the expression level of p53 protein was significantly decreased in OM-treated cells as compared with untreated cells. Northern blot analysis showed that p53 mRNA level was suppressed to a similar degree as the p53 protein in OM treated cells. Further studies by nuclear run-on assay show that the transcription of p53 gene was inhibited by OM.

To determine whether the p53 gene in these cancerous cells harbors mutations, genomic DNA was isolated and PCR reactions were carried out to isolate exons 2-11 of p53 gene. The PCR products then were subcloned into a TA cloning vector and sequenced. The sequence analysis showed that exon 5 carries a GTC to TTC transition. This missence mutation results in a change at codon 157 from amino acid valine to phenylalanine. The codon 157 resides in the sequence specific DNA binding domain of p53 and is located within one of the mutational hotspots (codon 151-164). The OM mediated down regulation of p53 protein and mRNA were not observed in MCF-7 and ZR75-1 cells. These cells contain normal p53 gene. At present, it is not clear whether OM only inhibits the transcription of the mutated p53 gene. The mechanism underlines the OM effect on p53 gene transcription is currently under investigation.

9. SUPPRESSION OF EGF-MEDIATED TYROSINE PHOSPHORYLATION BY PRETREATMENT OF BREAST CANCER CELLS WITH ONCOSTATIN M

Previously, we have shown that stimulation of H3922 cells with EGF increases cellular proliferation to 3-4-fold higher than that observed in untreated cells. Simultaneous treatment of H3922 cells with OM causes a time- and dose-dependent antagonistic effect on EGF-stimulated growth. In order to investigate the impact of OM on EGF-mediated signal transduction, we examined EGF-induced tyrosine phosphorylation events in H3922 cells that had been pretreated with OM for different lengths of times. Tyrosine phosphorylated proteins were immunoprecipitated from total cell lysates derived from H3922 cells treated under various conditions. Subsequent western blot analysis demonstrated that EGF stimulation transciently induces tyrosine phosphorylation of several cellular proteins including the EGF receptors (EGFR). Pretreatment of H3922 cells with OM for 6 to 48 hours prior to EGF stimulation did not affect tyrosine phosphorylation. However, 72 hours pretreatment of cells with OM severely diminished the tyrosine phosphorylation events induced by EGF (Figure 10). We also observed that the maximal inhibitory effect of OM on H3922 cell proliferation occurs after three days treatment. We are currently investigating the mechanism by which OM affects the cellular tyrosine phosphorylation event and the correlation between cell proliferation and tyrosine phosphorylation.

10. Detections of endogenous expressed OM in tumor tissue section.

One characteristic of the host response to tumor growth is the infiltration of tumors by macrophages and T lymphocytes. Production of tumor-inhibitory cytokines in a timely and locally released fashion may represent an important function of the host defense system to suppress tumor progression. From this prospective view, the inhibition of breast tumor growth by OM, a cytokine predominantly produced by activated T cells and macrophages may represent the tumor-host interaction. To examine this hypothesis, we have initiated a study to examine the OM expression in breast tumors by inflamatory cells. By utilizing anti-OM monoclonal antibodies, we examined OM expression in tissue sections derived from breast cancer and ovaian cancers. The preliminary results showed infiltrating macrophages and neutraphiles in breast tumors were positive with anti-OM mAbs. This immunostaining signals were blocked with addition of recombinant human OM in the incubation medium. Interestingly, high levels of anti-OM immunoreactivity were detected in mucinous epithelial cells in ovarian tumors. The expression of OM is associated with the low malignant potential of this type of ovarian tumor. Whether the high level expression of OM in ovarian tumors plays a functional role in mucinous epithelial cells transformation need to be further investigated. We currently in the process to conduct in situ hybridization to examine the mRNA expression of OM in breast and ovarian tumor sections.

D. CONCLUSION

The results described in this final report demonstrate that

1) OM, a cytokine produced by activated T cells and macrophages, has strong anti-proliferative activity against a broad spectrum of breast tumor cells. The presence of specific high-affinity OM receptors on breast cancer cells is essential for OM to exert its biological function.

2) OM has a inhibitory and different activities in both normal and malignant mammary epithelial cells.

3) The inhibitory mechanism in breast cancer cells involve antagonism to a variety of breast cancer mitogenes and down regulation of c-myc protooncogene. In addition, In OM treated cells, EGF no longer is able to induce the EGF receptor intrinsic tyrosine kinase activity. This may account for at least in part the mechanism by which OM antagonizes the EGF mitogenic activity.

4) OM activities can not be mimicked by LIF and other related cytokines. That suggest the OM-specific receptor not the OM/LIF shared receptor transduces the OM signal in normal and malignant mammary epithelial cells.

5) The transcription of a mutated p53 gene, but not the normal p53 gene, is inhibited by OM.

6) BCSG1 gene expression is associated with breast cancer development and progression.

7) BCSG1 is transcriptional suppressed by OM in a breast cancer cell line which is growth-inhibited by OM. That suggests BCSG1 expression may be involved in the process of OM-mediated growth inhibition.

8) The expression of OM by inflammatory cells that were infiltrating breast tumors suggest that OM may plays a functional role in vivo as a part of body defense system to inhibit tumor growth and progression.

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8. **Liu, J.**, Spence MJ, Zhang YL, Jia T., Shi E: Transcriptional suppression of the breast cancer specific gene (*BCSG1*) expression by the cytokine oncostatin M. Submitted to *Cancer Research.*
9. Ryan, R.E., **Liu, J.**, Wang, M., Sahin, A., Ma, Y., Streiff, R., and Vestal, R.E. Localization of oncostatin M in solid tumors by immunohistochemistry and in situ hybridization. Submitted to *Cancer.*
10. Spence MJ, Vestal RE, Ma Y, and **Liu J**: Suppression of EGF-mediated tyrosine phosphorylation in breast cancer cells by oncostatin M. Submitted to *Cytokines*
11. **Liu, J.**, Ahlborn, T., Spence, M.J., Lin, L., and Meng, L. Regulation of p53 gene transcription in breast cancer cells by oncostatin M. Manuscript in preparation.

G. AWARD

One of the two recipients for **Young Investigator Award**, International Cytokine Society, October 1996

H. A list of personnel receiving pay from this effort: Jingwen Liu

I. FIGURE LEGENDS

Figure 1. Effect of OM on proliferation of breast cancer cells. Breast cancer cells (1 x 10^3 cells/well) were incubated for 3 days in IMDM containing 2% FBS with indicated amount of purified human recombinant OM. Cells were then pulsed with [^{3}H]thymidine for 4 hours. The amount of radioactivity incorporated into cells was determined, and the data were expressed as the percentage of radioactivity incorporated compared to untreated cells.

Figure 2. OM antagonized EGF proliferative activity. H3922 cells (1 x 10^3 cells/well) were incubated for 3 days in IMDM containing 2% FBS supplemented with various amounts of purified human recombinant EGF (A), or with 0.6 ng/ml EGF plus the indicated amount of OM (B). Cells were pulsed with [^{3}H]thymidine for an additional 4 hours. The amount of radioactivity incorporated into cells was determined, and the data expressed as the percentage of radioactivity compared to cells that were not treated with factors.

Figure 3. Down regulation of the *c-myc* mRNA by OM in breast cancer cells. H3922 cells were cultured in the presence or absence of OM for 3 days. Then the cells were stimulated with EGF (10 ng/ml) for 4 hours before harvesting. Total RNAs were isolated from these cells and 20 μg/lane was analyzed by northern blot for the presence of the *c-myc* gene and *c-jun* gene transcript. The RNA blot was stripped and reprobed for *GADPH* subsequently to ensure the equal loading. The intensity of hybridization signal was quantitated by a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Lane 1, control; lane 2, OM for 3 days; lane 3, EGF for 4 h; lane 4, OM for 3 days plus EGF for 4 h.

Figure 4. Competition binding assays of ^{125}I-OM and ^{125}I-LIF. Cells were incubated with 2 nM of [^{125}I]OM or with 1 nM of [^{125}I]LIF in the presence of increasing concentrations of unlabeled OM, or unlabeled LIF. The total binding was determined as described in Table I.

Figure 5. Comparison of effects of OM related cytokines on *c-myc* gene transcription. Cells were incubated in 2% FBS IMDM containing the indicated cytokines (100 ng/ml) for 2 days, then stimulated with EGF (10 ng/ml) for 4 hours before harvesting. Total RNAs (20 μg/lane) were analyzed for *c-myc* mRNA by northern blot. Lane 1, EGF alone; Lane 2, EGF plus OM; Lane 3, EGF plus IL-6; Lane 4, EGF plus IL-11; Lane 5, EGF plus LIF.

Figure 6. Effect of OM on proliferation of HME cells. (A) DNA synthesis. HME cells (1.2 x 10^4 cells/well) were incubated for 3 days in MEGM with indicated amount of purified human recombinant OM. Cells were then pulsed with [^{3}H]thymidine for 4 hours. The amount of radioactivity incorporated into cells was determined by TCA precipitation method, and the data are expressed as the percentage of radioactivity incorporated compared to untreated cells. (B) Cell number count. HME cells were cultured in 4-well culture plates at a density of 1.7x10^5 cells/well in 5 ml MEGM supplemented with 100 ng/ml of OM. Three days later, cells were trypsinzed and trypan blue excluding cells were counted. Values are mean ±SD.

Figure 7. Northen blot analysis of BCSG1 expression in human breast cancer cell lines. Total RNA was isolated and analyzed by northern blot. Lane 1, H3396 (derived from pleural effusion); Lane 2, MCF-7 (derived from pleural effusion); Lane 3, SKBR-3 (pleural effusion); lane 4, MDAMB-231 (pleural effusion); Lane 5, H3914 (infiltrating ductal carcinoma); lane 6, H3922 (infiltrating ductal carcinoma); Lane 7, ZR-75-1 (infiltrating ductal carcinoma); Lane 8, T47D (infiltrating ductal carcinoma).

Figure 8. Time-dependent suppression of *BCSG1* mRNA expression by OM. Total RNA (20 μg/lane) was isolated from H3922 cells that were cultured in 2% FBS IMEM and treated with OM at a dose of 20 ng/ml for the indicated lengths of time. RNA samples were blotted onto a nylon membrane and hybridized to a ^{32}P-labeled 0.55 Kb *BCSG1* cDNA probe as described in "Materials and Methods". The blot was rehybridized under the same conditions with a ^{32}P-labeled human *GAPDH* probe. Radioactive signals were detected by autoradiography and quantified by densitometry.

Figure 9. Nuclear runon analysis of *BCSG1* transcription. Two slots were blotted onto each of two nylon membrane strips. One slot received 3 μg of the 0.3 kb fragment of the *BCSG1* cDNA which is the 3' end of the cDNA. This fragment was generated by cutting the *BCSG1* cDNA with the restriction endonuclease BstX1. The second slot was loaded with 5 μg of the *GAPDH* plasmid. One nylon strip was hybridized to a ^{32}P-radiolabeled nuclear runon reaction prepared from 16-h OM-treated H3922 cells. The second was hybridized to a labeled nuclear runon reaction prepared from control cells. Equal amount of radioactivity was used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis. Isolation of nuclei, preparation of nuclear runon reactions, hybridizations and washes were all as described in "Materials and Methods".

Figure 10. Oncostatin M has an antagonistic effect on EGF-mediated tyrosine phosphorylation of signaling proteins in H3922 cells. Tyrosine phosphorylated proteins immunoprecipitated from various H3922 cell extracts were detected on a western blot from an SDS-PAGE gel by enhanced chemiluminescence. Lane 1, untreated control cells; Lane 2, OM stimulated ten minutes; Lane 3, EGF stimulated ten minutes; Lane 4, both OM and EGF stimulated ten minutes; Lane 5, OM pretreated 72 hours, followed by EGF stimulation ten minutes. Ten-minute stimulation of H3922 cells with EGF induced tyrosine phosphorylation of many proteins (EGF 10′). Preteatment of H3922 cells with OM for 72 hours prior to stimulation with EGF (OM72h./EGF10′), reduced the number of detectable tyrosine phoshphorylated signals nearly to that observed in the control lane (C). These data demonstrated a clear antagonistic effect of OM on EGF-activated signaling components in H3922 cells.

Table I. Correlation of growth inhibitory effect of OM and [^{125}I]OM binding

Cells	Growth Response	[^{125}I]oncostatin M binding Affinity (Kd)	Sites/cell
H3396	inhibited	498 ± 65 pM	1,150 ± 103
H3477	none	ND	ND
H3630	inhibited	448 ± 49 pM	1,281 ± 115
H3680B	inhibited	306 ± 34 pM	1,489 ± 119
H3730	inhibited	826 ± 256 pM	3,140 ± 565
H3914	none	ND	ND
H3922	inhibited	582 ± 69 pM	10617 ± 849
HBT3464	none	ND	ND
MCF-7	inhibited	505 ± 107 pM	2,354 ± 322
ZR-75-1	inhibited	491 ± 135 pM	1,681 ± 316

Cells (1x10^5 cells/well) cultured in 48-well tissue culture plates were incubated with increasing concentrations of radiolabeled [^{125}I]OM as described in Materials and Methods. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled ligand. Specific binding was determined by subtraction of nonspecific binding from total binding. Scatchard analysis of the binding data was conducted by using Ligand, version 4. ND, not detected.

Table II

Analysis of OM receptor subunits mRNA by Quantitative PCR

Template	OMRβ	gp130	LIFR	DHFR	OM activity
H3922	639	471	68	500	Inhibition
H3630	186	542	3	500	Inhibition
H3396	205	246	37	500	Inhibition
H3477	---	280	---	500	No Response
H3914	---	---	---	500	No Response
HME	1154	1248	194	500	Inhibition
HME + OM	1711	990	81	500	Inhibition

A first strand cDNA synthesized from total RNA isolated from each cell line was used as a template for PCR amplification.

Data shown are thousands of pixels normalized for template efficiency. DHFR values indicate relative signal strength.

J. FIGURES

Figure 1

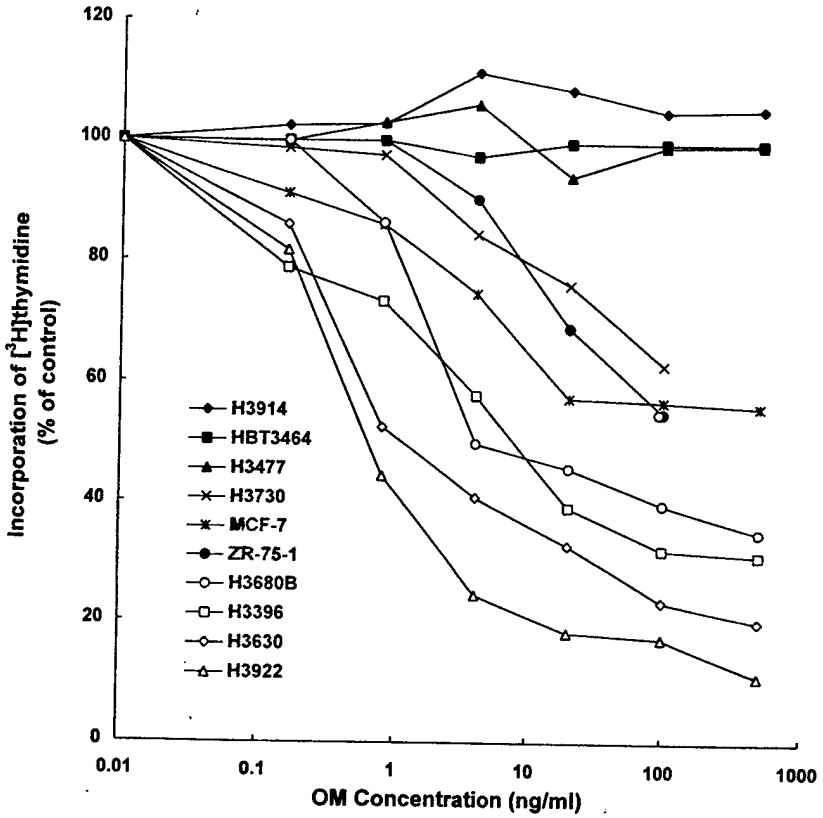
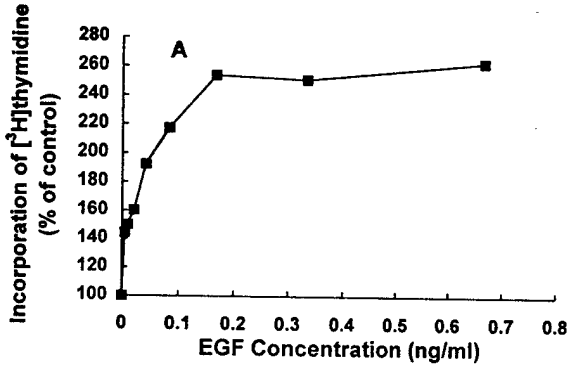


Figure 2



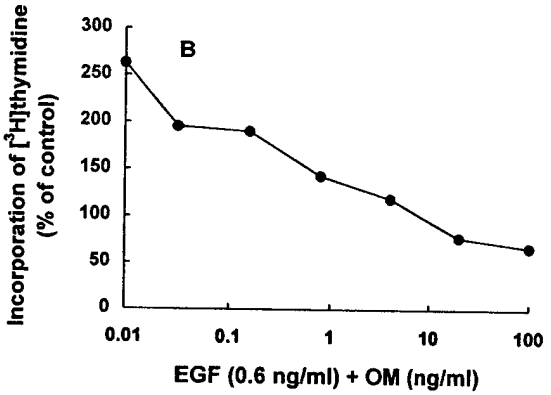


Figure 3

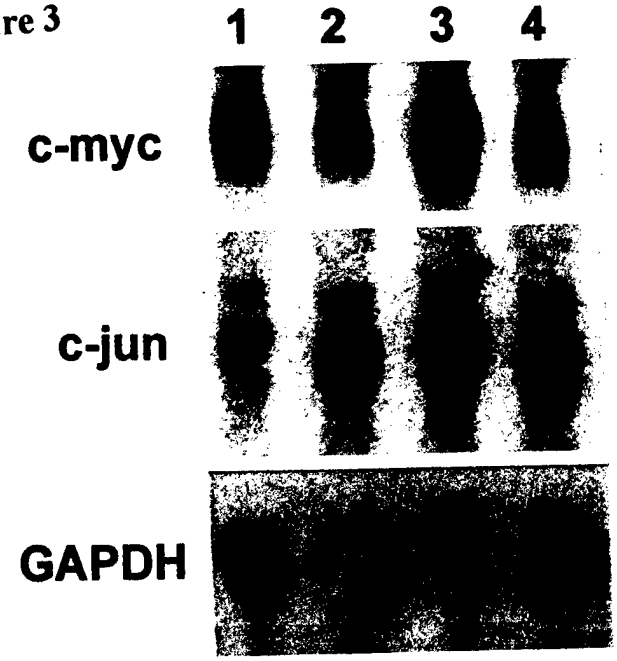
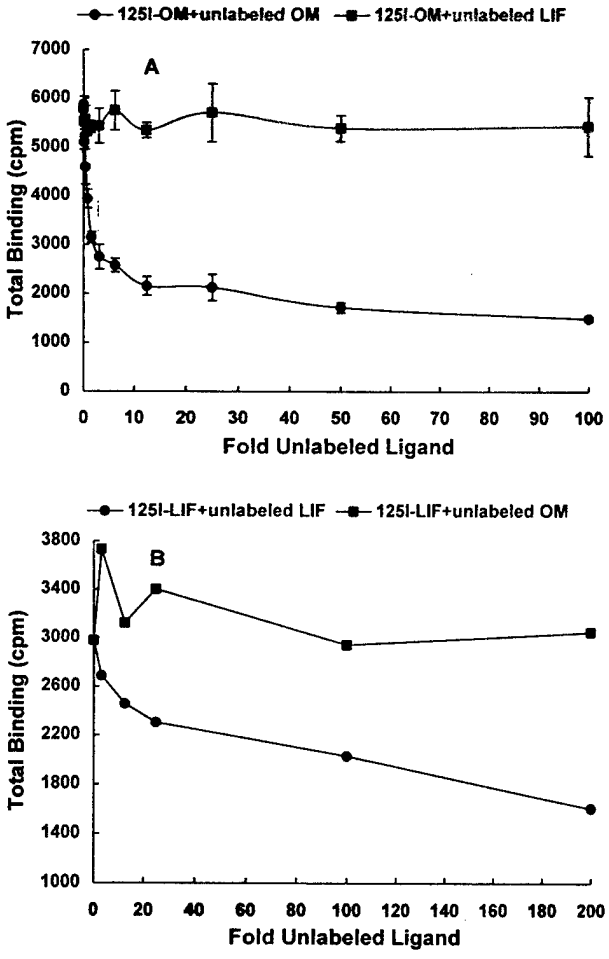


Figure 4



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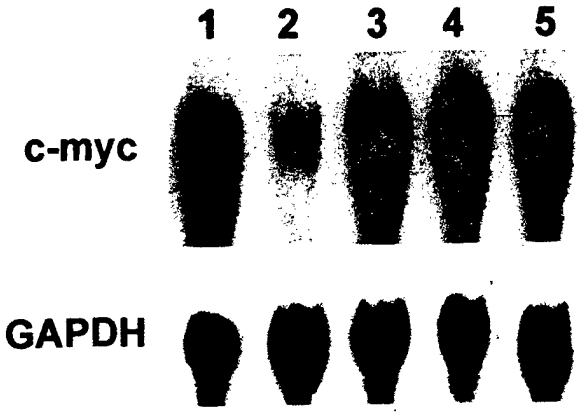

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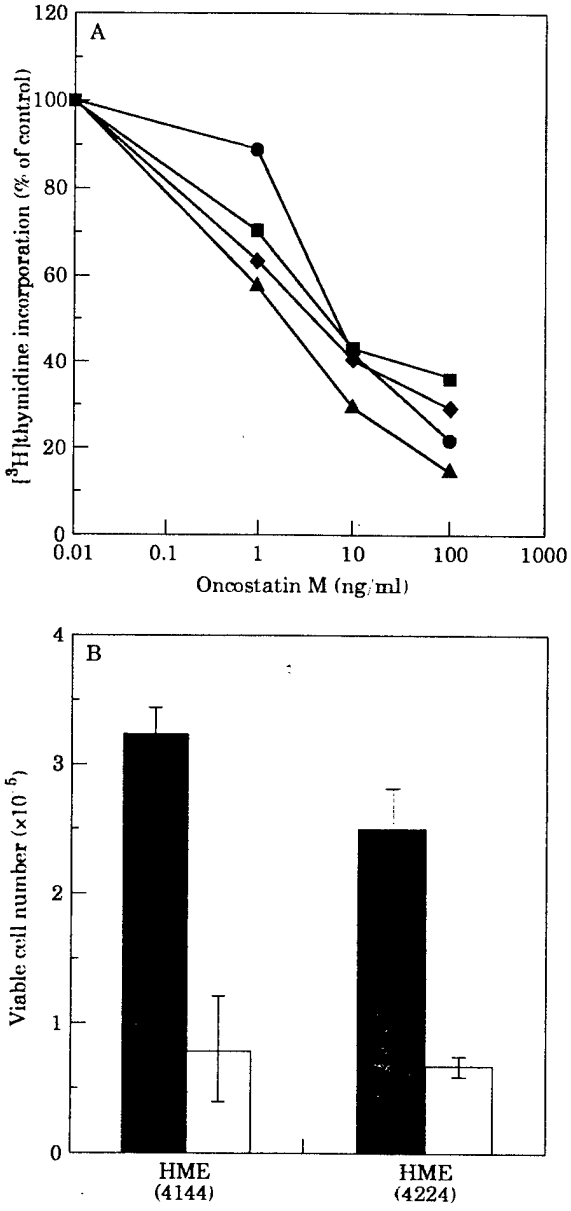

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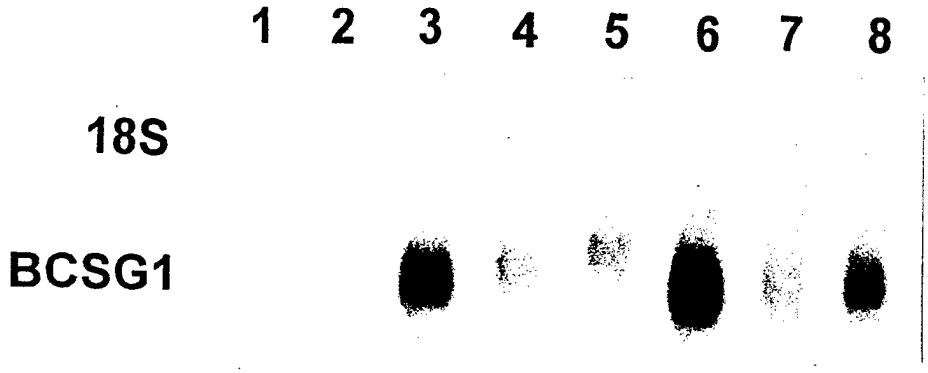

Figure 8

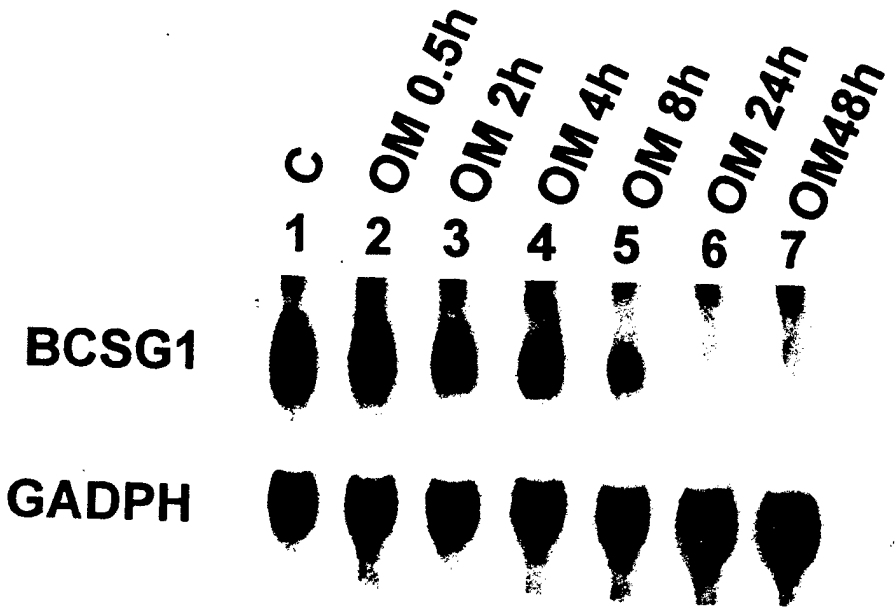

Figure 9

Figure 10