RESEARCH ARTICLE

Inhibition of Growth and Induction of Differentiation of SMMC-7721 Human Hepatocellular Carcinoma Cells by Oncostatin M

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Abstract

Oncostatin M (OSM) is a multifunctional cellular regulator acting on a wide variety of cells, which has potential roles in the regulation of gene activation, cell survival, proliferation and differentiation. Previous studies have shown that OSM can induce morphological and/or functional differentiation and maturation of many tumor cells. However, the action of OSM on the induction of differentiation of human hepatocellular carcinoma (HCC) has not been reported. Here, we investigated the effects of different concentrations of OSM on human HCC cell line SMMC-7721 growth, proliferation, cell cycling, apoptosis and differentiation *in vitro*. Cell growth was determined via MTT assay, proliferation by cell cycle analysis, apoptosis by flow cytometry, morphology by transmission electronic microscopy, and cell function by detection of biochemical markers. Our results demonstrated that OSM strongly inhibited the growth of SMMC-7721 cells in a dose-dependent manner, associated with decreased clonogenicity. Cell cycle analysis revealed a decreased proportion of cells in S phase, with arrest at G0/G1. The apotosis rate was increased after OSM treatment compared to the control. These changes were associated with striking changes in cellular morphology, toward a more mature hepatic phenotype, accompanied by significant reduction of the expression of AFP and specific activity of γ-GT, with remarkable increase in secretion of albumin and ALP activity. Taken together, our findings indicate that OSM could induce the differentiation and reduce cell viability of SMMC-7721 cells, suggesting that differentiation therapy with OSM offers the opportunity for therapeutic intervention in HCC.

Keywords: OSM - HCC - proliferation - apoptosis - differentiation

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Introduction

Human hepatocellular carcinoma (HCC) is one of the most common malignancies all around the world. It occurs with great frequency and is becoming more common as a complication of chronic Hepatitis C (Guan 2006; Zekri et al., 2012). Until now, many therapeutic approaches have been applied clinically such as surgery, interventional or micro-traumatic techniques, physical or chemical methods. But the high incidence of tumor recurrences, possibly from micrometastasis of tumor cells prior to curative surgery, further reduces patients' 5-year survival. And this cancer seems to be stubborn as it is resistant to any chemical agents developed until now and chemical therapies used to treat cancer are highly toxic and often nonspecific.

A number of studies have shown that HCC could be induced to differentiate by some substances such as Ginsenoside Rh2 (Zeng and Tu, 2003), sodium 4-phenylbutanoate (Wang et al., 2008), TGF (Damdinsuren et al., 2006), trichostatin A (Yamashita et al., 2003), among others. Up to now, searching for non-toxic and natural origin substances that induced the differentiation of cancer cells is a key for anticancer therapy.

Oncostatin M (OSM), a glycoprotein monomer of 28,000 Da, was originally isolated and purified from the conditioned media of phorbol 12-myristate 13-acetate (PMA)-stimulated human histiocytic lymphoma U937 cells by Zarling et al and was named by its activity to inhibit the proliferation of A375 melanoma cells (Tanaka and Miyajima, 2003). OSM is a multifunctional cellular regulator and can act on a wide variety of cells, which has potential roles in the regulation of gene activation, cell survival, proliferation and differentiation. Furthermore, OSM exhibits many unique biological activities in inflammation (Silver and Hunter, 2010), CNS (Morikawa, 2005; Weiss et al., 2006), fetal and adult hematopoiesis (Tanaka and Miyajima, 2003), osteogenesis (Sims and Walsh, 2010), immune system(Blais et al., 2006; Silver and Hunter, 2010). OSM can stimulate acute phase protein synthesis in hepatocytes (Luyckx et al., 2009), regulate the tissue metalloproteinases and

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tissue inhibitors of metalloproteinases (Li et al., 2001), stimulate the proliferation of human adipose tissue-derived mesenchymalstem cells (hATSCs) and inhibit their differentiation (Song et al., 2005).

OSM exhibits unique biological activities in liver. OSM promotes differentiation of hepatic cells and exhibits differentiation markers, which are accompanied by functional and morphological maturation. As the fetal liver matures, it gradually loses hematopoietic potential, and HSCs relocate to the bone marrow (Kinoshita et al., 2002). Roles of OSM in adult liver include regeneration, tissue remodeling (Okaya et al., 2005), regulating lipid metabolism (Kong et al., 2005), preventing hepatocytes apoptosis, prevention and treatment of liver injury (Hamada et al., 2007). There has been previously reported that OSM can induce morphology and/or function differentiation and maturation of many tumor cells, which are as follows: glioma cells (Chen et al., 2006), glioblastoma cells (Halfter et al., 2006), osteosarcoma cells (Brounais et al., 2009), breast cancer cells (West et al., 2012), lung adenocarcinoma cells (McCormick and Freshney, 2000), myeloid leukemia cells (Bruce et al., 1992). OSM can induce cells to differentiate into hepatocytes from embryonic stem cells (ES cells) (Hay et al., 2008), bone marrow-derived mesenchymal stem cells (Wei et al., 2008), umbilical cord blood-derived mesenchymal stem cells (Sensken et al., 2007), adipose tissue-derived stromal cells (hADSC) and hepatic stem cells in liver with cooperative effects of HGF (Kamiya et al., 2006).

The role of OSM in differentiation of hepatocellular carcinogenesis is less clear. According to unique biological activities in liver and induction of differentiation of some solid tumor cells by OSM, therefore, in the present study, we sought to identify that whether OSM could induce the differentiation of SMMC-7721 hepatoma cells towards a more mature hepatocytic phenotype. The results suggest that OSM significantly inhibits the growth, induces morphological changes, promotes apoptosis in SMMC-7721 cells.

Materials and Methods

Reagents and drugs

Recombinant human OSM was produced in the methylotrophic yeast Pichia pastoris X-33 and carried out the fermentation culture of OSM in 80 L fermentor in a fed-batch mode. The molecular mass and purity of the OSM were 28,000 Da and more than 95.0% respectively (Kong et al., 2009). MTT was purchased from Sigma and RPMI 1640 from GIBCO. FBS was purchased from Hyclone (Logan, UT). All other reagents were analytic reagents. Tissue culture plasticware was from Costar (Cambridge, MA).

Cells and cell culture

Human HCC cell line SMMC-7721 was provided by the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were maintained in RPMI 1640 medium supplemented with 10% inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.0 g/l $NaHCO_3$, at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were routinely seeded by 1×10^5 cells/100 cm^2 flask in 6 ml medium. The medium was changed every 2 days, and the cells were passaged when they reached confluence.

Cell viability analysis

The in vitro growth rate of SMMC-7721 cells were measured every 24 h for 6 consecutive days by MTT assay. Briefly, exponentially growing cells were seeded into a 96-well plate at 1×10^4 cells/ml in quadruple. After incubation for 24 h the cells were treated at concentrations varying from 1 ng/ml to 100 ng/ml OSM. The cells treated with culture medium only served as controls. On the day of harvest, 100 μl of spent medium was replaced with an equal volume of fresh medium containing 10% MTT 5 mg/ml stock. Plates were incubated at 37°C for 4 h, then 100 μl of DMSO (Sigma) was added to each well for solubilizing the formazan grains formed by viable cells and plates shaken at room temperature for 10 mins. The absorbance was measured at 570 nm.

Colony-forming assay

SMMC-7721 cells treated with OSM (1, 10, 100 ng/ml) at a density of 5×10^4 cells/ml were mixed with 1 ml MethoCult methylcellulose-based medium (StemCell, Vancouver, British Columbia, Canada) and plated in 6-well plates according to the manufacturer's instructions. After 7-10 days of incubation at 37°C, colonies (>50 cells) were counted using an inverted microscope.

Analysis of cell cycle and apoptosis

To analyze the proportion of cells in different phases, cellular DNA contents and apoptosis ratio were measured by flow cytometry after treatment for 5 days by 1, 10 and 100 ng/ml OSM. In brief, 5×10^6 cells were harvested and washed in phosphate buffered saline (PBS), then fixed in 70% alcohol for 30 mins at 4°C. After washing in cold PBS three times, cells were resuspended in 1 ml of PBS solution with 40 μg of propidium iodide (Sigma) and 100 μg of RNase A (Sigma) for 30 mins at 37°C in the dark. And the data were acquired with a BD FACScan™ cell sorting system (Becton-Dickinson, San Jose, CA). Cell-cycle analysis was carried out using ModFIT software (Becton-Dickinson). Apoptosis ratio of cultured cells were measured as percentage of hipodiploidic peak.

Morphological and supermicroscope observations

The cells seeded on slides, both control and treated with OSM for 5 days, were observed using Wright-Giemsa's staining method. The morphology of the cells was examined under a light microscope (Olympus IMT-2; Olympus Corp., Tokyo). Ultrastructure of the cells, both control and treated with 10 ng/ml and 100ng/ml OSM for 5 days, were fixed in 0.1 M PBS, 0.4% glutaraldehyde (pH 7.4) at 4 °C for 2 h and then 1% osmic acid for 1 h, dehydrated in ethanol series and replaced in propylene oxide.

The samples were examined by transmission electronic microscopy (CM-120, Philips Electron Optics, Eindhoven, the Netherlands).

Assays for gamma -glutamyl transpeptidase (γ-GT) and alkaline phosphatase activities

SMMC-7721 cells were grown at a density of 1×10^6 cells/ml and allowed to grow for 24 h before exposure to OSM (10 ng/ml and 100 ng/ml). Subconfluent growing cells were washed twice with cold PBS and collected by scraping. Cells were added into ice-cold lysis buffer containing 0.25% sodium deoxycholy and 1 mmol/l PMSF (phenylmethylsulfonyl fluoride), and left to lyse for 30 mins on ice before centrifugation for 20 mins at 12,000 rpm. In the supernatant, the activities of γ-GT and alkaline phosphatase were determined using commercially available reagent kits (Changzheng Medical Scientific Company, Shanghai, China). The protein concentrations in the samples were determined with Bradford protein assay using bovine serum albumin as the concentration standard.

Assays for the secretions of alpha -foetoprotein (AFP) and albumin

The culture supernatants collected at the indicated times after addition of 10 ng/ml or 100 ng/ml OSM were dried by vacuum-freezing and solubilized with PBS. The concentration of AFP secreted in the culture medium was determined by a radioimmunoassay (Chinese Atomic Energy Research Institute, Beijing, China) according to the manufacturer's instructions. The albumin content was determined using a Bromocresol Green Assay kit according the instructions by the manufacturer (Jian Cheng, Nanjing, Jiangsu, China).

Statistical analysis

Statistical analysis was performed by a one-way analysis of variance (ANOVA), and its significance was assessed by Dennett's post-hoc test. Data are presented as mean ± standard deviation (SD), and a value of $P < 0.05$ was considered statistically significant.

Results

OSM inhibits growth of SMMC-7721 cells in vitro

To address whether OSM could suppress proliferation and tumorigenicity of SMMC-7721 cells in vitro, we performed cell proliferation and colony formation assays. The cell lines were treated with different concentrations of OSM and viable cells were examined every 48 h for 6 days. As shown in Figure 1A, increased concentration of OSM significantly suppressed the proliferation of SMMC-7721 cells by the second day after treatment ($P<0.01$). This inhibition was observed at 10 ng/ml and 100 ng/ml of OSM. Remarkably, on the sixth day after treatment, the inhibition due to100 ng/ml of OSM resulted in an approximately 5-fold difference in total cell number. The suppressed proliferation by OSM was observed in a dose and time-dependent manner. In addition, as shown in Figure 1B, the cells treated with OSM formed fewer and smaller colonies than untreated controls.

OSM induces cell cycle arrest and apoptosis in SMMC-7721 cells

As observed above, OSM could inhibit the growth

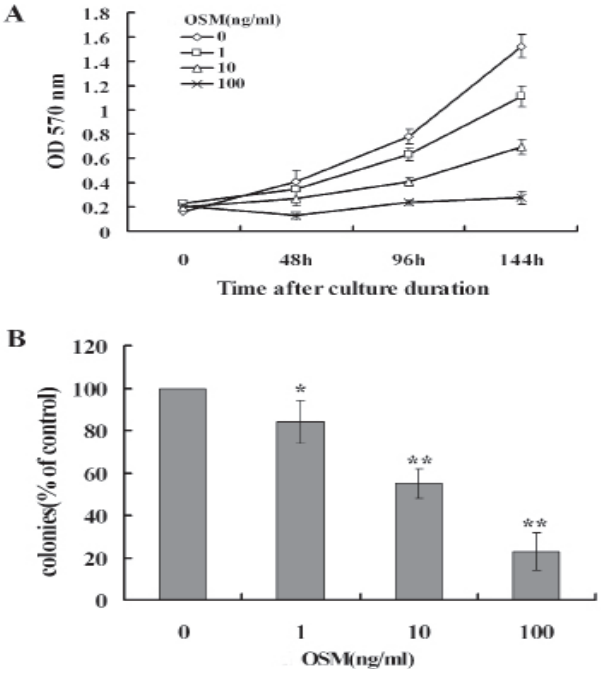

Figure 1. OSM Inhibited the Growth of Human HCC Cells in Vitro. (A) Growth of SMMC-7721 cells treated with OSM was detected by MTT assay. SMMC-7721 cells were incubated in the presence of the indicated concentrations of OSM for 144 h. SMMC-7721 cells had a significant decreased growth rate compared with the control cells ($P<0.01$). (B) Effects of OSM on clonal proliferation of SMMC-7721 cells. SMMC-7721 cells (5×10^3/well) were seeded into MethoCult methylcellulose-based medium. Colonies (>50 cells) were evaluated after 7 days of incubation. Results are expressed as the percentage of clone number compared with control group. The values are mean ±SD of four determinations. $^*P < 0.05$, $^{**}P < 0.01$ compared with control cells

of SMMC-7721 cells in vitro. We then explored the mechanisms underlying the growth inhibition by OSM. Because cell proliferation and death are closely linked to progression of the cell cycle and apoptosis, we analyzed cell cycle kinetics in SMMC-7721 cells. Representative cell cycle profiles of OSM-treated SMMC-7721 cells were shown as histograms in Figure 2, with data expressed as mean percentage of cells in each cell cycle phase, 5 days after OSM treatment. SMMC-7721 cell populations treated with OSM showed a higher proportion of cells in G0/G1 phase, compared with control SMMC-7721 cells, and a decrease in the proportion of cells in S phase relative to that observed in controls. There was no significant changing tendency between the groups in terms of cell percentage at phase G2/M. Cell cycle distribution analysis showed that OSM arrested the cell cycle in dose-dependent manners, and the blocking effect on cell progression of G1 to S phase was the most significant after treatment with 100 ng/ml OSM. The fraction of OSM-treated SMMC-7721 cells at concentration of 1,10,100 ng/ml that were apoptotic was greater than that observed in SMMC-7721 cultures, demonstrating that OSM could also induce apoptosis of SMMC-7721 cells.

Morphological and ultrastructure observations in OSM-treated SMMC-7721 cells

Typical micrographs of SMMC-7721 cells in

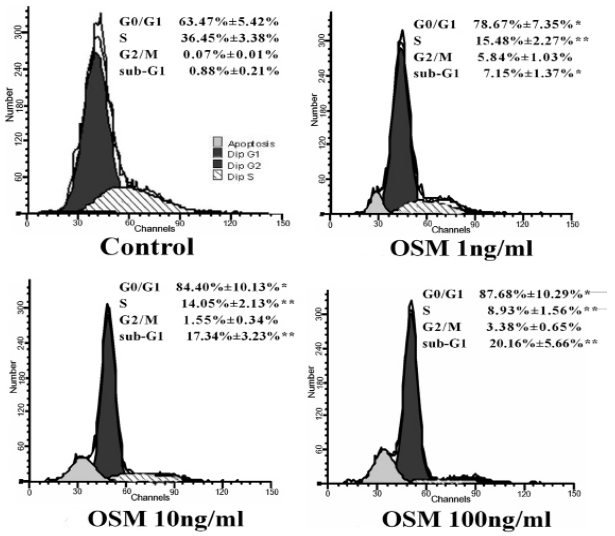

Figure 2. Effect of OSM on the Cell Cycle and Apoptosis. The effect of OSM on cell cycle profile was examined by flow cytometric analysis. SMMC-7721 cells were harvested 120 h after cultured with OSM, and propidium iodide staining was used to analyze cell cycle distribution. The apoptotic cell population was shown according to the sub-G1 fraction. Data are means of four independent experiments. $*P < 0.05$, $**P < 0.01$compared with control cells

monolayer culture were adherent and polygonal cells in a arrangement of pavement, with prominent intercellular adhesion along cell boundaries (Figure 3A1). They contained a large and irregular nucleus, loose chromatin, 3-4 distinct nucleoli, simple cytoplasmic organelles and possessed abundant microvilli on the surface (Figure 3B1 and C1,2). By contrast, SMMC-7721 cells treated with OSM for 12 h resulted in an increase in spacing between neighboring cells. Morphological changes of SMMC-7721 cells generally were not readily apparent until 4-6 days after OSM treatment. They lost their typical polygonal shape and became smaller and oval or round in shape (Figure 3A2). OSM made nucleus smaller, chromatin condensed, cytoplasm concentrated and dark-stained, together with a decrease in nucleus:cytoplasm ratio (Figure 3B2). There was an increase in the amount of heterochromatin and in the number of mitochondria in the OSM-treated cells. And moreover, Golgi complexes also grew larger and mature accompanied by proliferation of rough endoplasmic reticula and free ribosomes. These changes correlated with exposure concentrations of OSM. When the cells were treated with 100 ng/ml OSM (Figure 3C4), the morphological changes were more mature than 10 ng/ml OSM (Figure 3C3). These finding demonstrated that OSM could reverse the morphological features of SMMC-7721 cells towards those of normal hepatocytes.

Effect of OSM on γ-GT and alkaline phosphatase activities, AFP and albumin secretion in SMMC-7721 cells

To clarify the morphological changes and the effect of OSM on differentiation of SMMC-7721 cells, the expression of γ-GT, alkaline phosphatase, AFP, and albumin proteins was studied because γ-GT and AFP are known to be specific for transformed hepatocytes with a high grade of malignancy, and albumin secretion is recognized as one of the phenotypes specific for mature

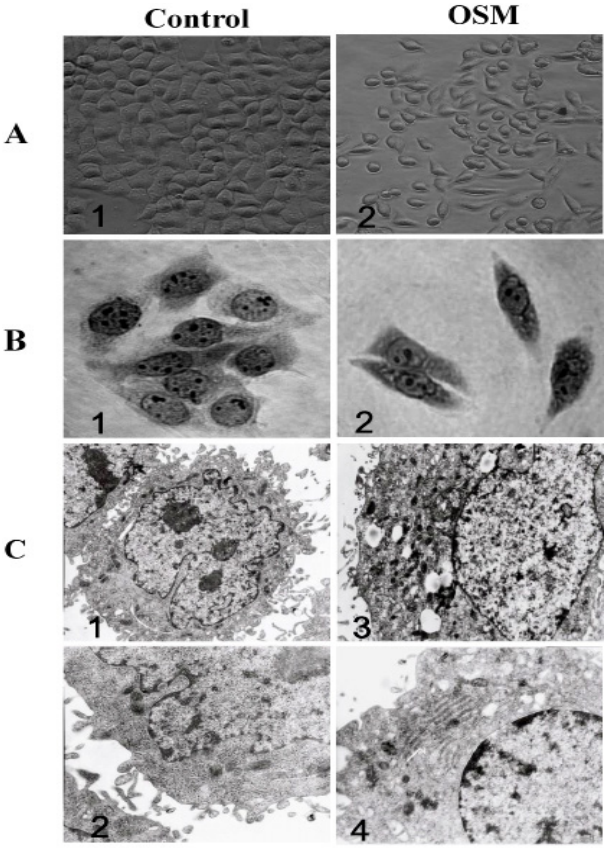

Figure 3. Effect of OSM on Morphological and Ultrastructural Changes of SMMC-7721 Cells. (A) The cells were observed under light microscopy after exposure to 100 ng/ml OSM for 5 days. OSM treatment resulted in smaller and oval or round in shape of SMMC-7721 cells, and increased their nucleus:cytoplasm ratio(A2). No such visible changes were observed in controls(A1). (B) SMMC-7721 cells stained with Wright-Giemsa before and after treatment with OSM. (B1) Control cells, big nucleus and loosed chromatin, 3-4 distinct nucleoli, large ratio of nucleus to plasm. (B2) OSM-treated cells, the volume of cells was small, dark-stained cytoplasm,1-2 nucleolus, decreased ratio of nucleus to plasm. (C) Ultrastructural features of SMMC-7721 cells before and after exposure to OSM for 5 days. Cells were harvested, and ultrastructure of the cells examined by electron microscope. (C1, 2) Control cells, large and irregular nucleus and much euchromatin, a little cytoplasm, simple cytoplasmic organoids, a lot of glucogens. (C3) 10 ng/ml OSM, nucleus diminished and cytoplasm augmented, increase of heterochromatin and mitochondria. (C4) 100 ng/ml OSM, round nucleus, less nucleoli, a lot of rough endoplasmic reticula and free ribosomes distributed around the nucleus layer by layer. Original magnification ×100 (A); ×400 (B);× 6,000 (C1); ×12,000 (C2,4); ×7,500 (C3)

hepatocytes. As shown in Figure 4A,C, a significantly higher levels of γ-GT and AFP were observed in control cells. However, after treating with OSM, a dose dependent decrease in both γ-GT and AFP expression was observed in the treated SMMC-7721 cells. The results also indicated that OSM treatment increased the expression of alkaline phosphatase and albumin in the treated SMMC-7721 cells compared with that of controls across time points examined (Figure 4B,D). OSM (100 ng/ml) caused a markedly greater increase in the alkaline phosphatase activity and albumin level, decrease in the γ-GT activity and AFP level in SMMC-7721 cells than OSM (10ng/ml)

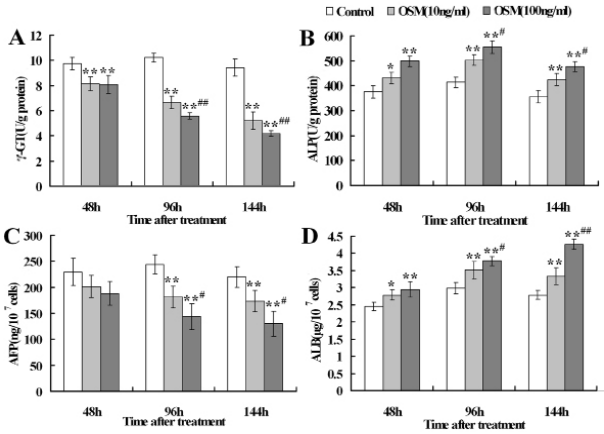

Figure 4. Effects of OSM on γ-GT, ALP, AFP and albumin expression in hepatoma cell lines. The cells were cultured with desired concentrations of OSM for 144 h and protein expression was measured in whole cell lysates by protein assays as described in Material and methods. OSM significantly decreased γ-GT activity and AFP level and significantly increased alkaline phosphatase activity and albumin concentration at any point during 6 culture days. Values are expressed as mean ± SD of four independent experiments. $*P < 0.05$, $**P < 0.01$compared with control cells. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with OSM (10 ng/ml) group

after treatment for 4 days. These effects appeared in time and dose-dependent manners. These finding indicated that OSM had the tendency to restore malignant human hepatocarcinoma cell lines to the normal phenotype.

Discussion

OSM is one of a group of growth factors operating through the gp130 receptor subunit. Others in the group include IL-11, ciliary neurotropic factor (CNTF), IL-6 and leukaemia inhibitory factory (LIF), granulocyte colony stimulating factor (G-CSF). Among those cytokines, the highest activity and greatest potency of inducing differentiation was found with OSM. Activities may be ranked as follows: OSM > IL-6 > LIF > IL-11 > G-CSF > CNTF (McCormick and Freshney, 2000).

In virtually all eukaryotic tissues, cell differentiation is associated with decreased proliferation. Our results from the MTT assay showed SMMC-7721 cells treated with OSM exhibited decreased growth in a dose-dependent manner. These observations were in line with the growth inhibitory effect of OSM on melanoma cell lines which led to the characterization of the protein (Tanaka and Miyajima, 2003). The number of SMMC-7721 cells in the subG1 phase increased when the OSM concentration increased. Analysis of cell cycle distribution showing an increase of cells in G1 phase and a decrease in S phase indicated that OSM leads to G1 arrest. These effects were accompanied by changes in cellular morphology. Thus, these results, in accordance with what has been previously reported for breast cancer cell and glioblastoma cells (Halfter et al., 2006), suggested that OSM suppresses G1 progression and prevents the G1-S transition in HCC cells SMMC-7721 cell line. Protein levels and kinase activities of cyclin A, cyclin D1, cyclin E, and cdk4 are significantly elevated in HCC (Masaki et al., 2003). In human HepG2 hepatoma cells, increased cellular levels of p21 (CIP1) and decreased levels of the hyperphosphorylated form of Rb and cyclinD1 were correlated with growth inhibition and G0-G1 cell cycle arrest (Halfter et al., 2006). The precise mechanisms of malignancy reversion through the differentiation of abnormally differentiated cancer cells into mature hepatocytes by OSM is unclear and we are currently investigating this possibility by using appropriate antibodies.

The results showed that OSM induced morphological and supermicroscope changes, as evidenced by cell changing from polygonal into oval-shaped, cell and nucleus turning small, chromosomal condensations, and cytoplasm augmenting accompanied by a proliferation of mitochondria, rough endoplasmic reticula, free ribosomes and Golgi complex. These characteristics are those of normal hepatic cells, and the results indicated that OSM has the ability to induce the morphology and ultrastructure of SMMC-7721 cells into the mature and normal form. To determine whether the morphological and ultrastructural alteration of SMMC-7721 cells was the result of differentiation, the modulation of some biochemical indices in SMMC-7721 cells by exogenous addition of OSM was examined. It was known that normal hepatocytes produce albumin while foetal hepatocytes produce AFP. The fact that some hepatoma cells can produce AFP suggests that malignant transformation is frequently associated with a change of cells towards the immature state, which usually corresponds to a certain developmental stage of the foetus. At the same time, the increase of γ-GT activity is the specific property related with hepatocyte malignancy, while the increase of alkaline phosphatase activity is the marker of hepatoma differentiation. Our results showed OSM markedly increased albumin secretion and alkaline phosphatase activity, while strikingly decreased AFP secretion, γ-GT activity. All the changes suggested that SMMC-7721 cells were inclined to differentiate.

Up to now, there have been no reports concerning differentiation therapy of HCC by OSM. We demonstrate that OSM modulates the biochemical markers of differentiation and malignancy, inhibits cell proliferation, induces cell morphological changes towards more mature forms of hepatocytes and blocks the cell cycle progression of SMMC-7721 cells, suggesting that the hepatocellular carcinoma cells can be channeled towards normalization by OSM. Taking these results into consideration, along with those arising from previous studies, our observations raise the possibility that OSM is a potential candidates for the inducing activity of HCC. These data have provided the foundation for further evaluation of OSM differentiation therapy in vivo. It is also of interest to know whether the modifications induced by OSM render the cells more vulnerable to conventional tumor therapy like X-ray-irradiation or treatment with cytoloxic agents. We believe that this OSM differentiation therapy could shed new light on HCC in the near future.

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