Inhibition effect of Oncostatin M on metastatic human lung cancer cells 95-D *in vitro* and on murine melanoma cells B16BL6 *in vivo*

Liming Ouyang[1], Li yun Shen[2], Ting Li[2] and Jianwen Liu[2]

[1] School of Bioengineering, State Key Laboratory of Bioreactor Engineering, Mailbox365#, and [2] School of Pharmacy, East China University of Science and Technology, Shanghai 200237, P.R. China

ABSTRACT

Oncostatin M (OSM) is a multifunctional regulator of cell growth and differentiation. It inhibits the growth of many types of tumor cells, but its role in metastasis is unknown. We studied the human OSM expressed and purified from reconstructed *E. Coli* on its activity of inhibiting metastasis of tumor cells by a series of assays *in vitro* and *in vivo*. Clone formation assay in soft agar was used to measure the inhibition activity of OSM on the proliferation of high metastatic human lung cancer cells 95-D. Cell attachment assay, cell migration assay and cell invasion assay were used to evaluate inhibition by OSM on 95-D cells of the adhesion ability, the migration ability, and the ability of cells to cross tissue barriers, respectively. Inhibition of OSM on secretion of MMP-2 and -9 secretion in 95-D cells was determined by Western blot. The *in vivo* inhibitory effect of OSM on metastasis of murine melanoma cells B16BL6 was examined in the pulmonary metastasis model. *In vitro* studies showed that OSM inhibited the proliferation of 95-D cells at low concentration. OSM also reduced the adhesion and invasion ability of 95-D cells and inhibited the secretion of MMP-2 and MMP-9 in OSM treated cells. *In vivo* results showed that OSM (20 µg/kg/d for 7 days) inhibited pulmonary metastasis at a rate of 20.7%. There were no differences in animal weights among the groups. These results suggest that OSM has the potential of being a clinical inhibitor on metastasis of some cancer cells.

Oncostatin M is a multifunctional cytokine produced by activated T-lymphocytes and monocytes (1, 7). It belongs to the interleukin (IL)-6-type cytokine subfamily because it is structurally and functionally related to the other members of the family including leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and IL-11. It has many unique functions and plays important roles for various biological systems such as inflammatory response, hematopoiesis, tissue remodeling, neurogenesis and osteogenesis (8, 13).

Address correspondence to: Dr. Jianwen Liu, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, P.R. China
Tel: +86-21-64252044, Fax: +86-21-64108253
E-mail: liujian@ecust.edu.cn

OSM can exert both stimulatory and inhibitory effects on cell proliferation. It stimulates the proliferation of fibroblasts, smooth muscle cells and Kaposi's sarcoma cells, but inhibits the growth of some normal and tumor cell lines, such as melanoma, glioma, cerebral meningioma, tumoral mammary. Furthermore, *in vitro* studies have shown that OSM upregulates the tissue inhibitor of metalloproteinases-1 (TIMP-1) (10). Because the balance between matrix metalloproteinases (MMPs) and TIMPs secreted from tumor cells is important for collagen degradation during metastasis of tumor cells, so it is suggested that OSM may have the activity of inhibiting cancer metastasis.

In this paper, we designed a series of experiments *in vitro* and *in vivo* to investigate if OSM has the ability to inhibit the metastasis of tumor cells and

analyzed the influence of OSM on MMPs secreted from 95-D high metastatic human lung cancer cells.

MATERIALS AND METHODS

Recombinant human Oncostatin M was prepared from genetic engineered *E.Coli* in our laboratory (the purity of the lyophilized protein is greater than 98%).

Cells and animals. Human highly metastatic lung carcinoma 95-D cells and the highly metastatic substrain B16BL6 of mouse melanoma B16 cells were obtained from Cell Bank of Chinese Academic of Science and were cultured in RPMI 1640 (GIBCO Industries Inc., Grand Island, USA) and 10% (v/v) dialyzed heat inactivated bovine serum (BS) (GIBCO) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Male C57BL/6 mice (6 weeks old) were obtained form the Animal Center of Chinese Academic of Science, and maintained on standard chow and water.

Clone formation in soft agar. The effects of OSM on soft agar colony formation of 95-D cells were investigated. Briefly, single-cell suspensions of 95-D cells were at first treated with or without different concentrations of OSM, and then mixed with agarose in a final concentration of 0.33%. Aliquots of 1.5 mL containing 10^3 cells and 10% fetal bovine serum (FBS) (Hyclone, Logan, USA) were plated in triplicate on 35-mm culture dishes (Nunc, Roskilde, Denmark) over a base layer of 0.6% agarose (Biowest, Miami, USA) and allowed to gel. Colonies of > 8 cells were counted after 21 days of incubation. This experiment was repeated three times.

Attachment assay. The adhesion of cells to the extracelluar matrix (ECM) was assayed as described by Kumagai *et al.* (5) with a slight modification. Briefly, 3 µg/well Matrigel (a reconstituted basement membrane) (Becton-Dickinson, Bedford, USA) was coated onto 96-well plates (Nunc) for 1 h at 37°C and washed by phosphate-buffered saline (PBS) for three times. Then, cells treated by OSM for 15 h or untreated cells resuspended by serum-free RPMI 1640 medium containing 0.1% bovine serum albumin (BSA) (Sigma, St Louis, USA) were seeded at a concentration of 8×10^4 cells/well and incubation was continued for 1 h at 37°C in 95% air and 5% CO_2. Nonattached cells after 4 h of incubation were removed by washing with PBS. Attached cells were assayed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphen-yltetrazolium bromide (MTT) (Biomol, Hamburg, Germany).

Cell migration assay. The migration assay was based on the method of Giancotti and Ruoslahti (2) and slightly modified. Briefly, after digestion by trypsin (Difco Laboratories, Detroit, USA), 1.2×10^5 cells were plated in a 24-well culture plate (Nunc). "Wounds" were made in subconfluent monolayers of the cells and the cells were allowed to migrate into the cell-free area. The wounds were then washed three times with PBS and treated for 12 h with medium with different concentration of OSM or only medium as controls. Cell migration was quantified by measuring the width of wounds with an ocular micrometer (Motic, Xiamen, China). The ocular micrometer was corrected by a stage micrometer with microscope. By measuring the differences in the width of wounds before and after treatment, the distances of the cells migrating into the cell-free area were determined. This experiment was repeated four times.

Cell invasion assay. The invasion assay was based on the method of "Improved Matrigel Invasion Chamber"(9). The 95-D cells were treated with different concentration of OSM for 24 h, 48 h, 72 h, 96 h and then digested and washed one time with PBS. Cells were seeded on the insert (Becton Dickinson Lab., Lincon Park, USA) covered with reconstructed basement membrane gel (Collaborative research, Bedford, USA) in a transwell chamber (Becton Dickinson Com., Franklin Lakes, USA) at a concentration of $2.5 \sim 5 \times 10^5$ cells/0.2 mL/chamber. The chambers were placed in 24-well-plate with DMEM medium in each well and then incubated for 8 h. After incubation the media was withdrawn and the cells on the upper surface of insert were wiped off with cotton swab. The cells on the lower surface were stained with Diff-Quick Stain Set (Baxter Scientific, Miami, USA) and counted. Inhibition ratio of invasion of tumor cells was calculated with the following equation: Invasion inhibition ratio = $(1 -$ mean amount of invasion cells/mean amount of invasion cells of the control) × 100%.

Western blotting of MMP-2 and MMP-9 secreted from tumor cells. Extraction of proteins secreted from tumor cells into the medium and western blotting of MMP-2 and MMP-9 were performed as described previously (3, 12). Briefly, 95-D cells were cultured in serum-free RPMI-1640 medium containing different concentration of OSM or non-OSM
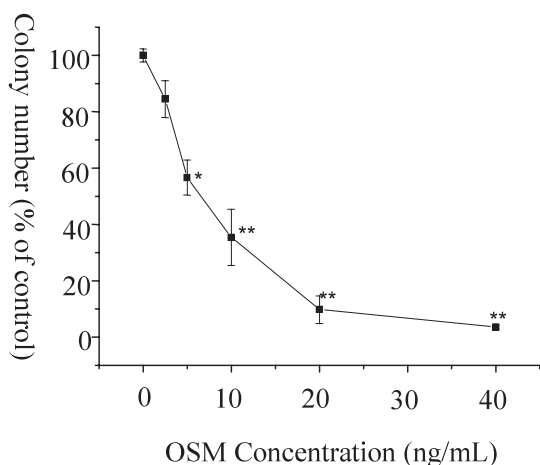
Fig. 1 Effect of OSM on colony formation ability of 95-D cells in soft agar. The figure shows that OSM inhibited the colony formation dose-dependently with complete inhibition at 40 ng/mL. (*$p < 0.05$, **$p < 0.01$)
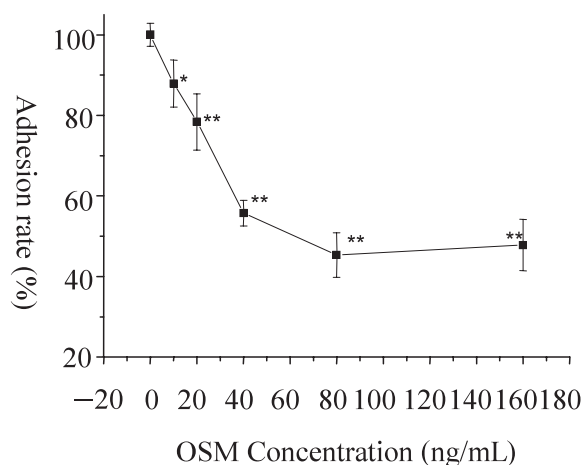

Fig. 2 Effect of OSM on adhesion ability of 95-D cells to basement membrane. The figure shows that OSM had an effect on the adhesion activity of 95-D cells at low concentrations. (*$p < 0.05$, **$p < 0.01$)
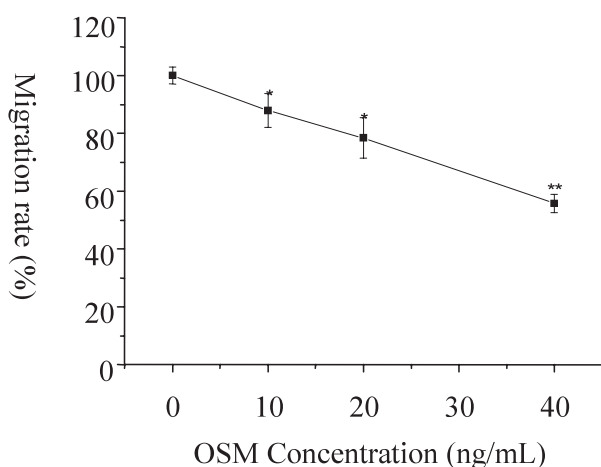

Fig. 3 Effect of OSM on migration ability of 95-D cells. The figure shows that OSM dose-dependently inhibited the migratory ability of 95-D cells and 40 ng/mL OSM could suppress migration by about 53%. (*$p < 0.05$, **$p < 0.01$)

medium as control. After 24 h, the supernatant was collected and run through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After blocking nonspecific reaction with 10% skim milk in PBS, the membrane was reacted at 4°C overnight with one of the following first antibodies: purified mouse monoclonal anti-human MMP-2 antibody (diluted 1 : 500; Santa Cruz Biotechnology, Santa Cruz, USA), purified mouse monoclonal anti-human MMP-9 (diluted 1 : 500; Santa Cruz Biotechnology) and followed by incubation with secondary antibody conjugated horseradish peroxidase (diluted 1 : 500; Santa Cruz Biotechnology). Then the blots

were visualized with enhanced chemiluminescence detection and imagined on KodakTM X-OMAT film.

Tumor Transplantation and Drug Administration. Monolayer B16BL6 cells cultures were harvested with trypsin and resuspended in PBS to provide a single-cell suspension. Each mouse was injected via the lateral tail vein with 5×10^5 viable cells in 0.2 mL. To avoid possible changes in cell viability, melanoma cells were injected into mice within 30 min after their collection. Tested reagents were injected via the lateral tail vein from the second day. Tumor-bearing mice were randomized into four groups each comprising eight mice. The study groups included: (1) animals injected with physiological saline as control; (2) animals injected with OSM (40 μg/kg/d) on days 1–7; (3) animals injected with OSM (20 μg/kg/d) on days 1–7; (4) animals injected with cisplatin (1 mg/kg/d) on days1–7. Animal weight and behavior were monitored every 3 days starting on the first day of treatment. After 3 weeks of drug administration, all the experiment mice were killed by cervical dislocation. Their lungs were excised and fixed in 10% phosphate-buffered formalin. The number of pulmonary tumors was determined by counting visible black foci under a dissecting microscope (14).

Statistical analysis. The scientific statistical software GraphPad Instat version 2.04 was used to evaluate the significance of differences between groups with statistical significance considered as $p < 0.05$,

p < 0.01 or *p* < 0.001.

RESULTS

Effect of OSM on clone formation ability of 95-D cells in soft agar

After 24 h treatment with OSM in different concentration, the colony formation inhibition of 95-D cells was determined and is shown in Fig. 1. OSM inhibited the colony formation dose-dependently with complete inhibition at 40 ng/mL.

Effect of OSM on adhesion ability of 95-D cells to basement membrane

OSM had an effect on the adhesion activity of 95-D cells at low concentrations. When concentration is 40 ng/mL, the inhibition rate reach 54%. And when concentration exceeds 80 ng/mL, the inhibition did not increase more (Fig. 2).

Effect of OSM on migration ability of 95-D cells

The present studies showed that OSM dose-dependently inhibited the migratory ability of 95-D cells and 40 ng/mL OSM could suppress migration by about 53% (Fig. 3).

Eeffect of OSM on invasion activity of 95-D cells

After OSM of different concentration treatment for 24 h, 48 h, 72 h and 96 h, the invasion activity of 95-D cells crossing through Matrigel was counted (Table 1). As showed, OSM had an effect on the invasion activity of 95-D cells at low concentrations. The IC_{50} is 14.29 ng/mL when 95-D cells were treated for 24 h. And as treatment time getting longer, the IC_{50} decreased significantly.

Effect of OSM on secretion of MMP-2, MMP-9 from 95-D cells

After treated with OSM of different concentration for 24 h, MMP-2 and MMP-9 secreted from 95-D cells decreased significantly in dose-dependent way as compared with control (Fig. 4A). And density scan showed that MMP-2 and MMP-9 secreted from 95-D cells decreased 40% and 33% respectively after treated with 10 ng/mL OSM (Fig. 4B, 4C).

Effect of OSM on Pulmonary Metastasis in Mice

Twenty-one days after tumor inoculation, all mice in the control group had pulmonary metastasis. After 9 days of treatment with OSM, the metastatic foci on the lung were significantly decreased as compared with the control group but the decreasing number is fewer than the cisplatin group. However, the body weight decreased in the cisplatin treatment group. The results showed that OSM inhibited B16BL6 pulmonary metastasis *in vivo* (Table 2). There were no significant differences in the inhibition effect between high and low dose OSM groups. There were no differences in animal weights among the groups treated with OSM and control group, and their behaviors were not abnormal, which showed that OSM of experimental dosage had no toxicity on the mice.

DISCUSSION

Previous studies have shown that OSM significantly inhibits proliferation of many types of tumor cells and doesn't inhibit or even stimulate the growth of fibroblasts and smooth muscle cells. Whereas the influence of OSM on metastasis and invasion of tumor cells is not reported yet. Metastasis is the mainly process that causes human death in cancer. And the mechanism of metastasis is complex and relative to many aspects including changes in cell adhesive ability, secretion of protease, and angiogenesis *etc*. MMPs are most frequently overexpressed in tumor cells, and are crucial in degrading the basement membrane and facilitating cell invasion. So inhibition of secretion of MMPs is one of the most important targets in cancer drugs. In the present experimental investigation, we demonstrated that OSM could effectively inhibit the multiplication

Table 1 *Effects of OSM on the invasive capacity of 95-D cells*

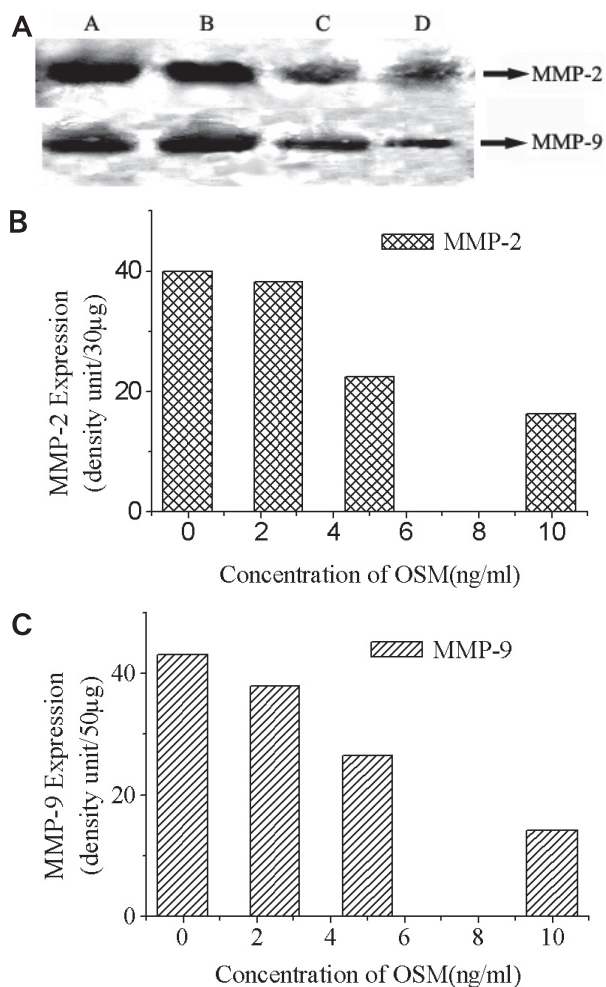| Concentration of OSM (ng/mL)	Inhibition (%) of different dose and treatment time			
	24 h	48 h	72 h	96 h
0	0 ± 0.123	0 ± 0.124	0 ± 0.184	0 ± 0.080
2.5	2.83 ± 0.134	3.45 ± 0.184	11.58 ± 0.116	11.73 ± 0.076
5	9.66 ± 0.146	14.69 ± 0.086	26.63 ± 0.102	31.37 ± 0.113
10	35.01 ± 0.097	47.13 ± 0.156	62.07 ± 0.125	79.10 ± 0.142
20	57.86 ± 0.104	66.71 ± 0.152	80.31 ± 0.096	84.63 ± 0.158
40	83.9 ± 0.097	83.96 ± 0.168	84.64 ± 0.059	86.20 ± 0.136
IC_{50}(ng/mL)	14.29	10.64	8.06	6.32

Fig. 4A Western blot result of MMP-2, 9 secreted from 95-D cells after treated with OSM. Lane A: Control; lane B: 2.5 ng/mL OSM; lane C: 5 ng/mL OSM; lane D: 10 ng/mL OSM. The figure shows that MMP-2 and MMP-9 secreted from 95-D cells decreased significantly in dose-dependent way as compared with control.

Fig. 4B Density scan result of MMP-2 Western blot band. Density scan showed that MMP-2 secreted from 95-D cells decreased 40% after treated with 10 ng/mL OSM.

Fig. 4C Density scan result of MMP-9 Western blot band. Density scan showed that MMP-9 secreted from 95-D cells decreased 40% after treated with 10 ng/mL OSM.

and metastases of metastatic human lung cancer cells 95-D *in vitro* by inhibiting secretion of MMP-2 and MMP-9 from tumor cells. Adhesive interactions are deeply involved in tumor growth, invasion and metastasis, and OSM interferes with adhesion of 95-D cells to laminin *in vitro*. High active motion ability of tumor cells is the characteristic of high metastatic tumor cells. Results of this study also show that OSM significantly inhibits the migration and invasion ability of 95-D cells.

Recent experiments focused on the actions of OSM to the balance between MMPs and TIMPs in inflammation, but the findings are inconsistent. A number of studies seem to support a predominant catabolic action of OSM by up-regulation of the synthesis of TIMPs (11), whereas those of other studies suggest a protective action by up-regulation of the synthesis of MMPs (4, 6). These divergent results may reflect the limitation of a particular *in vitro* or *in vivo* model system and may also reflect differences in the sources, purity, and specificity of the proteins tested, in addition to differences in experimental design.

In vivo experiments also demonstrated the inhibition role of OSM on metastases of murine melanoma cells B16BL6 without exhibiting cytotoxicity in nonmalignant cells. The relatively low inhibition effect of hOSM on the pulmonary metastasis *in vivo* modeled by B16BL6 murine melanoma cells may due to the affinity differences between human OSM and receptors of murine cells in this study. Correspondingly, cisplatin causes decrease in body weight although it displays better effect than OSM. Cisplatin and other platinum drugs are widely used for treating cancer for their effect of inducing cell death by the formation of chemical cross-links in DNA that interfere with DNA replication and transcription which, in turn, leads to cell death. While the effect is not selective so they cause serious side effect particularly nerve and kidney damages, which limit the use of platinum drugs. Comparing with the side ef-

Table 2 *Effect of OSM on Pulmonary Metastasis in Mice*

Group	Dose (μg/kg × d)	Body weight change (g)	Animal number		Total number of metastatic foci	Inhibition (%)
			begin	end		
Control		17.1 ± 2.1	10	10	70.4 ± 9.56	
Cisplatin	10×7	12.1 ± 1.8	8	8	$34.1 \pm 6.54^{**}$	51.5
OSM	20×7	16.6 ± 2.0	6	6	$54.2 \pm 8.86^{*}$	23.0
OSM	40×7	17.4 ± 2.9	6	6	$55.8 \pm 10.34^{*}$	20.7

OSM was given via tail vein for 7 days. $*p < 0.05$ and $**p < 0.01$ compared with control.

fect of cisplatin showed in this study, OSM is relatively safe and effective.

Considering the other reported advantages of OSM concerned to clinical use such as upregulating the expression of low density lipoprotein receptors, augmenting platelet counts, and inducing the expression of hematopoietic growth factors *etc*, it was suggested that OSM has the potential clinical value for human cancer by reducing proliferate and metastatic activity of tumor cells. While with the discovering of diversity of its biological activities, more details on the structures and mechanisms of domains of this molecule should be researched, which could make OSM an efficient and safe anticancer drug.

Acknowledgement

REFERENCES

1. Brown TJ, Lioubin MN and Marquardt H (1987) Purification and characterization of cytostatic lymphokines produced by activated human T lymphocytes. Synergistic antiproliferative activity of transforming growth factor beta 1, interferon-gamma, and oncostatin M for human melanoma cells. *J Immunol* **139**, 2977–2983.

2. Giancotti FG and Erkki R (1990) Elevated levels of the fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell* **60**, 849–859.

3. Heussen C and Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* **102**, 196–202.

4. Korzus E, Nagase H, Rydell R and Travis J (1997) The mitogen-activated protein kinase and JAK-STAT signaling pathways are required for an oncostatin M-responsive element-mediated activation of matrix metalloproteinase 1 gene expression. *J Biol Chem* **272**, 1188–1196.

5. Kumagai H, Tajima M, Ueno Y, Giga-Hama Y and Ohba M (1991) Effect of cyclic RGD peptide on cell adhesion and tumor metastasis. *Biochem Biophys Res Commun* **177**, 74–82.

6. Li WQ, Dehnade F and Zafarullah M (2001) Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires janus kinase/STAT signaling pathway. *J Immunol* **166**, 3491–3498.

7. Malik N, Kallestad JC, Gunderson NL, Austin SD, Neubauer MG, Ochs V, Marquardt H, Zarling JM, Shoyab M, Wei CM, Linsley PS and Rose TM (1989) Molecular cloning, sequence analysis, and functional expression of a novel growth regulator, oncostatin M. *Mol Cell Biol* **9**, 2847–2853.

8. Morikawa Y (2005) Oncostatin M in the development of the nervous system. *Anat Sci Int* **80**, 53–59.

9. Muller S, Migianu E, Lecouvey M, Kraemer M and Oudar O (2005) Alendronate inhibits proliferation and invasion of human epidermoid carcinoma cells in vitro. *Anticancer Res* **25**, 2655–2660.

10. Richards CD, Shoyab M, Brown TJ and Gauldie J (1993) Selective regulation of metalloproteinase inhibitor (TIMP-1) by oncostatin M in fibroblasts in culture. *J Immunol* **150**, 5596–5603.

11. Sohara N, Trojanowska M and Reuben A (2002) Oncostatin M stimulates tissue inhibitor of metalloproteinase-1 via a MEK-sensitive mechanism in human myofibroblasts. *J Hepatol* **36**, 191–199.

12. Sreenath T, Matrisian LM, Stetler-Stevenson W, Gattoni-Celli S and Pozzatti RO (1992) Expression of matrix metalloproteinase genes in transformed rat cell lines of high and low metastatic potential. *Cancer Res* **52**, 4942–4947.

13. Tanaka M and Miyajima A (2003) Oncostatin M, a multifunctional cytokine. *Rev Physiol Biochem Pharmacol* **149**, 39–52.

14. Yan L, Yee JA, McGuire MH and Graef GL (1997) Effect of dietary supplementation of selenite on pulmonary metastasis of melanoma cells in mice. *Nutr Cancer* **28**, 165–169.