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# Macrophage-colony stimulating factor inhibits the growth of human ovarian cancer cells *in vitro*

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**Abstract**

The effect of macrophage-colony stimulating factor (M-CSF), which regulates the growth and differentiation of haematopoietic progenitor cells on the growth of ovarian cancer cells was investigated in three ovarian cancer cell lines *in vitro*. The spontaneous growth of these cells was significantly inhibited by the addition of M-CSF in a concentration-dependent manner over 96 h of culturing. The maximum response was obtained with 10 ng/ml (3857 U/ml) of M-CSF by counting the viable cell number using the trypan blue exclusion assay. [$^3$H]-thymidine incorporation by these cells was also suppressed following a 96-h incubation with M-CSF. The inhibitory effect of M-CSF was reversed by the addition of anti-M-CSF monoclonal antibody. Flow cytometric analysis revealed that the treated ovarian cancer cells arrested at the G0/G1 phase of the cell cycle. These cells expressed M-CSF receptors on their surface as detected by Scatchard plot analysis using $^{125}$I-labelled M-CSF. These results indicate that M-CSF has an anti-tumour activity for ovarian cancer cells and suggest that it can be applied for the treatment of this disease. 

*Keywords:* M-CSF; Ovarian cancer; Growth inhibition; Cell cycle and M-CSF receptor

## 1. Introduction

Ovarian cancer has the lowest overall survival rate of all the gynecological malignancies. Frequently, at the time of diagnosis most patients already have advanced disease that has spread beyond the pelvis and into the peritoneal cavity. Cytoreductive surgery and combination chemotherapy are mainly performed as an annual treatment, but can not improve the prognosis. Therefore, it is necessary to understand the mechanism of growth regulation of the tumour cells and develop novel strategies for therapy. In ovarian cancer, it has been reported that a variety of cytokines and haematopoietic factors, including interleukin (IL)-1α, β, IL-6, IL-10, IL-11, interferon (IFN)-γ, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-α, are expressed and are attributed to either progression or regression of ovarian cancer cells [1–8].

Macrophage-colony stimulating factor (M-CSF) belongs to a family of glycoprotein growth factors and is mainly produced by stromal and immunocompetent cells. M-CSF induces the proliferation, differentiation and survival of haematopoietic cells [9]. Recently, it was reported that the *c-fms* proto-oncogene product is related to the M-CSF receptor and overexpression of M-CSF and its receptor led to the proliferation of ovarian carcinoma cells [10,11]. Elevated serum or ascitic M-CSF levels and overexpression of M-CSF and its receptor in patients with ovarian carcinoma is associated with poor prognosis [12–15]. Moreover, M-CSF also induces the maturation of some leukaemic cells and solid tumour cells [16–19].

In gynecological malignancies, M-CSF could be used to treat or to prevent myelosuppression during chemotherapy. However, the effect of M-CSF on the growth of gynecological malignant tumour cells has not been extensively evaluated. The purpose of this current

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investigation was to examine the biological role of M-CSF and to understand the growth mechanism of ovarian cancer cells.

## 2. Materials and methods

### 2.1. Cell culture

Two human ovarian cancer cell lines; MCAS and TYK-nu [20,21] (Health Science Research Resources Bank JCRB0240, JCRB0234) were cultured in Eagle's Modified Essential medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 292 μg/ml L-glutamine (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan), and 20% or 10% heat-inactivated fetal bovine serum (FBS) (ICN Biomedicals Japan Co. Ltd, Tokyo, Japan), respectively, 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO BRL, Grand Island, NY, USA). Another ovarian cancer cell line; RMUG-S [22] (Institute for Fermentation IFO5050320) was cultured in Ham's F-12 medium (GIBCO BRL) supplemented with L-glutamine, 10% heat-inactivated FBS and penicillin–streptomycin (GIBCO BRL). All cell lines were maintained under conditions of humidified 5% $CO_2$ in air at 37°C. Medium was changed every 3 days and the cells were subcultured once a week. For subculturing and the experiments, monolayers were detached by treating with 0.25% trypsin–0.02% EDTA in phosphate-buffered saline (PBS).

### 2.2. Cytokines and anti-cytokine antibodies

Human M-CSF purified from urine was donated by Green Cross Co. Ltd (Osaka, Japan). The activity of M-CSF was 385.7 U/μg protein, which was determined from stimulating the activity of monocyte/macrophage colony formation from human monocyte precursors *in vitro*. Anti-human M-CSF monoclonal antibody (anti-M-CSF MAb) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The 50% neutralisation dose of M-CSF MAb is approximately 5–20 ng/ml in the presence of 2.5 ng/ml of M-CSF according to manufacturer's instructions (R&D Systems, Inc.). Purified mouse IgG2α immunoglobulin as an isotype control was purchased from Pharmingen (San Diego, CA, USA).

### 2.3. Detection of cell growth

MCAS, RMUG-S ($5\times10^3$/well) and TYK-nu cells ($1\times10^4$/well) in Ham's F-12/Dulbecco's Modified Eagle medium (DMEM) (Nissui Pharmaceutical Co. Ltd) with 2% (for MCAS) or 1% (for others) FBS were plated into 96-well flat bottomed microtitre plates (Falcon 3072, Beckton Dickinson Co., NJ, USA) with various concentrations (0.1–10 ng/ml: 38.57–3857 U/ml) of M-CSF at 37°C for up to 96 h in 5% $CO_2$ and 95% air. The growth of cells was detected by counting the number of viable cells in the culture plates using 0.1% trypan blue in PBS in triplicate cultures. To measure DNA synthesis, the cells were pulsed for the last 20 h with 0.2 μCi of tritiated thymidine ([$^3$H]-TdR, specific activity of 5 Ci/mM, Amersham International plc, Amersham, Bucks, UK), were harvested with the aid of an automated cell harvester (Abekagaku Co., Funabashi, Japan) and the radioactivities incorporated into the cell were counted by a liquid scintillation counter (Aloka Co., Tokyo, Japan). The results were expressed as the mean±standard error of the mean (SEM) of the counts per minute (cpm) of [$^3$H]-TdR incorporation into the cells in triplicate cultures.

### 2.4. Neutralisation of M-CSF bioactivity

MCAS and RMUG-S cells ($5\times10^3$) were plated into 96-well flat-bottomed microtitre plates. After 24 h, 1 ng/ml M-CSF alone or 1 ng/ml M-CSF preincubated with 100 ng/ml (for MCAS) or 1000 ng/ml (for RMUG-S) anti-M-CSF MAb at 37°C for 30 min was added to each well and cultured for a further 48 h. M-CSF preincubated with mouse IgG2α was also added as the control. Cell proliferation was measured by [$^3$H]-TdR incorporation as described above. The results were shown from triplicate cultures.

### 2.5. Analysis of cell cycle by flow cytometry

MCAS cells ($5\times10^4$/well) in Ham's F-12/DMEM with 2% FBS were plated in a 60-mm dish (Falcon 3002) in the presence of 10 ng/ml of M-CSF at 37°C in 5% $CO_2$ and 95% air. After a 48-h incubation, the cells were collected by trypsinisation, washed with PBS, fixed with 70% ethanol and stored at 4°C for more than 3 h. They were collected and washed with PBS and re-suspended in 0.25 mg/ml RNase A (Sigma Chemical Co. St Louis, MO, USA) at 37°C for 1 h. Just before analysis, 1 mg/ml of propidium iodide solution (PI) (Wako Pure Chemical Industries Ltd, Osaka, Japan) was added to the cell samples. The PI fluorescence of individual nuclei was measured using an EPICS-XL flow cytometer (Coulter Co., Healeah, FL, USA) and the data obtained were analysed. The results shown are from a single experiment representative of three independent experiments with similar results.

### 2.6. Radiolabelling of M-CSF

M-CSF was radiolabelled as previously described in Ref. [23]. 1000 ng of M-CSF in 0.1 M borate buffer (pH 8.5) was added to 1 mCi of $^{125}$I-labelled Bolton Hunter reagent (4000 Ci/mmol; Amersham International plc) which had been evaporated by a gentle stream of dry nitrogen. The reaction was allowed to proceed on ice for

1 h and was terminated by addition of 5 μl of 1 M glycine ethyl ester. 30 μl of 2% gelatin in PBS was added as a carrier, and the labelled M-CSF was separated by chromatography on a 1 ml column of Biogel P-10 (Bio-Rad Laboratories, Richmond, CA, USA). Aliquots (100 μl) were collected and fractions containing protein-bound radioactivity were pooled. The specific activity of $^{125}$I-labelled M-CSF was $1.67\times10^5$ cpm/fmol protein. The radiolabelled M-CSF migrated as a single Mr 85 000 Da band when electrophoresed on a sodium dodecyl sulphate-polyacrylamide gel.

### 2.7. Binding assay of $^{125}$I-labelled M-CSF to ovarian cancer cells

M-CSF receptor on ovarian cancer cells was assayed as previously reported in [24]. Briefly, cells ($5\times10^4$) were cultured in 24-well flat-bottomed microtitre plates (Falcon 3024) and grown to confluence in conditioned medium with 20% (for MCAS) or 10% (for others) FBS. The cells were then washed twice with conditioned medium containing 1% FBS (binding buffer), and 200 μl of binding buffer containing various concentrations of $^{125}$I-M-CSF (1.0–30 pM) was added to each well. After incubation at 4°C for 4 h, the cells were washed with binding buffer, were detached by treating with 0.25% trypsin–0.02% EDTA in PBS and the amount of radioactivity bound to the cells was counted by a γ-counter (Aloka Co.). The non-specific binding of [$^{25}$I]-M-CSF was determined by incubating the cells with labelled M-CSF in the presence of a 100-fold excess of cold M-CSF. The results were expressed as the mean cpm (specific binding) of duplicate cultures in which non-specific binding was subtracted.

### 2.8. Statistical analysis

Statistical analysis was performed by Student's *t*-test. A *P* value below 0.05 ($P<0.05$) was regarded as significant.

## 3. Results

### 3.1. Growth inhibition of ovarian cancer cells by exogenous M-CSF

We initially examined the effect of M-CSF on the growth of the three ovarian cancer cell lines. As shown in Fig. 1, 1 ng/ml M-CSF significantly inhibited the growth of MCAS, TYK-nu and RMUG-S cells. By counting the viable cell number, M-CSF inhibited the cell growth approximately 58.5% in MCAS, 79.4% in TYK-nu and 51.0% in RMUG-S. A concentration-dependent inhibition was observed. The maximum effect in each cell line (78.6% inhibition in MCAS, 86.2% in TYK-nu and 61.5% in RMUG-S) was observed by culturing in 10 ng/ml of M-CSF for 72 h (Fig. 2). Cell viability did not differ between the control and the inhibited group (data not shown).

M-CSF also decreased [$^3$H]-TdR incorporation of these cell lines particularly after 96 h in culture (Fig. 3). These results indicate that M-CSF can inhibit cell growth and suppress DNA synthesis of these cells.

### 3.2. Effect of neutralising Ab to M-CSF on the growth inhibition of ovarian cancer cells by exogenous M-CSF

To determine whether M-CSF specifically inhibits the growth of ovarian cancer cells, a neutralisation study was carried out using anti-M-CSF MAb. As demonstrated in Fig. 4, antibody sufficient to neutralise 1 ng/ml M-CSF significantly blocked the antiproliferative effect of M-CSF detected by [$^3$H]-TdR incorporation, whereas control mouse IgG2α did not.

### 3.3. Effect of M-CSF on the cell cycle of ovarian cancer cells

To elucidate the mechanism of the antiproliferative effect of M-CSF, we examined the cell cycle distribution

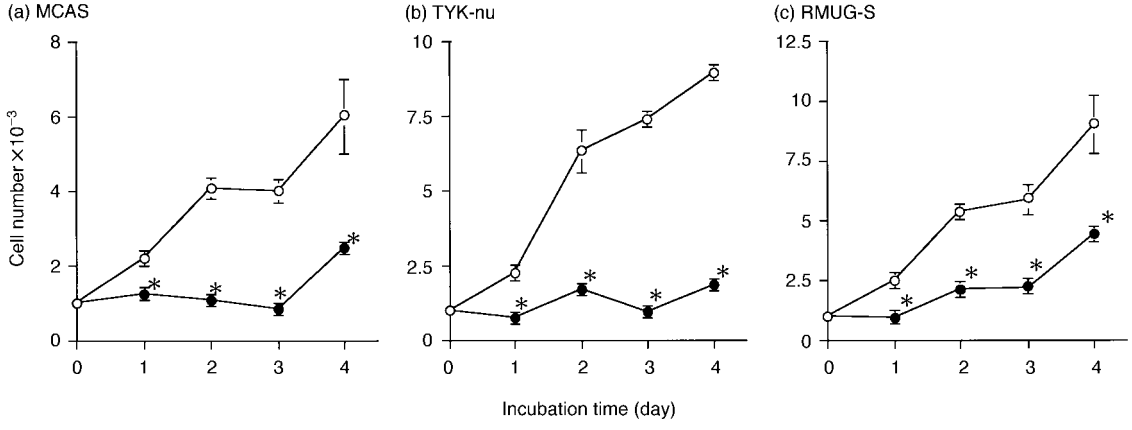


Fig. 1. Time course of inhibitory effect of exogenous macrophage-colony stimulating factor (M-CSF) on three ovarian cancer cell lines, (a) MCAS, (b) TYK-nu and (c) RMUG-S. Each point represents the mean±SEM of cell number in triplicate cultures. *Significantly inhibited.

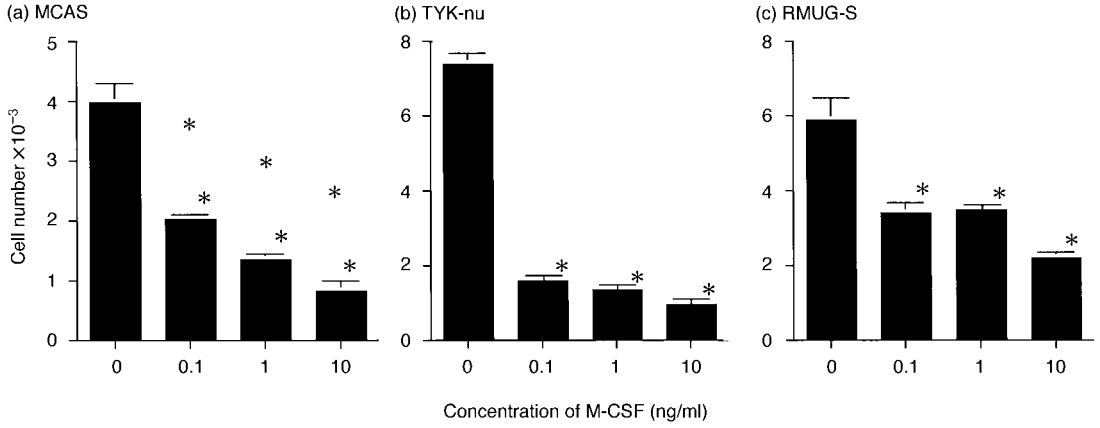


Fig. 2. Dose–response of macrophage-colony stimuating factor (M-CSF) on the growth of ovarian cancer cell lines, (a) MCAS, (b) TYK-nu and (c) RMUG-S. Cells ($5\times10^3$ of MCAS and RMUG-S cells and $1\times10^4$ of TYK-nu cells) were cultured for 72 h with M-CSF at the concentrations indicated. Results are expressed as the mean±SEM of cell number in triplicate cultures. *Significantly inhibited.

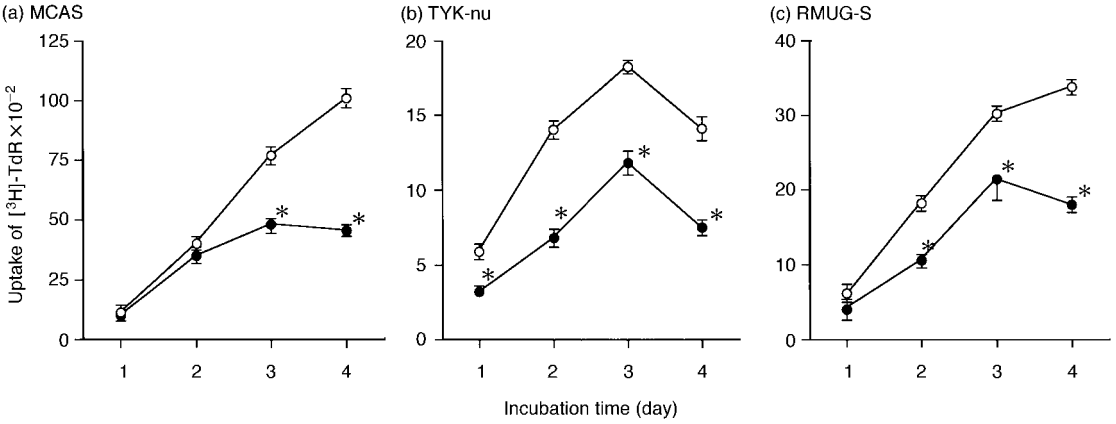


Fig. 3. Effect of macrophage-colony stimulating factor (M-CSF) on DNA synthesis of three ovarian cancer cell lines. Cells (a) MCAS, (b) TYK-nu and(c) RMUG-S were cultured for 96 h in the absence (○) or the presence of 1 ng/ml (385.7 U/ml) M-CSF (●) and labelled with [$^3$H]-TdR for the last 20 h before each time-point. Results are expressed as cpm of [$^3$H]-TdR uptake of tumour cells (mean±SEM of triplicate cultures). *Significantly inhibited.

of the ovarian cancer cells by flow cytometry. Fig. 5 shows a representative result using MCAS cells. Without treatment, 52.0% of cells were in the G0/G1 phase and 43.4% of the cells were in the S + G2/M phase. Following treatment with M-CSF, cells in the G0/G1 phase were increased to 63.2% and cells in the S + G2/M phases were decreased to 31.3%. These results indicate that M-CSF treatment induces a G0/G1 arrest of ovarian cancer cells.

### 3.4. Detection of M-CSF receptor on ovarian cancer cells

A binding assay with $^{125}$I-labelled M-CSF was carried out to determine the presence of specific receptors for M-CSF on MCAS, TYK-nu, and RMUG-S cells. The specific binding of $^{125}$I-labelled M-CSF is displayed in Fig. 6(a). The number of M-CSF receptors and their dissociation constants ($K_d$) were calculated by Scatchard plot analysis. The binding data revealed one linear regression line, suggesting the presence of one class of M-CSF receptors in each cell line. The $K_d$ was $8.5\times10^{-13}$ M for MCAS cells, $1.8\times10^{-10}$ M for TYK-nu cells and $4.0\times10^{-12}$ M for RMUG-S cells. The binding sites were $1.04\times10^2$ sites/cell in MCAS, $1.84\times10^4$ sites/cell in TYK-nu and $6.72\times10^1$ sites/cell in RMUG-S cells (Fig. 6b).

## 4. Discussion

There have been several reports that human haematopoietic growth factors support the growth of haematopoietic or non-haematopoietic malignant cells. Berdel and colleagues described that granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF stimulated the clonal growth of three colon adenocarcinoma cell lines [25]. In ovarian cancer, a few studies have reported that GM-CSF is a growth factor while G-CSF is an inhibitor [26,27]. However, the effect of M-CSF on the growth of malignant cells has not been investigated extensively. In contrast, the secretion of M-CSF and the expression of its receptor have been confirmed

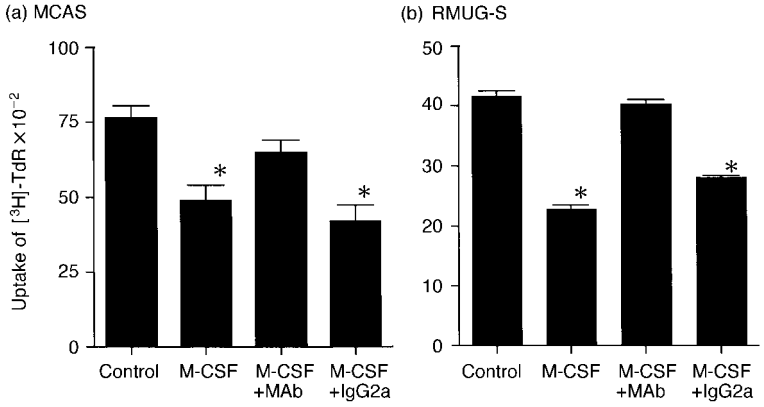


Fig. 4. Effect of anti-macrophage-colony stimulating factor (M-CSF) MAb on DNA synthesis of ovarian cancer cell lines, (a) MCAS and (b) RMUG-S. Cells were cultured and labelled with [$^3$H]-TdR as described in the Methods. Results are expressed as cpm of [$^3$H]-TdR uptake of tumour cells (mean±SEM of triplicate cultures). *Significantly inhibited.

on various malignant cells *in vitro* and *in vivo*. Only one report described CSFs, including M-CSF, as part of a network of autocrine or paracrine loops involved in prostate carcinoma cell proliferation [28]. Thus, the effect of M-CSF on tumour cell lines remains to be elucidated. This is the first report that M-CSF has a direct antiproliferative activity on *in vitro* cultured cell lines. The exogenous addition of M-CSF inhibited the growth of three ovarian cancer cell lines, MCAS, TYK-nu and RMUG-S cells. The effect was concentration-dependent using 0.1–10 ng/ml of M-CSF and the maximum inhibition was obtained at a concentration of 10 ng/ml. This corresponds to 38.57–3857 U/ml of M-CSF. Commonly, $8\times10^6$ U/body M-CSF is administered to a patient that weighs 50 kg and the theoretical concentration of exogenous M-CSF is approximately $1.6\times10^2$ U/ml. Thus we consider that the concentration of 38.57–385.7 U/ml of M-CSF which showed an inhibitory effect in this study is a clinically achievable level. In our experiments, we used 1–2% FBS. Using higher FBS conditions, 20% (MCAS) or 10% (the others), the growth-inhibitory effect of M-CSF was still observed, but was not significant. We consider that some chemical agents in FBS blocked the inhibitory effect of M-CSF. Interestingly, this antiproliferative activity of M-CSF can be specifically reversed by the addition of its neutralising Ab. However, the anti-proliferative activity of M-CSF is not a direct cytotoxic activity, because the cell viability of the inhibited group was not changed from that of the control group. Furthermore, DNA synthesis of these cells was inhibited by the addition of M-CSF.

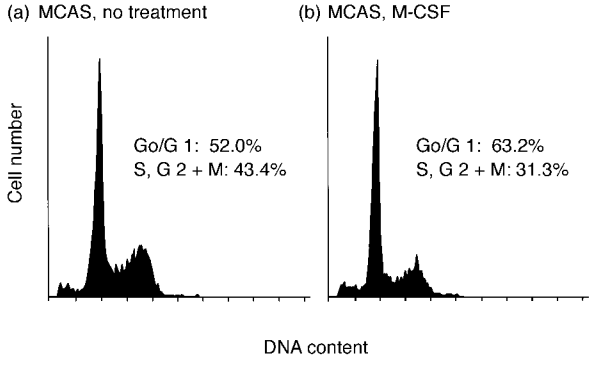


Fig. 5. Effect of exogenous macrophage-colony stimulating factor (M-CSF) on the cell cycle of ovarian cancer cell lines. MCAS cells were cultured for 24 h in the presence (b) or absence (a) of M-CSF (10 ng/ml) and the cell cycle was analysed by flow cytometry. Data are presented as a histogram in which cell number (*y* axis) is plotted against DNA content (*x* axis). The numbers show the percentage of cells in the different phases of the cell cycle. The first peak contains cells with diploid DNA in G0/G1, the cells in the second peak with twice propidium iodide (PI)-fluorescence intensity are tetraploid in G2/M, and the area found between the two peaks shows the cells in S phase.

Recently, the mechanism of cell cycle progression has been extensively studied. We, therefore, analysed the cell cycle profile by flow cytometry to study the molecular events involved in the growth inhibition of ovarian cancer cells induced by M-CSF treatment. The addition of M-CSF to MCAS cells reduced the percentage of the cells in the S + G2/M phases as these cells arrested at G0/G1 prior to the onset of DNA synthesis. This suggests that the mechanism of the antiproliferative effect of M-CSF involves cell cycle arrest.

We detected one class of M-CSF receptors on the ovarian cancer cells. This suggests that M-CSF mediates its growth-inhibitory effects on ovarian cancer cells by binding to M-CSF receptors.

To date, the activity of M-CSF has been mainly focused on its ability to induce proliferation and differentiation of haematopoietic cells. However, the evidence presented here will provide new insight into the activity of M-CSF. Although the antiproliferative effect of M-CSF on ovarian cancer cells *in vitro* is circumstantial at present, the work presented in this report substantiates that, at least *in vitro*, M-CSF can inhibit the growth of ovarian cancer cell lines. Detailed studies and a better understanding of the mechanism of this effect may provide us with a useful new approach not only to control

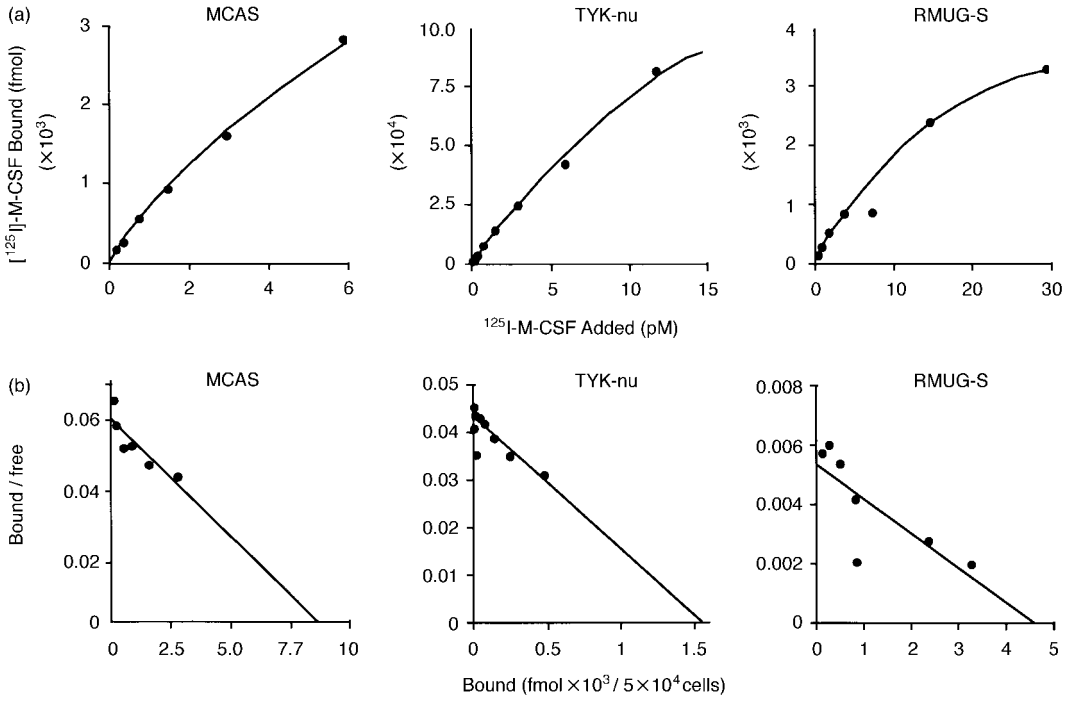


Fig. 6. Detection of macrophage-colony stimulating factor (M-CSF) receptor on ovarian cancer cell lines. (a) Specific binding of radiollabelled M-CSF to MCAS, TYK-nu and RMUG-S cell lines. Specific binding is the difference between the binding of [$^{125}$I]-M-CSF in the presence or absence of 40 nM unlabelled M-CSF. Each point represents the mean of duplicate measurements. (b) Scatchard plot analysis of [$^{125}$I]-M-CSF bound on each cell line.

the growth of cancer cells, but also to treat, at least some, ovarian cancer patients. It is necessary that the interactions of other haematopoietic factors, cytokines and their receptors in these diseases are clarified in the future.

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