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Failure of GM-CSF to Influence the Growth of Small Cell and Non-small Cell Lung Cancer Cell Lines *in vitro*

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The effects of human recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) 1–1000 U/ml on the growth of human lung cancer cell lines have been studied *in vitro*. A panel of 10 small cell, 1 adenocarcinoma and 1 large cell lines was used with multidrug resistant sublines of 3 of the panel. The MTT assay was used to quantify cell numbers after 6–8 days' growth in the presence of GM-CSF. Neither growth inhibition nor stimulation of any of the cell lines in the presence of GM-CSF was observed. Any effects of this agent on residual tumour cells may not therefore present a problem during its clinical use to stimulate marrow regeneration after high-dose chemotherapy for lung cancer.
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INTRODUCTION

GRANULOCYTE–MACROPHAGE colony-stimulating factor (GM-CSF) is a glycoprotein involved *in vivo* in the regulation of haemopoietic precursor cell proliferation [1, 2]. GM-CSF is undergoing clinical trial for its ability to stimulate regeneration of the bone marrow in patients who have received cytotoxic chemotherapy [3]. It was originally believed that the presence of GM-CSF cell surface receptors and the effects of this agent upon cell proliferation were confined to haemopoietic cells. Recent reports, however, have described effects upon a variety of human tumour cell lines, including small cell lung cancer (SCLC) [4–7]. The presence of GM-CSF receptors on SCLC cells has now been reported [7]. Such findings raise the possibility that the use of this agent in SCLC patients receiving chemotherapy may produce effects in residual tumour cells as well as in the haemopoietic precursor cell population.

In addition, there are implications for the histogenesis of SCLC. Although it is generally believed that SCLC derives from the lung epithelium and shares a common stem cell with the other types of lung cancer [8], an alternative has been proposed. This hypothesis suggests SCLC to be of haemopoietic stem cell origin, deriving from transformation of macrophages that migrate to damaged bronchial epithelium [9]. The theory is supported by the presence of "macrophage-specific" antigens on the surface of some SCLC cells [9], and by the observation of the presence of a 3p chromosome deletion in the cells of erythroleukaemias occurring in two SCLC patients after chemotherapy [10]. The presence of GM-CSF receptors and the responsiveness of SCLC cells to the GM-CSF is obviously relevant to this theory.

We describe here experiments designed to examine the effects of GM-CSF on a panel of lung cancer cell lines of both small cell and non-small cell origin.

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MATERIALS AND METHODS

Cell lines

We used a panel of 10 SCLC lines. NCI-H69/P was kindly provided by Dr A. Gazdar and Dr D. Carney of the NCI/Navy Medical Oncology Branch, Bethesda. The other SCLC lines were all derived in this laboratory [11]. NCI-H69/LX4 is a multidrug resistant (MDR) subline of NCI-H69/P that hyperexpresses P-glycoprotein and was derived by *in vitro* exposure to doxorubicin [12, 13].

The large cell lung cancer line COR-L23/P was derived in this laboratory and the adenocarcinoma line MOR was kindly supplied by Dr Morag Ellison of the Ludwig Institute, Sutton. MDR sublines (COR-L23/R and MOR/R) were also derived from each of these lines by *in vitro* exposure to doxorubicin [12]. In contrast to NCI-H69/LX4, however, neither of these lines hyperexpresses P-glycoprotein, although each shows reduced accumulation of MDR-type drugs [12–14].

All the SCLC lines grow as floating aggregates except COR-L88, which grows loosely attached to plastic. COR-L23 and MOR and their MDR sublines grow as adherent monolayers on plastic.

Culture conditions

All cell lines are routinely maintained in RPMI 1640 plus 10% fetal calf serum, penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively). NCI-H69/LX4, COR-L23/R and MOR/R are maintained in 0.4, 0.2 and 0.2 μg/ml doxorubicin, respectively. The drug is, however, removed 2 days before the use of these cells in response experiments. Cells are maintained at 37°C in 8% CO_2 in air. Cells are routinely screened for mycoplasma: the results were negative throughout this study.

Control blood cells

As a positive control for the effects of GM-CSF, we used peripheral blood cells from patients with chronic lymphocytic

Table 1. Effect of GM-CSF on growth of human lung cancer cell lines

Cell line	Final optical density as fraction of control*									
	300 U/ml						1000 U/ml			
NCI-H69/P	0.98†	1.03†	0.98	0.86	—	—	1.01‡	1.08	0.93	—
NCI-H69/LX4	0.99	1.06	1.01	1.12	—	—	0.99	1.41	0.81	—
COR-L23/P	0.87	0.98	0.94	0.97	—	—	0.96	1.27	—	—
COR-L23/R	1.01	1.01	1.01	0.89	—	—	0.97	0.97	—	—
MOR	1.10	1.16	0.97	0.83	—	—	0.90	1.00	—	—
MOR/R	0.85	1.03	0.99	0.83	—	—	1.02	1.02	—	—
COR-L24	1.06	1.02	1.04	0.90	0.76	0.89	0.89	1.01	0.63	0.93
COR-L32	—	1.30	1.22	0.99	0.98	1.01	1.18	1.03	0.71	1.03
COR-L42	—	—	0.88	—	1.07	—	0.81	—	0.91	—
COR-L47	0.91	0.99	1.00	0.82	1.14	—	1.07	0.96	1.01	—
COR-L51	1.74	1.06	1.26	1.01	1.00	—	1.10	1.23	0.94	—
COR-L88	1.12	1.04	0.96	1.01	1.30	0.94	0.93	1.07	0.92	0.99
COR-L103	0.83	1.12	0.80	—	0.95	—	0.82	—	0.91	—
COR-L266	—	—	1.05	—	1.06	—	1.05	—	1.07	—
COR-L279	—	—	0.96	—	0.78	1.07	0.95	—	0.80	1.04

*Figures in same vertical column are from same experiment. Typical standard error on group of four wells is 5%, hence typical standard error on fractional optical density is 7%.

†First two columns of results at 300 U/ml were obtained with GM-CSF from Boehringer Mannhein: all other data were obtained with GM-CSF from Amersham.

‡Single experiment with 3000 U/ml gave value for NCI-H69/P of 0.92.

leukaemia (CLL). 10 ml blood was obtained from each of five patients with CLL and a white blood cell (WBC) count over 3×10^{10}/l. The white cells were separated on a Ficoll–Histopaque gradient and resuspended in RPMI 1640 with 10% fetal calf serum as above. Cells were counted on a haemocytometer and diluted in medium.

GM-CSF

GM-CSF from two sources was used. Early experiments were done with human recombinant GM-CSF from Boehringer Mannheim (activity over 6×10^7 U/mg). Later experiments used recombinant human GM-CSF from Amersham International (manufactured by Amgen Biologicals) activity 5×10^7 U/mg.

Response assay

The response of the cell lines or peripheral WBC to GM-CSF was measured in the MTT colorimetric assay [15–17]. For the SCLC lines, suspensions containing single cells and small aggregates were prepared mechanically from stock cultures in the exponential phase of growth with a syringe and 21 G needle. After counting, dilutions were prepared in medium and cells were plated onto 96-well tissue culture plates (Falcon) at 200 μl per well. Cell numbers were selected to give a final optical density in the range 0.6–1.2 after an incubation of 8 days (except for the more rapidly growing H69 lines for which the incubation period was 6 days) and ranged from 4×10^3 to 2×10^4 per well. Single cell suspensions of the COR-L23 and MOR lines were prepared with a 15 min incubation in 0.4% trypsin and 0.02% EDTA in phosphate-buffered saline. A 6 day incubation was used for these cell lines, which were plated at between 10^3 and 5×10^3 per well. Peripheral WBC from patients with CLL were plated at 4×10^5 per well and a 6 day incubation was also used. After inoculation of the cell suspensions, the plates were incubated at 37°C for 2 h and GM-CSF, diluted in medium, was added in 20 μl to give final concentrations of 1.0–1000 U/ml.

After the appropriate incubation period, the plates containing SCLC cells or WBC were centrifuged (5 min at 200 *g*) to pack the cells on the bottom of the wells. The bulk of the medium was removed and replaced with 200 μl fresh medium containing 0.5 mg/ml MTT (Sigma). (This medium-replacement procedure was used to avoid the artefact in which results are perturbed by the exposure of cells to the MTT in medium that had been variably depleted by the supporting of cell growth [17].) The plates were returned to the incubator for 4 h and removed and re-centrifuged (for SCLC lines and WBC). The medium was aspirated and 200 μl of 100% DMSO was added to each well. The plates were agitated for 5 min on a shaker and then read on a Titertek Multiskan MCC at 540 nm with a reference wavelength of 690 nm.

In each experiment, four replicate wells per GM-CSF dose were used, with the exception of the first experiment with Boehringer Mannheim GM-CSF in which only two wells were used. The mean optical density of each set of treated wells was expressed as a fraction of the mean optical density of the control wells.

RESULTS

The data for the response of the cell lines to the two batches of GM-CSF are shown in Table 1. Only data for 300 and 1000 U/ml are shown. Overall, the GM-CSF failed to produce changes in the growth of any of the cell lines. This was also true for concentrations in the range 1–100 U/ml (data not shown).

In contrast, a response was seen for four out of five specimens of peripheral WBC from patients with CLL (Table 2). The fifth sample (CLL4) showed only a marginal response.

DISCUSSION

No effects of GM-CSF were detected in the panel of human lung cancer cell lines that we studied. These results are therefore in contrast to several studies that examined effects on smaller

Table 2. Effect of GM-CSF on peripheral blood leucocytes from CLL patients

Cells	Final optical density as fraction of control	
	300 U/ml	1000 U/ml
NCI-H69/P*	0.87, 1.07, 0.95 (0.96 [0.10])†	0.95, 1.09, 0.92 (0.99 [0.09])†
CLL1	1.84	1.87
CLL2	1.65	1.66
CLL3	1.25	1.70
CLL4	1.25	1.14
CLL5	1.49	1.51
	(1.50 [0.26])‡	(1.58 [0.27])‡

*Data for NCI-H69/P are from the three separate experiments in which the various CLL cells were studied.
†(Mean [S.D.]) value for NCI-H69/P in three experiments.
‡(Mean [S.D.]) value for 5 CLL specimens.

numbers of lines. 4 SCLC lines were studied by Ruff *et al.* [4] who measured the response to 0–2500 U/ml GM-CSF with tritiated thymidine (^{3}H-TdR) incorporation after 5 days' incubation. 2 of the cell lines showed a 20% inhibition of ^{3}H-TdR incorporation at 1250 U/ml while 2 others (including NCI-H69) showed a 40% inhibition. Inhibition of colony formation in soft agar (50–70%) was also seen for 3 of the lines (no data were presented for NCI-H69). The GM-CSF used in this study was a partly purified preparation from Genzyme. In contrast to this study, a report by Baldwin *et al.* [7] showed that GM-CSF enhanced colony formation in soft agar for 2 SCLC lines (NCI-H69 and NCI-H128) and also enhanced ^{3}H-TdR uptake by NCI-H128 (9 day assay): these effects were seen at 10–100 pmol/l (about 10–100 U/ml). These investigators used human recombinant GM-CSF from either Genetics Institute Inc. or from Sandoz, and stated that these preparations could be used interchangeably. Savaraj *et al.* [18] have reported that GM-CSF (Sandoz) failed to effect the growth of 3 SCLC lines over 72 h. The reasons for the discrepancies between these various data are unclear. It is noteworthy that results with NCI-H69 indicate stimulation [7], inhibition [4] and now no effect of GM-CSF. It seems unlikely that the differences are due to the different assay systems used. Any such differences would be a more reasonable explanation of quantitative rather than qualitative differences. The description by Ruff *et al.* [4] of their GM-CSF preparation as partly purified must leave open the possibility that residual impurities, rather than the GM-CSF itself, were the cause of the inhibiting effects seen in these studies. Whether or not recombinant human GM-CSFs from different suppliers have different biological effects is unknown, but a comparative study in a range of cell lines would be useful.

There have been several reports suggesting that MDR sublines of various human cell lines show an enhanced response to biological agents [19, 20]. Our unpublished data show the MDR subline, COR-L23/R, to be more sensitive to growth inhibition by γ-interferon than the parent line (COR-L23/P) while MOR/R is more responsive to tumour necrosis factor than its parent line. The results presented here for the MDR variants of 3 of our cell lines indicated this was not the case for the GM-CSF that we studied.

In conclusion, our results in a panel of 10 SCLC and 2 non-SCLC cell lines, with MDR sublines of 3 of them, showed no effects of GM-CSF on cell growth over 6–8 days. Effects of this agent upon residual lung cancer cells may not therefore be a major concern for its use during or after cytotoxic chemotherapy.

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