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In Vivo and *In Vitro* Production of Haemopoietic Colony-stimulating Activity by Murine Cell Lines of Different Origin: a Frequent Finding

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Abstract—*The production of colony-stimulating factor (CSF) by murine transformed cells was investigated in* 10 *cell lines derived from spontaneous or chemically induced tumours and from cells transformed by SV40 or Moloney-MSV; histologic types included carcinomas, sarcomas and melanoma. Nine of* 10 *supernatants contained CSF activity as judged by* in vitro *proliferation and differentiation of normal murine monocytic and granulocytic progenitors in agar cultures. Tumours induced with CSF-producing cells caused alterations of haemopoiesis which can include leukocytosis, granulocytosis and splenomegaly. Haemopoietic alterations were also evident in the absence of a local tumour in mice bearing large experimental lung metastases. Production of CSF seems to be a frequent finding among murine cell lines, and its biological and immunological consequences on host–tumour relationships should be taken into account.*

INTRODUCTION

LEUKOCYTOSIS is observed at the time of diagnosis in 15–30% of patients bearing non-haematologic malignancies; the incidence is high in lung (56%) and colorectal (40%) carcinomas and almost negligible in other tumours, like melanoma (5%) [1].

Tumour-induced haemopoietic alterations have been documented in animals bearing experimental tumours [2–10], and in immuno depressed mice transplanted with human tumours [11–17]. The production of colony-stimulating factors (CSF) by tumour cells [3, 6–10, 16, 18–20] is probably the main cause of this phenomenon. Since the majority of studies investigated CSF production in single cell lines, it is still unclear whether this pleiotropic event is rare or common.

The purpose of our work was therefore to study the frequency of CSF production among a variety of transformed murine cell lines of different origin (spontaneous mammary adenocarcinomas, methylcolanthrene-induced fibrosarcomas, SV40- and Moloney murine sarcoma virus-transformed cells). In addition, since some of these lines are metastatic, we investigated the haematologic alterations induced in mice bearing experimental metastases, in the absence of a primary tumour mass.

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MATERIALS AND METHODS

Mice

Eight–12-week-old BALB/cAnNCrlBR mice (hereafter referred to as BALB/c) and C57BL/6NCrlBR (C57BL/6) purchased from Charles River, Calco, Italy, were used throughout the study.

Cells

All the cells were routinely cultured in Dulbecco's modified Eagle medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (referred to as DMEM) and with 10% heat-inactivated foetal calf serum (FCS, Gibco, Paisley, U.K.) in a 5% CO_2 humidified atmosphere at 37°C.

Assays for CSF activity

Assays for detection of CSF production were made on conditioned media obtained as follows [21]: 0.75–3 × 10^6 cells were seeded in 75 cm^2 flasks (Falcon Plastics, Oxnard, U.S.A.) in DMEM + 10% FCS. After 1 day serum-containing

medium was discarded and replaced by DMEM supplemented with 30 nM selenium dioxide, 5 μg/ml insulin and 5 μg/ml transferrin (referred to as ITS); 3 days later supernatants were collected, centrifuged at 1800 RCF for 20 min and filtered through 0.45 micron Millex filters (Millipore), while the cell monolayer was processed to determine cell number/flask. The ability of conditioned media to induce bone marrow proliferation in agar cultures was tested as follows: 2×10^5 bone marrow cells from tibias and femurs of 2-month-old BALB/c or C57BL/6 mice were plated in 35 mm Petri dishes in Iscove's medium (Gibco) supplemented with 20% FCS, 0.3% agar, and with lung conditioned medium (obtained according to [22]) for the positive control cultures, or with 10% conditioned media under test. Granulocytic–macrophagic colonies (colony-forming unit–granulocyte monocyte, CFU-GM) were scored after 10 days of incubation. Megakaryocytic colonies were scored using the cytoenzymatic technique for acetylcholinesterase, directly performed in the dishes [23].

In vivo *studies*

Mice were given subcutaneous (s.c.) injections of 10^5 to 10^7 viable cells; 1–2 months later the following parameters were examined: tumour volume (calculated as: $\pi \cdot [\sqrt{(a \cdot b)}]^3/_6$ where a = maximal tumour diameter and b = tumour diameter perpendicular to a), peripheral leukocytes (number/mm^3), percentage of peripheral lymphocytes, spleen weight and lung metastases. For studying lung colonization, mice were given intravenous (i.v.) injection of 10^5 cells in the lateral tail vein and sacrificed 21 days later. Lung metastases and colonies were identified according to Wexler [24]. Briefly, the trachea was transected well above the carina and injected with 2 ml of 15% black India ink in deionized water (two drops of ammonia water per 100 ml were added to this solution to facilitate dispersion of ink particles). The lungs were then dissected, placed for a few minutes in water and finally fixed in Fekete's solution (87% of 70% alcohol, 8.7% of formaldehyde, 4.3% of glacial acetic acid). Lung nodules were counted after at least 24 h with the aid of a dissecting microscope.

Statistical evaluation

Statistical evaluation was performed by Student's t test.

RESULTS

In vitro *CSF production*

The presence of factors inducing proliferation and differentiation of normal murine bone marrow progenitors was investigated in serum-free media conditioned by cell lines of different origin, some of which are widely used or commercially available (see Table 1).

Supernatants of all the cell lines except B16-A were able to induce in bone marrow agar cultures macrophagic colonies along with granulocytic and/or macrophagic-granulocytic colonies (Fig. 1a). Megakaryocytic colonies were never detected. A normalized evaluation of CSF production was calculated as number of bone marrow colonies/numbers of medium-conditioning transformed cells (Fig. 1b). Also in this case mammary adenocarcinomas showed the highest activity.

The possibility that B16-A cells produce inhibitors of CSF (e.g. PGE_2) has been considered. PGE_2 production was not detected in supernatants of B16-A cells, whereas it was observed in conditioned media of some other cell lines concomitantly to CSF production. For instance, supernatants from E1, MN/MCA-1 and M-MSV cells showed PGE_2 levels higher than 2000 pg/ml, as measured by radioimmunoassay.

In vivo *haemopoietic alterations*

The effects on haemopoiesis *in vivo* were studied in tumour bearing syngeneic mice; the results obtained with nontumorigenic C57SV and KB/cSV cell lines are also reported as additional controls. Tumorigenic cell lines showed quite different *in vivo* growth rates: E1 and M-MSV grew very rapidly, whereas a very slow growth was observed with TS/B and mKSA-Tu5. Therefore, mice bearing TS/B or mKSA-Tu5 tumours were sacrificed 70–80 days after cell injection, whereas mice bearing fast-growing tumours were sacrificed 30–40 days after cell injection.

All the tumorigenic cell lines producing CSF *in vitro* induced significant alterations *in vivo* (Table 2). Mice bearing tumours induced with E1, TS/B, SV-T2, M-MSV, and MN/MCA-1 developed progressive leukocytosis and splenomegaly; leukocytosis was mainly due to the enlargement of the granulocytic population. A kinetic study showed that a significant increase of tumour volume is accompanied by a significant increase of peripheral leukocyte count (Fig. 2). A strong decrease of lymphocyte percentage without leukocytosis was evident in animals bearing CE-2 and mKSA-Tu5 tumours (Table 2). Lymphocyte number was significantly decreased in mice with E1, CE-2, mKSA-Tu5, and SV-T2 tumours (data not shown). It should be noted that most tumorigenic cell lines (E1, CE-2, mKSA-Tu5, SV-T2, MN/MCA-1) gave rise to lung metastases in tumour bearing animals (Table 2).

In the literature, the effects of CSF-producing cells on haemopoiesis have always been investigated *in vivo* in animals bearing local tumours. Two cell lines used in this study, E1 and MN/MCA-1, were able to produce several large experimental lung

Table 1. *Origin of cell lines*

Cell line	Origin	Transformation	Mouse strain	Reference
E1	TS/A mammary carcinoma clone	Spontaneous	BALB/c	[31]
TS/B	Mammary carcinoma	Spontaneous	BALB/c	Our unpublished results
CE-2	Fibrosarcoma	Methylcolanthrene	BALB/c	[32]
KB/cSV*	Kidney cells	SV40	BALB/c	[33]
mKSA-Tu5	Kidney cells	SV40	BALB/c	[34]
SV-T2	3T3 clone A31	SV40	BALB/c	American Type Culture Collection
M-MSV	3T3 clone A31	Moloney sarcoma virus	BALB/c	American Type Culture Collection
MN/MCA-1	Fibrosarcoma	Methylcolanthrene	C57BL/6	[35]
B16-A	Melanoma	Spontaneous	C57BL/6	[36]
C57SV*	Embryo fibroblasts	SV40	C57BL/6	[33]

*Not tumorigenic in immunocompetent mice.

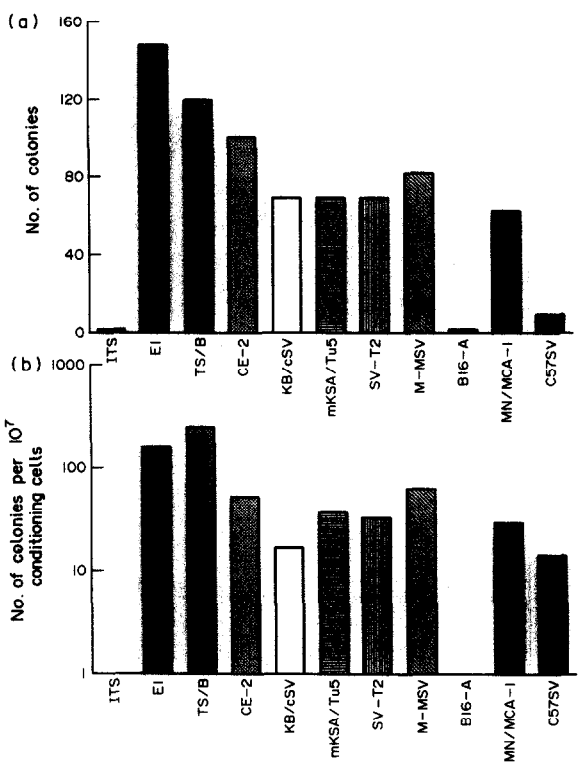

Fig. 1. *Macrophagic, granulocytic and macrophagic/granulocytic colonies of normal bone marrow cells* (2×10^5 *cells*/35 mm *Petri dish*) *induced by serum-free supernatants*: (A) *number of colonies*; (B) *number of colonies per* 10^7 *conditioning cells. ITS = control medium. A representative experiment of at least three experiments is shown; in positive controls with lung-conditioned medium* 75 *colonies were observed.*

metastases after intravenous injection, therefore we took the opportunity of studying haematological parameters also in the absence of a local mass. Table 3 shows that E1 and MN/MCA-1 metastases were able to induce significant reductions of lymphocyte percentage.

DISCUSSION

The main conclusion which can be drawn from our study is that the production of CSFs and the ability to induce alterations of haemopoiesis appear to be quite common properties among murine cell lines: nine of 10 lines in our panel were found positive, regardless of their origin, transforming agent or histotype. Since CSF-producing cells, when injected *in vivo*, can alter many host parameters, a CSF production assay should be included in the characterization of all murine cell lines used for *in vivo* studies. A lack of *in vitro* production by B16 melanoma is in agreement with previous data from our [8] and other laboratories [9] and was not due to a production of PGE_2. However, the possibility that other inhibitors of CSF could be present in B16-A supernatants cannot be ruled out.

The possibility that CSF production could be a result of the adaptation to the *in vitro* environment should not be ruled out; however, at least for the mammary carcinoma clone E1, this did not appear to be the case, since the parental tumour transplanted *in vivo* and never passaged *in vitro* was able to induce haematological alterations like peripheral granulocytosis and splenomegaly (data not shown). The possibility of constitutive CSF gene-expression being a part of the transformation process could be suggested by the breadth of this phenomenon. It should be noted that CSF production can be detected also in supernatants of immortalized, non-tumorigenic 3T3 cells (our unpublished data and [25, 26]).

The occurrence of macrophagic colonies along with granulocytic and/or macrophagic–granulocytic colonies and the absence of other colonies, observed with all active conditioned media, rules out the possibility that cell lines produce IL-3 and

Table 2. In vivo *growth and haematological alterations*

Cell line	Tumour volume (cm^3)	Leukocytes (per mm^3)	Lymphocytes (%)	Spleen weight (mg)	Lung metastases Incidence	Median	Range
*Mice: BALB/c**							
None	–	5550 ± 518	76 ± 2	102 ± 3			
KB/cSV	0	7200 ± 695	69 ± 4	138 ± 25			
E1	5.6 ± 0.6	62357 ± 14203	6 ± 1	392 ± 56	7/7	121	71 to >200
TS/B	2.3 ± 0.3	48561 ± 15924	18 ± 2	383 ± 54	3/9	0	0–3
CE-2	3.6 ± 0.6	6450 ± 931	30 ± 8	211 ± 30	7/8	77	0 to >200
mKSA-Tu5	2.6 ± 0.2	4778 ± 424	35 ± 8	121 ± 14	8/8	5	1–14
SV-T2	3.7 ± 0.3	15486 ± 2465	14 ± 2	476 ± 58	7/7	61	9–135
M-MSV	8.8 ± 1.1	20087 ± 4565	29 ± 3	428 ± 31	0/9	0	
*Mice: C57BL/6**							
None	–	10115 ± 790	80 ± 2	84 ± 5			
C57SV	0	11236 ± 1175	75 ± 1	98 ± 9			
MN/MCA-1	6.7 ± 0.6	44379 ± 5432	20 ± 2	231 ± 20	7/7	74	47–127
B16-A	3.7 ± 0.2	12691 ± 1497	58 ± 2	129 ± 25	3/10	0	0–7

*Groups of 7–15 mice each.
Underlined values are significantly different ($P < 0.01$) from controls.

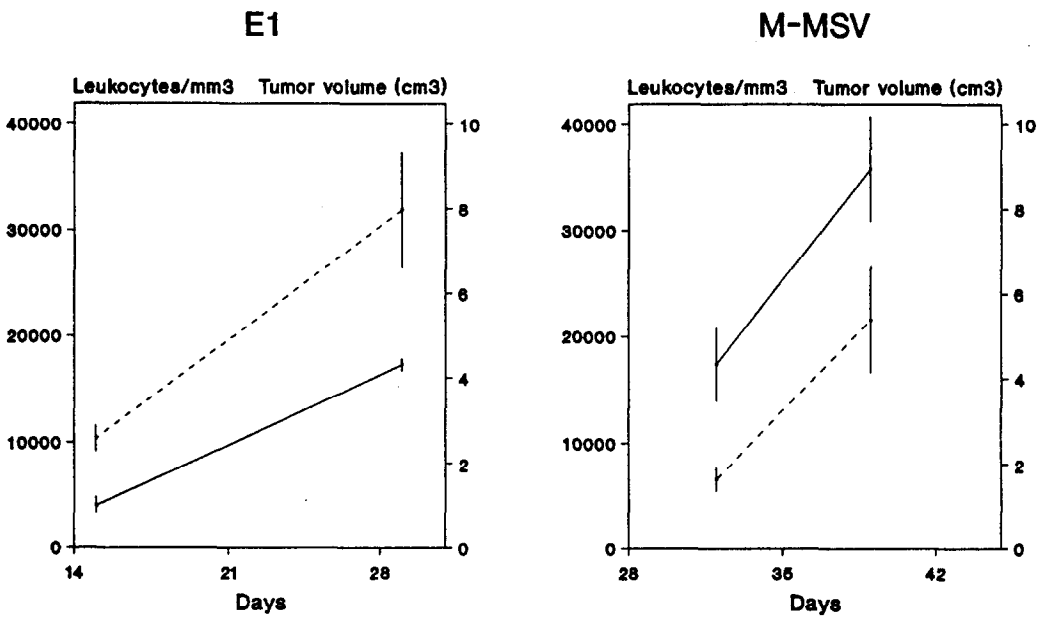

Fig. 2. *Kinetics of peripheral leukocyte count (dashed line) and tumour volume (solid line) in mice bearing tumours induced by E1 and M-MSV cells. Vertical bars represent standard errors* (5–10 *mice per group*).

Table 3. *Haemopoietic alterations in mice bearing experimental lung metastases*

Mice	Cell line	Leukocytes (per mm^3)	Lymphocytes (%)	Spleen weight (mg)	Lung colonies* Incidence	Median	Range
BALB/c	None	4960 ± 258	73 ± 1	106 ± 3			
	E1	5446 ± 630	45 ± 2	138 ± 10	7/7	103	22–149
C57BL/6	None	6880 ± 597	73 ± 4	83 ± 6			
	MN/MCA-1	5540 ± 991	46 ± 5	80 ± 6	7/7	>200	>200

*10^5 cells i.v.
Significant difference from controls: $P < 0.01$ solid underlines; $P < 0.05$ dashed underlines.

suggests that the production of GM-CSF alone or a mixture of M-CSF and G-CSF or GM-CSF occurs. The variable production of colony stimulating activity by the different cell lines here studied could also be explained by a concomitant production of other factors, such as IL-1.

A problem associated with CSF production is the correlation between *in vivo* and *in vitro* results [8, 21, 27, 28]. Our data showed a good agreement from a qualitative point of view, since all the cell lines whose supernatants induced proliferation of normal bone marrow in agar cultures also significantly altered haemopoiesis *in vivo*; minor discrepancies in the extent of haematological alterations were observed. This could be either due to a different stability of the factor(s) produced or be the consequence of *in vitro* culture conditions on CSF production. For example, C57SV cells in serum-containing medium (data not shown) showed a higher activity than in serum-free medium, while negligible differences were found for other cell lines. Also, it is possible that differences between *in vivo* and *in vitro* could be due to the presence of host–tumour interactions *in vivo*, including the presence of other factors (e.g. IL-1) that can augment the effect of CSF [29].

We have shown here that experimental lung metastases of CSF-producing cells can induce detectable haemopoietic alterations in the absence of a local tumour; in addition we [8, 21] and other authors [9, 18] have previously reported that CSF production could play a role in the metastatic process. However the relationship between CSF and metastasis is not completely understood.

Murine tumours producing CSF could be invaluable tools for studying long term effects of a continuous, high dose CSF administration *in vivo*, in particular adverse actions on the immune system, like the induction of thymus aplasia [8, 30] or of suppressor cells [10]. The therapeutic use of cytokines endowed with a CSF activity [29] could share some of these effects.

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