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Expression of pp60$^{c\text{-}src}$ in Human Small Cell and Non-small Cell Lung Carcinomas

Natalia N. Mazurenko, Eugenia A. Kogan, Irina B. Zborovskaya and Fjodor L. Kisseljov

c-*src* protein was found in 60% of lung carcinomas (20 of 33 cases or primary tumours) by immunoblotting with a monoclonal antibody (Mab 327) and immunohistochemistry with serum from rabbits bearing tumours induced by Rous sarcoma virus. *src* protein expression was assessed in 4 small cell lung carcinomas and in an atypical carcinoid of neuroendocrine origin. However, pp60$^{c\text{-}src}$ was also found in non-small cell lung carcinomas: in 60–80% of adenocarcinomas and bronchiolo-alveolar cancers and in 50% of squamous cell carcinomas. In the squamous cell carcinomas, *src* protein was expressed more frequently in poorly differentiated than in well and moderately differentiated carcinomas. Expression of pp60$^{c\text{-}src}$ was not found in epithelial cells of histologically unchanged lung tissues. These results show that pp60$^{c\text{-}src}$ may be activated in human lung carcinomas of different histopathological types.
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INTRODUCTION

THE PROTOONCOGENE c-*src* is the normal cellular homologue of the Rous sarcoma virus transforming gene v-*src*, which codes pp60$^{c\text{-}src}$ membrane-associated phosphoprotein with endogenous tyrosine-specific protein kinase activity [1]. The highest levels of pp60$^{c\text{-}src}$ have been found in the brain [2], platelets [3], and peripheral blood lymphocytes [3]. A study on differentiation and development of the neural retina showed elevated pp60$^{c\text{-}src}$, with high c-*src* kinase activity levels [4]. Whereas some oncogenes (*myc, fos, ras*) are widely expressed in human tumours, mRNA analysis [5, 6] shows that c-*src* activation is restricted to tumours of neuroendocrine origin, especially neuroblastomas [7, 8]. pp60$^{c\text{-}src}$ kinase activity was also found in mammary [9] and colon [10] carcinomas.

The two main histological groups of lung cancer—small cell (SCLC) and non-small cell lung carcinomas (NSCLC)—are distinguished by morphology, tendency to metastasise, hormone secretion, and responsiveness to chemotherapy and radiotherapy [11]. Neuroblastoma and SCLC of neuroendocrine origin have many common histochemical and biochemical characteristics [12]. Expression of c-*src* in cultured human neuroblastoma and some SCLC cell lines correlated with neurocrine differentiation [13]. These results prompted our study into pp60$^{c\text{-}src}$ expression in primary tumours of patients with SCLC or NSCLC.

MATERIALS AND METHODS

Fresh tumour and adjacent lung tissues were obtained from the Cancer Research Center and Moscow Medical Academy after surgery on 47 patients with different morphological types of lung cancer. Samples from patients with carcinoid, fibroma, lymphosarcoma and tuberculoma were also studied. None of the patients had received chemotherapy or radiotherapy before surgery. All tissues were stored in liquid nitrogen. Tumour histology and level of differentiation were examined in paraffin-embedded sections by haematoxylin and eosin staining, and by electron microscopy and the Kreiberg method [14]. Neuroendocrine differentiation of cells was also assessed by the Grimelius reaction.

For immunoblotting, frozen tissue samples were disrupted with a micro-dismembrantor II (B. Braun, Melsungen AG, Germany) and whole cell protein lysates were obtained with radioimmunoprecipitation assay (RIPA) buffer (50 mmol/l Tris–HCl pH 7.2, 100 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS], 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l aprotinin). Another cell lysate preparation was obtained with GuTC buffer (6M guanidine izothiocyanate, 50 mmol/l Tris–HCl, 5 mmol/l sodium acetate, 3% mercaptoethanol and 0.5% *N*-lauroylsarcosine, sodium salt) followed by CsCl centrifugation and dialysis against 100 mmol/l NaCl and 1 mmol/l ethylenediaminetetraacetic acid, sodium salt. Protein content was determined by a modified Lowry procedure [15], and 100 μg protein samples were analysed by 10% SDS polyacrylamide gel electrophoresis, followed by blotting on a Transphor apparatus (LKB) with nitrocellulose filter paper (Schleicher and Schull). The filters were blocked with 5% dry milk solution before incubation overnight at 6°C with mouse monoclonal antibody Mab 327 [16] diluted 1:200 in phosphate buffered saline (PBS). Mab 327 were provided by Dr S. Pahlman of the Pathology Department at Uppsala University. After washing with PBS containing 0.05% Tween-20, the filters were incubated with anti-mouse rabbit immunoglobulin, and conjugated with peroxidase (Dacopatt) for 2 h. After washing with PBS and 0.05% Tween-20, the reaction was developed with diethylaminobenzidine.

For protein kinase assay [17], lung tissue lysates were prepared with RIPA buffer as described. Each protein lysate was incubated overnight with 5 μl serum from tumour bearing rabbits (TBR), inoculated with Praha-C and Schmidt-Ruppin strains of Rous sarcoma and D6 strain of avian sarcoma viruses [18]. Immunoprecipitates were pelleted with protein-A Sepharose and washed 4 times with RIPA buffer. 185 KBq [γ-^{32}P] ATP in kinase buffer (20 mmol/l Tris–HCl, pH 7.2, 5 mmol/l $MnCl_2$, 0.5% aprotinin) were added to each sample and incubated at 37°C for 15 min. After electrophoresis in 10% SDS polyacrylamide gel, the proteins were autoradiographed.

Immunohistochemistry was done by the indirect immunoperoxidase method [19] with TBR serum. The χ^2 test was used for statistical analysis.

Table 1. pp60$^{c\text{-}src}$ expression in human lung cancer

Specimens	Immunoblot Group I*	Immunoblot Group II†	Immunoblot I+II	Immunohistochemistry Group II
NSCLC	8/14	10/17	18/31	14/26
Adenocarcinomas + bronchiolo-alveolar cancer	5/6	3/4	8/10	6/10
Squamous cell carcinomas	3/8	6/10	9/18	6/11
Poorly-differentiated	3/5	4/4	7/9	3/4
Moderate and well-differentiated	0/3	2/6	2/9	3/7
Metastases	1/1	1/2	2/3	—
Adenosquamous carcinomas	—	1/2	1/2	1/2
Large cell carcinomas	—	0/1	0/1	1/3
Small cell lung carcinomas	—	2/2	2/2	4/4
Atypical carcinoid	—	—	—	1/1
Typical carcinoid	—	—	—	0/1
Lymphosarcoma	—	0/1	0/1	1/1
Fibroma	—	0/1	0/1	0/2
Tuberculoma	—	0/1	0/1	0/1
Adjacent normal lung tissues	5/13	0/11	5/24	0/44
Morphologically unchanged tissues	—	0/11	0/11	0/44

Buffer: * GuTC, † RIPA.

RESULTS

By immunoblotting with anti-*src* monoclonal antibodies we studied two groups of lung tumour samples from different clinics. The first group (I) was obtained from 14 patients with NSCLC (Table 1), with 1 case of metastasis of squamous cell carcinoma to lymph node. 13 samples of apparently unaffected lung tissues adjacent to these tumours were also examined. All specimens were prepared with GuTC buffer. The immunoblot detection of c-*src* protein with Mab 327 showed pp60$^{c\text{-}src}$ in 8 of 14 cases of NSCLC, in this group (Table 1); 5 of these cases were adenocarcinomas and bronchiolo-alveolar cancers, and 3 more were poorly differentiated squamous cell carcinomas. In all of these samples, Mab 327 recognised a protein with molecular weight 60 kD corresponding to pp60$^{c\text{-}src}$ (Fig. 1). In a few lysates, another protein with a molecular weight of around 46 kD was detected, but its nature was unknown. *src* protein was also expressed in 1 case of metastasis of squamous cell carcinoma to lymph nodes. In 5 of 13 samples of adjacent lung tissues pp60$^{c\text{-}src}$ was detected.

The second group (II) of lung tumour samples was studied by immunoblotting and immunohistochemistry. The immunoblot analysis of 2 cases of SCLC, 17 cases of NSCLC and 2 metastases of squamous cell carcinomas to lymph nodes is summarised in Table 1. All specimens were prepared with RIPA buffer. Mab 327 recognised pp60$^{c\text{-}src}$ in both cases of SCLC, in 10 of 17 cases

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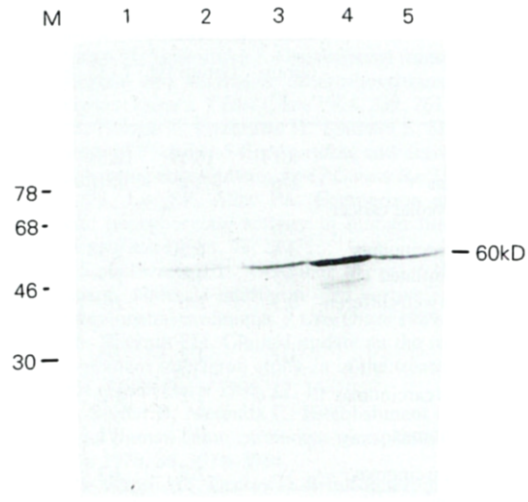


Fig. 1. pp60$^{c\text{-}src}$ expression in protein fractions from patients with lung tumours (group I): 1, adjacent lung tissue (patient 5); 2, adjacent lung tissue (4); 3, adenocarcinoma (3); 4, poorly differentiated squamous cell carcinoma (4); 5, adenocarcinoma (5). M—molecular weight markers (kD).

of NSCLC and in 1 of 2 cases of squamous cell carcinoma metastasised to lymph nodes (Fig. 2). In some cases *src*-related protein 46 kD was also detected.

In groups I and II the same results were obtained: pp60$^{c\text{-}src}$ expression was detected in about 60% of primary NSCLC [in 8 of 14 cases (57%), group I; in 10 of 17 cases (59%), group II],

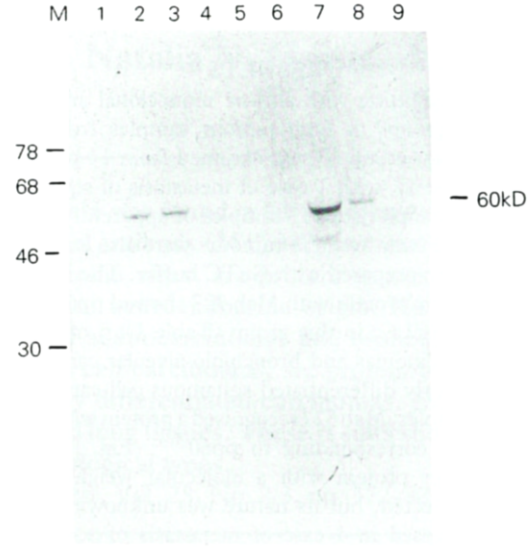


Fig. 2. pp60$^{c\text{-}src}$ expression in whole cell protein lysates from patients with lung tumours (group II): 1, metastasis of squamous cell carcinoma (8); 2, primary squamous cell carcinoma (8); 3, bronchiolo-alveolar cancer (7); 4, adjacent morphologically unchanged lung tissue (7); 5, lung lymphosarcoma (12); 6, adjacent morphologically unchanged lung tissue (12); 7, bronchiolo-alveolar cancer (2); 8, adenocarcinoma (1); 9, adjacent morphologically unchanged lung tissue (1).

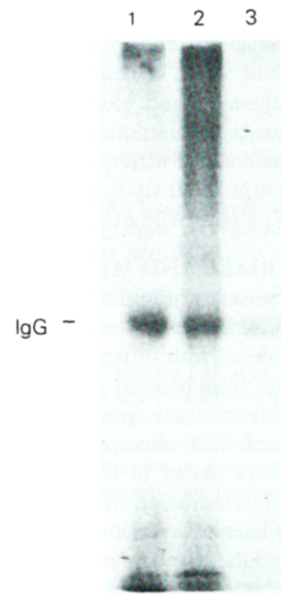


Fig. 3. Protein kinase assay of pp60$^{c\text{-}src}$ in immuno-complexes with TBR serum from lung tissues: 1, adenocarcinoma (1); 2, bronchiolo-alveolar cancer (2); 3, adjacent morphologically unchanged lung tissue (1).

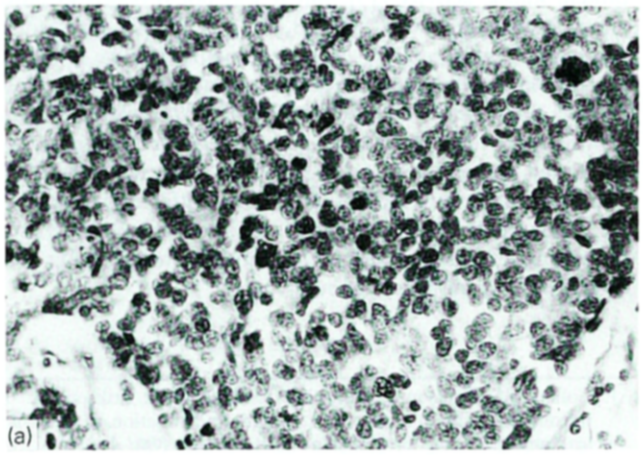
meaning that both methods for protein preparation are equally efficient for the analysis, and the results obtained for the both groups of samples can be not only compared but also summarised. pp60$^{c\text{-}src}$ was expressed in 60% of tested lung carcinomas (22 of 36 cases), in 20 of 33 cases of primary tumours; 2 of 3 cases of metastases to lymph nodes.

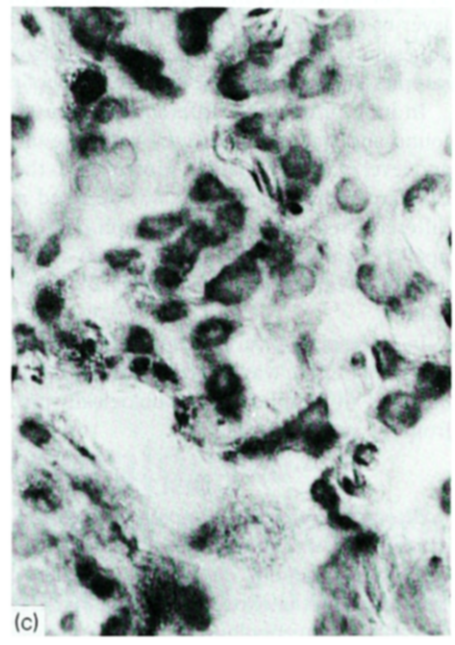
According to morphological types of tumours pp60$^{c\text{-}src}$ was found in 8 of 10 cases of adenocarcinomas and bronchiolo-alveolar cancers and in 9 of 18 cases of squamous cell carcinomas. pp60$^{c\text{-}src}$ was more often expressed in poorly differentiated (7/9) than in moderately and well-differentiated squamous cell carcinomas (2/9), and this difference was significant ($P < 0.05$). Therefore, there is a tendency towards negative correlation between pp60$^{c\text{-}src}$ expression and the level of squamous cell carcinoma differentiation.

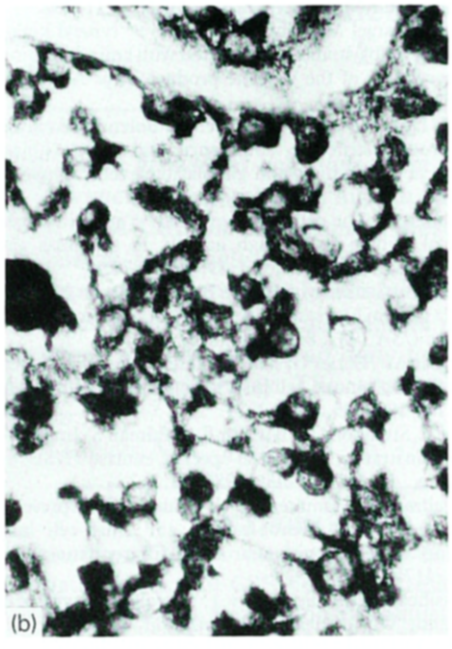
In group II, all tissue samples from patients with lymphosarcoma, fibroma and tuberculoma were negative for pp60$^{c\text{-}src}$ expression (Table 1). In addition, 11 samples of adjacent lung tissues from the same lung cancer patients were studied. All tissues were checked microscopically and immunohistochemically with TBR serum. pp60$^{c\text{-}src}$ was observed in none of 11 morphologically unchanged lung tissues, indicating that pp60$^{c\text{-}src}$ is not expressed in normal tissue.

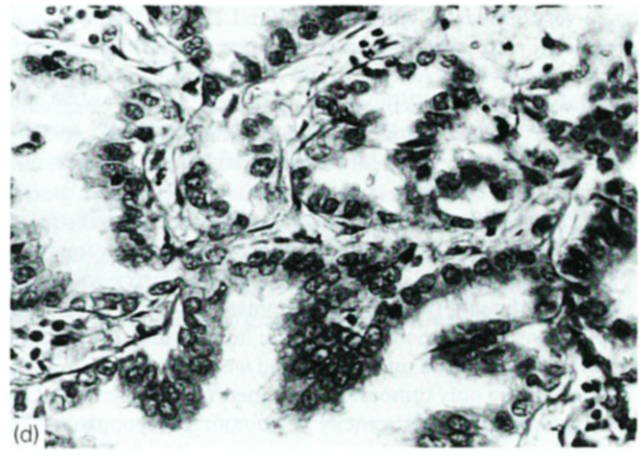
To show the pp60$^{c\text{-}src}$ protein kinase activity, the immune-complex protein kinase assay was used. The incubation of TBR immunoprecipitates from lung carcinomas with [γ-^{32}P] ATP resulted in phosphorylation of the IgG heavy chain (Fig. 3). Kinase activity of pp60$^{c\text{-}src}$ in malignant tissues was greater than in normal tissues, perhaps due to pp60$^{c\text{-}src}$ abundance in carcinomas.

Immunohistochemical analysis of pp60$^{c\text{-}src}$ expression in 30 lung carcinoma and 44 samples of morphologically unchanged lung tissues was done with TBR serum. *src* protein was identified









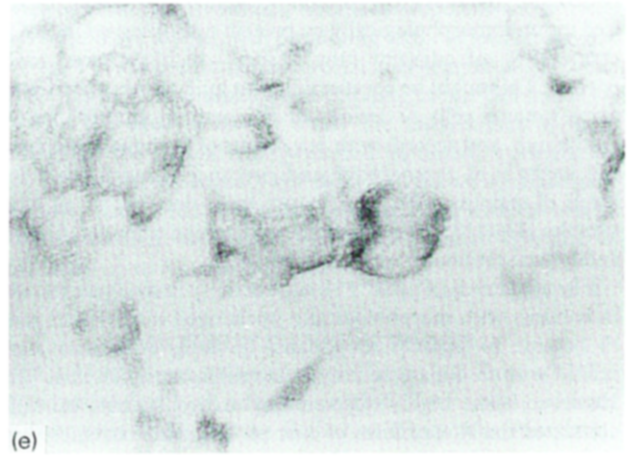


Fig. 4. Immunohistochemical detection of pp60$^{c\text{-}src}$ in parenchyma of lung cancer. (a–c) Small cell lung carcinoma with neurocrine differentiation; (d,e) bronchiolo-alveolar carcinoma. Staining with haematoxylin–eosin (a,d), × 400, indirect immunoperoxidase method (c,e), × 1000; Grimelius reaction (b), × 1000.

in 19 of 31 tested tumours (61%), in cytoplasm near the inner layer of the cell membrane in the plaques of cell contacts and adhesion (Fig. 4). The malignant cells from the same tumour samples have revealed the different level of pp60$^{c\text{-}src}$ expression.

In lung tumours of different histogenesis *src* expression was found and was not connected with size of tumour, metastases and level of differentiation. However, in the group of squamous cell carcinomas, *src* protein was detected most often in poorly differentiated tumours with lymph node metastases (Table 1). pp60$^{c\text{-}src}$ was expressed in all 4 cases of SCLC and the highest level of *src* expression was in cells with neuroendocrine differentiation.

In 1 case of atypical carcinoid—the tumour of neuroendocrine origin *src* protein was also detected. *src* expression was not found in any of 44 samples of morphologically unchanged lung tissues from the same patients.

DISCUSSION

Elevated expression of pp60$^{c\text{-}src}$ in lung tumours of different histogenesis was found: pp60$^{c\text{-}src}$ was observed in all cases of SCLC of neuroendocrine origin, and in 60% of NSCLC tumours. This result was unexpected because SCLC differs from NSCLC not only in morphology, ability to metastasise and hormone secretion, but also in expression of other oncogenes (L-*myc*, N-*myc*, N-*ras*, Ki-*ras*, *erb*-B) [20, 21].

We compared pp60$^{c\text{-}src}$ expression in protein fractions and whole-cell tissue lysates prepared from two groups of tumour samples obtained from different clinics. NSCLC samples results were very similar in both groups I and II: pp60$^{c\text{-}src}$ was most frequently expressed in adenocarcinomas (80%), especially in bronchiolo-alveolar cancer. In the group of squamous cell lung carcinomas, c-*src* protein was expressed in 50% of cases only. There was a tendency towards correlation between pp60$^{c\text{-}src}$ activation and the level of differentiation of squamous cell carcinomas: *src* protein was observed more often in poorly differentiated than in well or moderately differentiated squamous cell carcinomas. The same results were obtained by immunoblotting with Mab 327 and immunohistochemistry with TBR serum. Although the lung carcinomas were not 'pure' cell populations, all tumour samples in the second group were examined by electron microscopy and we tried to include in the NSCLC group only tumours without neuroendocrine cells. Our results show the heterogeneity of tumour cell populations in expression of c-*src*, and that some tumour cells may produce *src* protein regularly, but in variable amounts.

pp60$^{c\text{-}src}$ expression was not found in morphologically unchanged lung epithelial tissues from the second group of patients. In the first group of samples which were not characterised immunomorphologically *src* protein was observed in 5 of 13 cases (38%) of adjacent lung tissues which appeared to be normal. This might be because adjacent lung tissues may contain some tumour cells or lesions of regenerative or proliferative epithelium, and accords with our results of immuno-histochemical analysis of regenerative and precancer lung lesions: low levels of staining with TBR serum were detected in 14 of 42 samples (33%) [22]. Tuberculoma and benign tumours (fibroma and typical carcinoid) were *src* negative.

Elevated levels of pp60$^{c\text{-}src}$ kinase activity in malignant tissues in contrast with morphologically unchanged lung tissues seem to be due to pp60$^{c\text{-}src}$ abundance in lung carcinomas, and accord with *src*-kinase activity in neuroblastoma cell lines [23]. However, some highly differentiated neuroblastoma cell lines contained the altered form of c-*src* protein with extremely high protein kinase activity [13], but it was not revealed in other neuroblastoma and SCLC lines [8, 13, 23]. We did not test this abnormal form of pp60$^{c\text{-}src}$ in lung tumours, but we found an elevated level of c-*src* kinase activity which may be due to a greater abundance of pp60$^{c\text{-}src}$ protein. Apparently c-*src* expression in lung carcinomas may be regulated on transcriptional and translational levels. Recently, v-*src* related mRNA was found in all tested NSCLC cell lines of different histogenesis [24], thus elevated *src* expression is an important characteristic of tumours of this type and perhaps may influence the growth control of lung epithelial tumours.

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Effect of Interleukin-3 and Granulocyte–macrophage Colony-stimulating Factor on Growth of Xenotransplanted Human Tumour Cell Lines in Nude Mice

Wolfgang E. Berdel, Monica Zafferani, Reingard Senekowitsch, Ernst D. Kreuser and Eckhard Thiel

The clonal growth of cell lines from some human solid tumours can be stimulated by haematopoietic growth factors such as recombinant human (rh) interleukin-3 (IL-3) and rh granulocyte–macrophage colony-stimulating factor (GM-CSF) *in vitro*. Among these cell lines are the human colorectal adenocarcinoma cell line HTB 38 and the human small-cell lung cancer cell line HTB 119. Here we report on a series of experiments studying the influence of subcutaneously administered rhIL-3 and rhGM-CSF on the *in vivo* growth of HTB 38 and HTB 119 cell lines as xenografts in athymic nu/nu BALB/c mice. Beginning 1 day after transplantation of the tumour the cytokines were administered daily for 20 days as a subcutaneous bolus distant from the tumour lesion at dose levels up to 1 mg/m^2/day. The cytokines caused no significant and reproducible growth modulation of the tumours *in vivo*.
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INTRODUCTION

INTERLEUKIN-3 (IL-3) AND granulocyte–macrophage colony-stimulating factor (GM-CSF) belong to a family of glycoproteins, that control survival, growth and differentiation of haematopoietic progenitor cells and modulate function of mature haematopoietic cells [1]. The genes of these haematopoietins have been molecularly cloned, and recombinant human (rh) factors are available. Some of those factors are currently being studied in clinical trials. Among various other possible indications for their clinical use, rhGM-CSF and rhIL-3 are being studied clinically in patients with malignant tumours to prevent and decrease myelosuppression and accelerate bone marrow recovery after cytotoxic chemotherapy as well as after high-dose chemotherapy followed by bone marrow transplantation [2].

There is increasing interest in the extrahaematopoietic activity of some of these CSF on tumour cells. This area has been reviewed recently [3]. Among non-haematopoietic tumour cell lines responsive for a growth promoting effect of haematopoietic CSF *in vitro* are the human colorectal adenocarcinoma cell line HTB 38 [4] and the human small-cell lung cancer cell line HTB 119 [5]. In order to further study the implications of these findings for the clinical trials with CSF in tumour patients, we have xenotransplanted both cell lines into Balb/c athymic mice and have studied the tumour growth with and without subcutaneous treatment of the mice with rhIL-3 and rh-GM-CSF at daily doses up to 1 mg/m^2.

MATERIALS AND METHODS

HTB 38, a human colon adenocarcinoma cell line and HTB 119, a human small-cell lung cancer cell line were obtained from the American Type Culture Collection (Rockville, Maryland,

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