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Acknowledgement—This study was supported by the Stifterverband für die Deutsche Wissenschaft (ZE 858 NW).

Pergamon

European Journal of Cancer Vol. 30A, No. 14, pp. 2125–2129, 1994

0959–8049/94 $7.00+0.00

0959-8049(94)00364-5

Constitutive Secretion of Bioactive Transforming Growth Factor β_1 by Small Cell Lung Cancer Cell Lines

J.R. Fischer, H. Darjes, H. Lahm, M. Schindel, P. Drings and P.H. Krammer

We investigated effects of soluble mediators secreted by small cell lung cancer (SCLC) cell lines on modulation of cytokine-induced growth of lymphocytes. We found that interleukin-2 (IL-2)-mediated T-cell growth was inhibited by a cytokine constitutively secreted by the SCLC cell line, NCI-N417. Of several cytokines tested, only transforming growth factor β_1 (TGFβ_1) severely suppressed IL-2-dependent T-cell growth. Using a specific anti-TGFβ_1 antibody, we found that this antibody blocked the immunosuppressive activity secreted by NCI-N417. Thus, the NCI-N417-derived immunosuppressive molecule was serologically identified as TGFβ_1. Further experiments showed that TGFβ_1 was secreted by four of eight SCLC lines tested. mRNA for TGFβ_1 was expressed in NCI-N417 and in SCLC-22H. Constitutive secretion of biologically active TGFβ_1 by SCLC lines suggests that tumour-derived immunosuppression may have clinical relevance.

Key words: TGFβ_1, SCLC, immunosuppression
Eur J Cancer, Vol. 30A, No. 14, pp. 2125–2129, 1994

INTRODUCTION

IN RECENT years, many control mechanisms that regulate activation, proliferation and differentiation of normal lymphocytes by cytokines have been described. In contrast, mechanisms that downregulate immune functions are less well characterised. Interestingly, one factor, transforming growth factor (TGF)β_1, that was identified as inhibiting immune functions is not only released by lymphocytes, but also by a variety of tumour cells [1–4]. Particularly in patients with malignant tumours, immunosuppression is a common feature [1–4], and it has been speculated that the release of immunosuppressive factors might be an important step in malignant evolution [1]. Thus, tumour

cells may escape from immune surveillance by secretion of factors that impair the function of the immune system.

Clinical observations suggest an immunocompromised state in patients with small cell lung cancer [5]. Immunosuppression in such patients may explain impaired delayed cutaneous hypersensitivity, impaired response of lymphocytes to mitogens, reduced activity of natural killer cells and altered macrophage functions [5]. We have previously reported secretion of an immunosuppressive factor and an autocrine growth factor by the small cell lung cancer (SLCL) line NCI-N417 [6]. In addition, we showed that cytokine secretion is selectively suppressed in SCLC patients at the time of diagnosis, and that the same cytokine profile that was found in these patients could be induced by $TGF\beta_1$ in immunocompetent cells from normal healthy individuals [7, 8].

In the present study, we have addressed the question of whether SCLC lines secrete cytokines that inhibit interleukin (IL)-2-mediated T-cell growth. We demonstrate differential secretion of an immunosuppressive factor by SCLC lines that blocked IL-2-mediated growth of a T-cell line. Using a specific antibody directed against $TGF\beta_1$ and a $TGF\beta_1$ ELISA, this immunosuppressive molecule was identified as $TGF\beta_1$. We further show that bioactive $TGF\beta_1$ was constitutively secreted by four of eight SCLC lines tested.

MATERIALS AND METHODS

Cell lines and culture conditions

The small cell lung cancer cell lines NCI-N417, NCI-H69, NCI-N592, SCLC-16HV, SCLC-21H, SCLC-22H, SCLC-24H and SW-210.5 were kindly provided by Dr G. Bepler (Duke University Medical Center, Durham, North Carolina 27710, U.S.A.). These cell lines, the breast cancer cell line MCF-7 and the IL-2-dependent murine T-cell line W2 were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, New York, U.S.A.) supplemented with HEPES (10 mM final concentration), L-glutamine (2 mM final concentration), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum.

Cytokine preparations

Supernatants (SN) of SCLC lines were produced by incubating 5×10^5 cells/ml in serum-free medium at 37°C and 5% CO_2 in air for 72 h. The SN was harvested and stored at 4°C until further use. Highly purified human $TGF\beta_1$ and anti-$TGF\beta_1$ antibody were purchased from British Biotechnology (Oxford, U.K.). IL-2 was obtained from Biogen (Geneva, Switzerland).

SN concentration

SN was concentrated 100-fold by ultrafiltration using a YM-10 membrane (Amicon Corporation, Scientific Division, Danvers, Massachusetts, U.S.A.), filter-sterilised and stored at 4°C.

Interleukin-2 assay for detection of $TGF\beta_1$ bioactivity

The IL-2-dependent murine T-cell line W2 was used for the detection of factors that inhibit IL-2-induced T-cell growth. Serial dilutions of $TGF\beta_1$, NCI-N417- and H69-derived SN were added to the cells incubated with IL-2 (1 U/ml). In neutralisation experiments, $TGF\beta_1$ or SCLC-derived cytokines and anti-$TGF\beta_1$ antibody were incubated together for 1 h prior to the addition of cells. Proliferation was determined by measuring [^{3}H]thymidine ([^{3}H]TdR) incorporation after pulsing the cells with 0.5 μCi [^{3}H]TdR for the last 4 h of a 24-h culture period. Cells were harvested on to filter papers. The filters were dried and radioactivity was determined using a liquid scintillation counter. All results were confirmed in at least three independent experiments.

$TGF\beta_1$ ELISA

An enzyme-linked immunosorbent assay (Genzyme Corporation, Cambridge, Massachusetts, U.S.A.) was used to detect $TGF\beta_1$ secreted by SCLC lines and used as recommended by the manufacturer. The specific anti-$TGF\beta_1$ antibody used in this ELISA did not crossreact with $TGF\beta_2$ or $TGF\beta_3$.

Northern blot analysis

Total RNA was isolated from SCLC cell lines NCI-N417, NCI-H69, NCI-N592, SCLC-16HV, SCLC-21H, SCLC-22H, SCLC-24H, SW-210.5 and breast cancer cell line MCF7 during the log phase of growth by the method of Gough [9]. After determination of purity and concentration, 10 μg/lane of total RNA were separated by electrophoresis in a 1.3% agarose/formaldehyde denaturing gel, transferred to a GeneScreen™ membrane (Du Pont-NEN, Regensdorf, Switzerland) and baked for 2 h at 80°C under vacuum. The plasmid pm$TGF\beta_1$-A, containing cDNA for $TGF\beta_1$, was a generous gift of Dr H.L. Moses (Nashville, Tennessee, U.S.A.). pm$TGF\beta_1$-A was linearised with HindIII and an α[^{32}P] UTP (Amersham, U.K.) labelled m$TGF\beta_1$ riboprobe was produced using the T7 polymerase (Boehringer, Germany). Membranes were prehybridised for 4 h at 65°C in 5 × SSC, 50 mM TRIS, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrolidone, 0.2% Ficoll, 5 mM EDTA, 50% deionized formamide and 150 μg/ml of salmon testis DNA. The radiolabelled probe was added at 1×10^6 cpm/ml and hybridisation was performed for 20 h under the same conditions. Maximal washing stringency of the membranes was twice for 15 min at 65°C in 0.1 × SSC/0.1% SDS. Blots were autoradiographed with Hyperfilm MP (Amersham) at −70°C with intensifying screens. Autoradiographs were developed after 7 days. Ethidium bromide staining of the gel confirmed that comparable amounts of total RNA had been loaded in each lane.

Mycoplasma testing

All cell lines were tested every 8 weeks for mycoplasma contamination by fluorescent bisbenzimidazole staining and standard culture procedures, and were always free of mycoplasma.

RESULTS

A cytokine derived from NCI-N417, but not from NCI-H69, inhibits IL-2-dependent T-cell growth

Conditioned medium of NCI-N417 was found to contain a cytokine that inhibits IL-2-mediated growth of T-cells. As shown in Figure 1a, this NCI-N417-derived cytokine suppressed IL-2-mediated growth of the IL-2-dependent T-cell line W2 dose dependently. This factor was dialysable, sensitive to temperature of 80°C and the activity was destroyed by proteinase K and chymotrypsin (data not shown). In contrast, no immunosup-

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Revised 8 Aug. 1994; accepted 24 Aug. 1994.

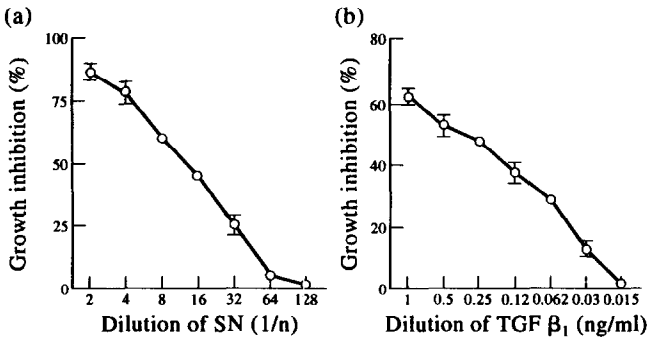

Figure 1. NCI-N417-derived factor and TGFβ1 inhibit IL-2-mediated growth of W2 cells. Cells were cultured in the presence of serial dilutions of 100-fold NCI-N417-derived factor (SN) or TGFβ1. [3H]TdR uptake was 1116 ± 34 cpm in the presence of culture medium and 13309 ± 710 cpm in the presence of IL-2 (1 U/ml). [3H]TdR uptake was 1734 ± 68 cpm in the presence of IL-2 (1 U/ml) and NCI-N417-SN (100-fold) (a) and 5029 ± 642 cpm in the presence of IL-2 (1 U/ml) and TGFβ1 (1 ng/ml) (b), respectively. [3H]TdR uptake in the presence of IL-2 was not suppressed by the addition of NCI-H69-SN (100-fold) (data not shown). Data are the mean of duplicates. A representative experiment of five independent experiments is shown.

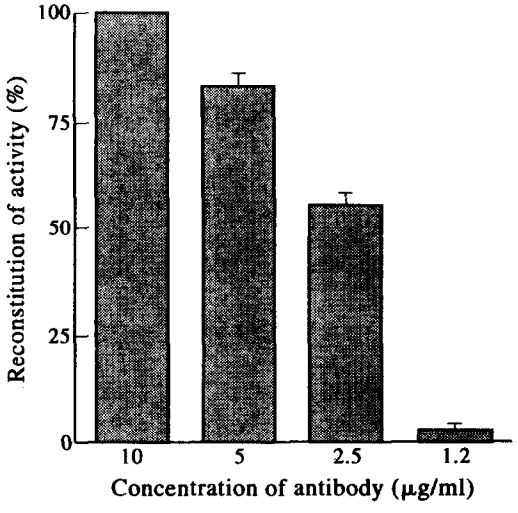

Figure 2. TGFβ1-induced inhibition of IL-2-mediated growth is reversed by anti-TGFβ1 antibodies. W2 cells were cultured in the presence of IL-2 (1 U/ml), TGFβ1 (1 ng/ml) and serial dilutions of anti-TGFβ1 antibodies. [3H]TdR uptake was 259 ± 29 cpm in the presence of culture medium, 15331 ± 426 cpm in the presence of IL-2 (1 U/ml) and 8007 ± 353 cpm in the presence of IL-2 (1 U/ml) and TGFβ1 (1 ng/ml). Data are the mean of duplicates. A representative experiment of three independent experiments is shown.

pressive activity blocking IL-2-mediated growth of T-cells was found in the SN of NCI-H69 (data not shown).

TGFβ1 inhibits IL-2-dependent T-cell growth

To determine the immunosuppressive activity secreted by NCI-N417, we tested a variety of mediators known to be secreted by solid tumour cell lines. These mediators include insulin-like growth factor I (IGF I), epidermal growth factor (EGF), TGFα, TGFβ1 and bombesin. We found that only TGFβ1 inhibited IL-2-mediated growth of the T-cell line W2 dose dependently (Figure 1b). Thus, IL-2-mediated growth of the T-cell line W2 was blocked by NCI-N417-derived factor and TGFβ1 in a similar manner.

Anti-TGFβ1 antibodies neutralise the immunosuppressive activity secreted by NCI-N417

Since TGFβ1 was the only factor tested that blocked growth of W2 in a way similar to the effect exerted by the factor secreted by NCI-N417, we tested whether NCI-N417 secreted TGFβ1 or TGFβ1-like activity. For this purpose, we used an antibody directed against highly purified human TGFβ1. Figure 2 shows that immunosuppression mediated by highly purified human TGFβ1 was abrogated by the addition of these antibodies.

Figure 3 demonstrates that the anti-TGFβ1 antibodies also blocked the immunosuppressive activity mediated by the NCI-N417-derived factor. IL-2-mediated growth of W2 cells, inhibited by addition of NCI-N417-derived immunosuppressive factor, was reconstituted after addition of the anti-TGFβ1 antibodies. Thus, the inhibitory activity secreted by NCI-N417 was serologically identical to TGFβ1.

Moreover, inhibition of IL-2-dependent T-cell proliferation by TGFβ1 secreted from NCI-N417 was not a non-specific effect, since addition of excess IL-2 did partially overcome the inhibitory activities of both highly purified human TGFβ1 and NCI-N417 derived TGFβ1 (data not shown).

Further experiments demonstrated that secretion of TGFβ1 by NCI-N417 is not an isolated finding. Table 1 shows that four of eight SCLC lines tested were found to secrete TGFβ1 as

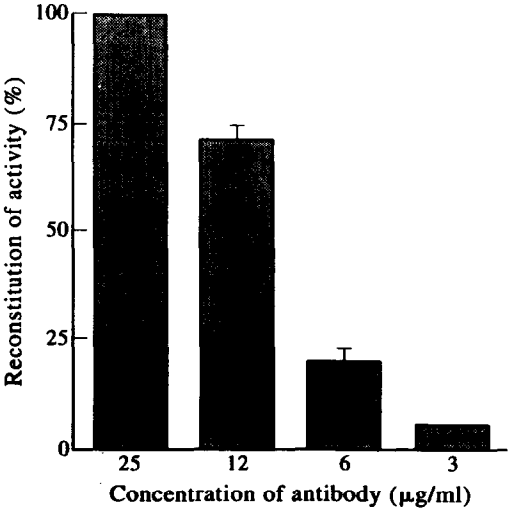

Figure 3. NCI-N417-SN-induced inhibition of IL-2-mediated growth is reversed by anti-TGFβ1 antibodies. W2 cells were cultured in the presence of IL-2 (1 U/ml), NCI-N417-SN (100-fold) and serial dilutions of anti-TGFβ1 antibodies. [3H]TdR uptake was 473 ± 22 cpm in the presence of culture medium, 18174 ± 517 cpm in the presence of IL-2 (1 U/ml) and 10537 ± 398 cpm in the presence of IL-2 (1 U/ml) and NCI-N417-SN (100-fold). Data are the mean of duplicates. A representative experiment of five independent experiments is shown.

measured by TGFβ1 ELISA with a specific anti-TGFβ1 antibody.

Expression of mRNA for TGFβ1 in NCI-N417

To further investigate TGFβ1 secretion by four of eight SCLC lines, RNA was isolated from the eight lines to examine TGFβ1 mRNA expression. The breast cancer cell line MCF 7, known to produce TGFβ1, was used as a positive control. Using a specific probe for TGFβ1, we demonstrated the presence of

Table 1. TGFβ$_1$ is secreted by four of eight SCLC lines tested

SCLC line	TGFβ$_1$ activity (ng/ml)
NCI-N417	1.6
NCI-H69	—
NCI-N592	1.3
SCLC-16HV	—
SCLC-21H	—
SCLC-22H	4.0
SCLC-24H	—
SW-210.5	0.8

TGFβ$_1$ was assessed by ELISA specific for TGFβ$_1$. One representative experiment out of two is shown. Data represent the mean of duplicates. Deviation between parallel measures was within 10%. — below detection limit of 0.05 ng/ml.

mRNA for TGFβ$_1$ in NCI-N417 and in SCLC-22H (Figure 4). The latter line was found to secrete relatively high amounts of TGFβ$_1$ (Table 1). In two other SCLC lines, NCI-N592 and SW-210.5, which secrete lower amounts of TGFβ$_1$-protein, no mRNA could be detected in the northern blot. In four cell lines which do not secrete TGFβ$_1$, no TGFβ$_1$ mRNA was detected.

DISCUSSION

We have previously reported secretion of an immunosuppressive factor and an autocrine growth factor by the SCLC line NCI-N417 [6]. In the present paper, we addressed the question of whether SCLC cell lines secrete factors that inhibit IL-2-mediated T-cell growth. We found that the SCLC line NCI-N417 secretes such a factor. The immunosuppressive activity was overcome by the addition of anti-TGFβ$_1$ antibodies. Thus, the immunosuppressive factor secreted by NCI-N417 is serologically identified as TGFβ$_1$. NCI-N417 expresses both mRNA for TGFβ$_1$ and bioactive TGFβ$_1$, whereas another cell line, NCI-H69, does not express TGFβ$_1$ mRNA and does not secrete TGFβ$_1$. Further investigation showed that four of eight SCLC lines secrete TGFβ$_1$ protein. Further mRNA analysis demonstrated the presence of TGFβ$_1$-specific mRNA in the TGFβ$_1$-secreting line SCLC-22H but failed to detect TGFβ$_1$-specific mRNA in low TGFβ$_1$-secreting lines NCI-N592 and SW-210.5. No mRNA for TGFβ$_1$ was detected in the four cell lines that did not secrete any detectable TGFβ$_1$. SCLC-22H appears to express less mRNA than NCI-N417, although the amount of secreted TGFβ$_1$ is higher than in NCI-N417. The reason for this discrepancy could be either greater mRNA stability in NCI-N417 or increased translation efficiency in SCLC-22H. The SCLC lines, NCI-N592 and SW-210.5, secreted lower amounts of TGFβ$_1$ than NCI-N417 and SCLC-22H. Therefore, as seen in other reports for macrophage colony-stimulating factor, IL-6 and IGF II [10–12], the amounts of TGFβ$_1$ mRNA may be below the level of detection, and thus could not be detected by northern blotting.

A recent investigation reported expression of TGFβ$_1$ mRNA in 10 of 21 SCLC lines [13]. Secretion of bioactive TGFβ$_1$ was not examined in this study. However, as expression of mRNA

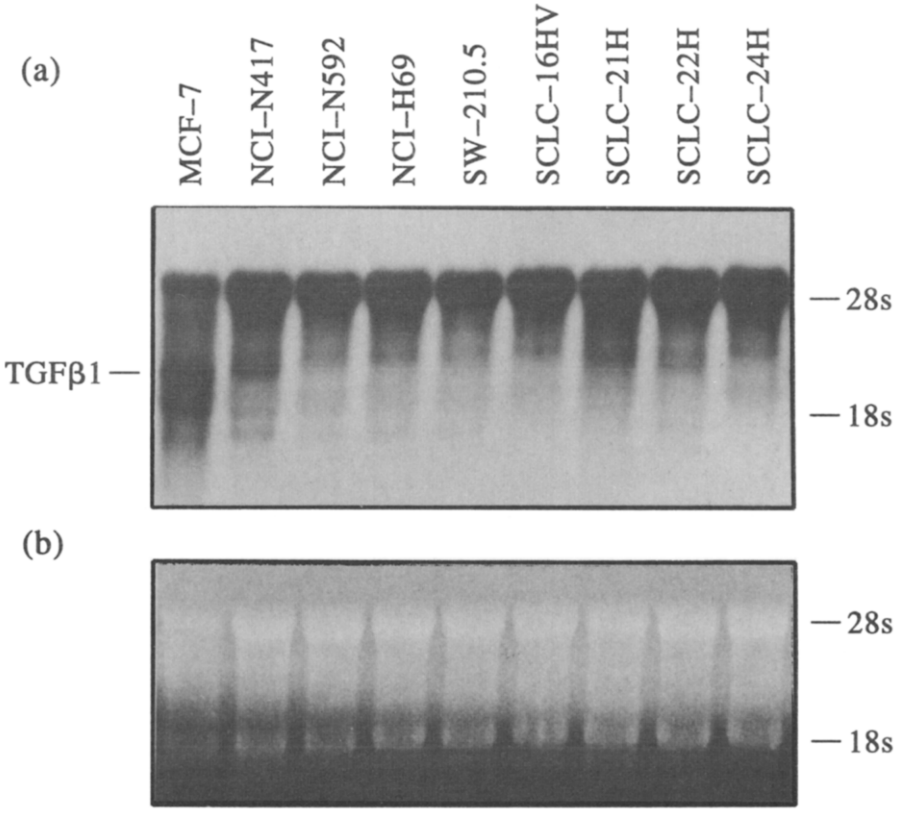

Figure 4. mRNA for TGFβ$_1$ was differentially expressed in SCLC cell lines. (a) Northern blot analysis demonstrates differential expression of mRNA for TGFβ$_1$ in SCLC cell lines. TGFβ$_1$-producing MCF7 cells were used as a positive control. Ten micrograms of RNA were loaded per lane. (b) Ethidium bromide staining of the gel confirmed that comparable amounts of total RNA had been loaded in each lane. X-ray film was exposed for 7 days.

may not always indicate that the bioactive protein is being secreted, the examination of secreted protein is important with respect to the possible interactions between tumour cells and immune functions, which may also have clinical implications.

It has been shown that $TGF\beta_1$ suppresses a variety of cytokine-mediated immune reactions, including proliferation of peripheral T lymphocytes [14–16], generation of cytotoxic T-cells [17–19], IL-1-dependent proliferation of thymocytes [20, 21], proliferation of B lymphocytes [22, 23] or inhibition of cytokine production [24]. We have demonstrated that $TGF\beta_1$ suppresses secretion of IL-2, IFNα, IFNγ and TNFα, but not of IL-1α and IL-1β in whole blood cell culture [7, 8]. Thus, $TGF\beta_1$ blocks the function of the immune system on different levels and impairs secretion of cytokines and their effects.

The fact that SCLC cell lines constitutively secrete an immunosuppressive factor is intriguing. Immunosuppressive cytokines secreted by SCLC may influence proliferation of the malignant cells in their environment by inhibition of immunoreactions directed against the tumour cells. In fact, tumours transfected with $TGF\beta_1$ cDNA have been shown to escape immune surveillance [25]. We have studied cytokine profiles of SCLC patients at the time of diagnosis. We found that, in comparison to normal controls, secretion of cytokines in these patients is selectively impaired. The impaired cytokine profile found in these patients is similar to the cytokine profile induced by $TGF\beta_1$ in whole blood cell culture from healthy individuals [7, 8]. Secretion of $TGF\beta_1$ by SCLC lines and selective suppression of cytokine secretion in SCLC patients may suggest that secretion of $TGF\beta_1$ by SCLC lines is clinically relevant. Thus, secretion of an immunosuppressive factor may be important for malignant growth of SCLC *in vivo*, and suppression of immune functions may influence the course of the malignant disease.

Our results may improve the immunological understanding of the interactions between SCLC and immune effector reactions. These findings may serve as a basis for future clinical investigations that take into account tumour-derived suppression of a variety of cytokines. Stimulation of cytokine secretion as well as blocking of the activity of immunosuppressive factors, such as $TGF\beta_1$, may improve the prognosis of SCLC patients.

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Acknowledgements——We thank Dr P. Amstad for his advice and discussion. We gratefully acknowledge the excellent assistance of M. Allegrini in preparing the photographic artwork. This work was supported in part by the Tumour-Centre Heidelberg–Mannheim.