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Human ex vivo carcinoma cells produce transforming growth factor β and thereby can inhibit lymphocyte functions in vitro

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Abstract We tested 20 human carcinoma samples for the production of transforming growth factor β (TGFβ) in vitro. Tumour cell suspensions without obvious contamination with non-malignant cells were kept in culture conditions for 16 h and their supernatants were added to CCL-64 cells. The proliferation of these cells is inhibited by TGFβ. According to this assay, the supernatants contained both active and latent TGFβ. In addition, the supernatants were found to suppress the spontaneous cytotoxic function and activation of T-cell-enriched lymphocyte populations. A specific monoclonal antibody (mAb) counteracted these effects and therefore we concluded that they were mediated to a large extent by TGFβ. In line with the results obtained with the supernatants, activation of lymphocytes could also be inhibited by tumour cells and their inhibitory effect was weaker in the presence of the TGFβ-specific mAb. It is important to note that, when TGFβ-specific mAb was added to autologous mixed lymphocyte/tumour cell cultures, lymphocyte activation occurred more often. These results thus substantiate the assumption that production of TGFβ may help the survival of potentially immunogenic tumour cells in immunocompetent patients.

Key words TGFβ · Lymphocyte functions · Human carcinomas

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Introduction

The demonstration of specific tumour rejection responses induced by immunisation in experimental systems motivated the search for immune responses against tumours in patients. In vitro assays detecting lymphocytes that reacted with the tumour cells [17, 35, 41], antibodies specific for the tumour [28], and the temporary regressions achieved in immunotherapeutic protocols in certain tumour types indicate that tumours can elicit an immune response in humans. However, the results of immunotherapy are still unsatisfactory.

Motivated by the knowledge that MHC molecules present the antigenic epitopes, much attention has been directed to quantitative alterations in their expression and to the selective down-regulation of certain alleles on tumour cells [11, 25, 37]. Similarly, the expression of adhesion molecules has also been studied on tumour cells because they contribute to the interaction with lymphocytes [13].

Several findings, such as alteration in the signal-transducing molecules on the surface of the patients' lymphocytes [24] and the impaired immune function of lymphocytes resident at the tumour site, which can recover after incubation in vitro, indicated the presence of suppressive factors in the tumour environment [21, 24, 40]. It was indeed demonstrated that tumour cells can release immunosuppressive factors such as prostaglandin E2 and transforming growth factor β (TGFβ) [31]. The latter may have direct consequence because it can inhibit the generation of cytotoxic T lymphocytes and the function of natural killer cells [1, 15]. TGFβ can inhibit cell proliferation and thereby can control tumour growth. However, malignant cells are often less sensitive or they are resistant to the effect of TGFβ. Therefore, it can be assumed that production of TGFβ is advantageous for the tumour because it inhibits immune responses [27, 29].

TGFβ is constitutively produced by almost all cell types mainly in an inactive form. Several cell lines established from malignant tissues have been shown to secrete active molecules as well [4, 10, 18]. The latent form is a molecular

complex from which active TGFβ can be released by cell-membrane-associated proteases [23]. Experimentally it can be activated by heat treatment or by low pH. Only the active molecule can bind to the specific cell membrane receptors. Thus the effect of TGFβ is regulated on several levels by the quantity and quality of the molecule produced by the cell, by the activator capacity and by the expression of specific receptors on the target cell membrane. Through modulations of these latter parameters, cells can acquire resistance to TGFβ.

Elevated amounts of TGFβ mRNA were detected in tumour tissues compared to their non-malignant counterparts. Several tumours were found to be resistant to the growth-inhibitory effect of TGFβ [4, 8–10, 14, 18, 27, 30] and therefore it was proposed that a critical feature of the malignant phenotype is acquisition of resistance to TGFβ.

Earlier studies on the production of TGFβ have been performed on tumour cell lines. We studied ex vivo carcinoma cells for production of TGFβ paying special attention to its inhibitory effect on lymphocyte functions.

Materials and methods

Tumour cell suspensions

Tumour cell suspensions were prepared from surgical specimens of 3 human lung and 16 ovarian carcinomas and of 1 malignant melanoma, either by mechanical dispersion or by treatment of the finely minced tumour tissue with collagenase (3 mg/ml) and DNase (0.2 mg/ml) for 30 min. The tumour cells were separated by a combination of velocity and density sedimentation and by exploitation of the difference in adhesive properties of the various cell types. Sedimented cells of malignant effusions were processed similarly. The procedure described earlier was followed [36]. The cell suspensions used for the experiments contained over 88% viable tumour cells. Contamination with non-malignant cells was estimated by screening 800 cells on smears and by immunofluorescence using mAb specific for T, B cell and monocyte markers. Contamination with these cells was usually under 3%. Ovarian carcinomas were stained with the mAb MOv18 (see below). Except for three patients with ovarian carcinomas, the patients did not receive immunosuppressive treatment before the tests. Frozen stored tumour cells were used as targets in the cytotoxicity assays performed with lymphocytes cultured with tumour cells for 5 days.

Cell lines

Growth of CCL-64, a mink lung epithel cell line (American Tissue Culture Collection, Rockville, Md., USA), is inhibited by TGFβ. Daudi is a Burkitt-lymphoma-derived cell line, which is sensitive to natural and activated killer lymphocytes. Both were propagated in RPMI-1640 medium, containing 10% heat-inactivated fetal bovine serum.

Preparation of lymphocytes

Lymphocytes were collected from heparinized blood after centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway) gradients [6]. The plastic- and nylon-wool-adherent cells were removed [16]. About 91% of the lymphocytes that passed through the nylon-wool column reacted with OKT3, 5% with NKH-1 and 5%–7% with anti-Tac mAb, directed against CD3, CD56 and CD25 respectively. We will refer to this population as NCp.

Activation of lymphocytes by cross-linkage of the CD3 complex

NCp lymphocytes (2×10^5/well) were cultured in 200 µl RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) for 16 h in U-bottom 96-well micro-culture plates (A/S Nunc Delta, Kamstrup, DK-4000 Roskilde, Denmark) coated with CD3 mAb. Lymphocytes incubated in uncoated wells served as controls. Induction of DNA synthesis was estimated by the level of [^{3}H]thymidine incorporation during a 4-h pulse at the end of the culture period [38]. The cytotoxic activity against Daudi and/or ex vivo tumour cells was tested in a 5-h ^{51}Cr-release assay.

Activation of lymphocytes by exposure to autologous tumour cells (mixed lymphocyte tumour cell culture, MLTC)

Aliquots of lymphocytes (5×10^5/well) and mitomycin-C-treated tumour cells were cultured (at ratios between 40:1 and 5:1) in wells of U-bottomed 96-well micro-culture plates in 200 µl medium. Lymphocytes cultured alone served as controls. The plates were centrifuged for 3 min at 200 g and incubated for the indicated period. Cytotoxic activity was tested on day 6 in a 5-h ^{51}Cr-release assay as described earlier [34].

Reagents

rTGFβ was purchased from Calbiochem Novabiochem; the IgG2b anti-TGFβ mAb (2G7) and a non-specific IgG2 mAb were kindly provided by R. Narayanan (Division of Oncology, Roche Research Center, Hoffmann-La Roche Inc, Nutley, N.J.). The anti-TGFβ mAb neutralises the three major isotypes of human TGFβ (TGFβ1, -β2 and -β3), and 1 µg 2G7 mAb neutralises the activity of 0.15–0.5 ng TGFβ1, in the mink lung epithelial cell growth-inhibition assay. The mAb MOv18 [22] is specific for and reacts with 72% of ovarian carcinoma cases. It was used for assessment of the composition of ovarian carcinoma cell suspensions. mAb OKT3 and DAKO-CD21, reacting with T and B lymphocytes respectively, were purchased from Dakopatts A/S, Denmark; anti-leu M5, reacting with CD11c present on all monocytes, was purchased from Becton Dickinson Immunocytometry Systems, California, USA.

Medium

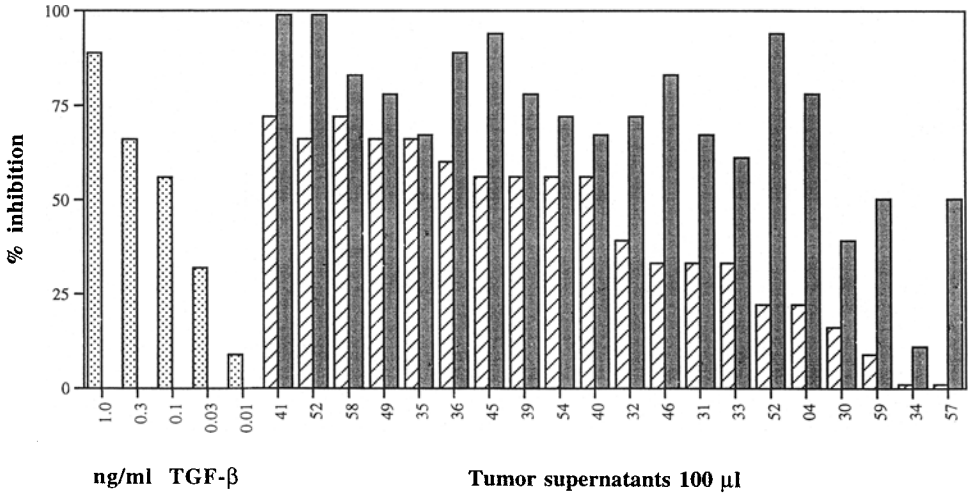
RPMI-1640 medium (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland), supplemented with L-glutamine (200 mM solution, 1% by volume), benzylpenicillin (100 U/ml), spreptomycin sulphate (100 mg/ml), HEPES buffer (0.1 mM) and 10% heat-inactivated fetal bovine serum, was used.

Tumour cell supernatants

Tumour cell suspensions were washed and incubated (10^6/ml) in RPMI-1640 medium supplemented with 0.5% FBS and 0.5% human serum albumin (HSA) for 16–20 h in a total volume of 15 ml in Falcon tissue-culture flasks. Cell viability was controlled by staining with trypan blue. The proportion of dead cells varied in the different samples. Supernatants were collected if fewer than 15% of cells were stained (crude supernatant). For activation of latent TGFβ in the supernatants, one aliquot was heated for 10 min at 85 °C (heated supernatant) and thereafter cooled in ice [5]. The supernatants were used either freshly or after frozen storage.

CCL-64 mink lung epithelial cell growth-inhibition assay

The method described by Cone was followed [7] with minor modifications. CCL-64 cells were harvested with trypsin/EDTA (Gibco) and 2.5×10^4 cells/well were seeded into flat-bottomed 96-well microtitre plates in RPMI-1640 medium. The plates were incubated at 37 °C for



4 h. After two washes with serum-free medium, aliquots of the supernatants were introduced in a total volume of 100 µl RPMI-1640 medium supplemented with 0.5% FBS and 0.5% HSA (AB Kabi, Stockholm, Sweden). The plates were incubated in culture conditions and pulsed for 4 h with 1 µCi [^{3}H]thymidine at the end of the 16-h incubation period. The cells were then washed, exposed to trypsin/EDTA solution and harvested on glass-fibre filters. The radioactivity was measured in a liquid-scintillation counter (model 1900, Packard Instrument). In similar experiments known amounts of rTGFβ were added to the cells.

Fig. 1 Tumour cell supernatants inhibit the growth of CCL-64 cells. Percentage inhibition of [^{3}H]thymidine incorporation during a 4-h pulse at the end of 16 h incubation. Tumour cells were incubated in RPMI-1640 medium containing 0.5% fetal calf serum and 0.5% human serum albumin for 16 h at 37 °C, and the supernatants were collected. A 100 µl aliquot of supernatant was added to semi-confluent CCL-64 cells. *Dotted bars* recombinant tumour growth factor β (rTGFβ), *hatched bars* crude supernatant, *grey bars* heated supernatant. The supernatant samples were tested in five experiments (which all included titration of rTGFβ) and some of them were tested three times. The radioactivities in the five control cultures were between 18 × 10^4 cpm and 32 × 10^4 cpm

Results

Detection of TGFβ in the tumour cell supernatants

TGFβ was detected (1) by its growth-inhibitory effect on the mink lung epithelial cell line (CCL-64), (2) by inhibition of lymphocyte-mediated cytotoxicity and (3) by inhibition of lymphocyte activation induced either through cross-linkage of the CD3 complex or by exposure to autologous tumour cells. The fact that TGFβ present in the supernatants contributed to these effects was substantiated by inhibition with the specific mAb.

Growth inhibition of CCL-64 cells

Out of 20 supernatants collected from the incubated tumour cell suspensions, 18 inhibited the DNA synthesis of CCL-64 cells (Fig. 1). The levels of the inhibitory effect varied.

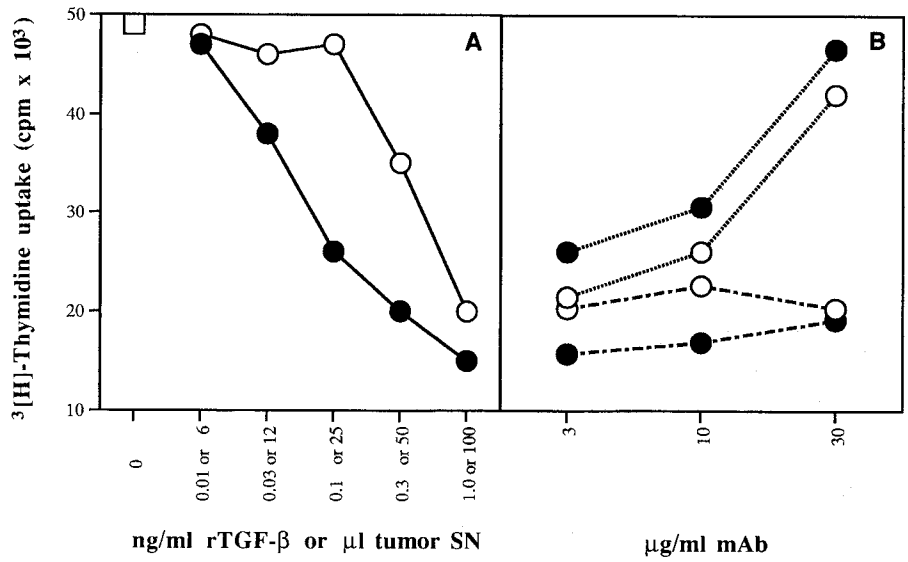


Fig. 2A, B Effect of tumour supernatant 32 on the growth of CCL-64 cells. **A** Dose response of the growth inhibition. Growth inhibition was assayed as in Fig. 1. □ Control culture, ● rTGFβ, ○ tumour supernatant (SN). **B** Abrogation of inhibition by mAb against TGFβ. ●···● rTGFβ (0.3 ng/ml) and mAb against TGFβ, ●–·–● rTGFβ and control mAb, ○···○ heated supernatant (100 µl) and mAb against TGFβ, ○–·–○ heated supernatant and control mAb (○–·–·○)

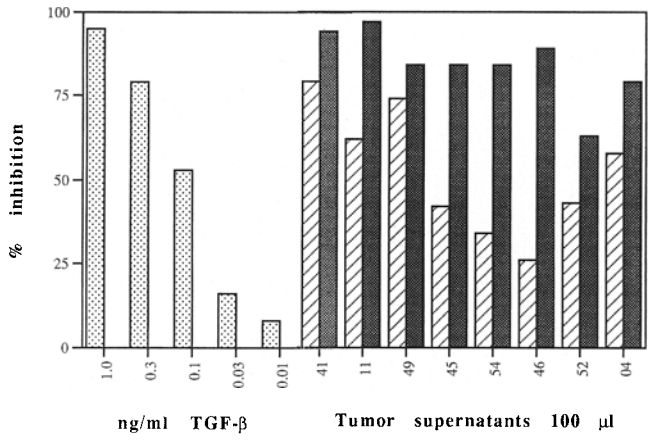


Fig. 3 Tumour cell supernatants inhibit activation of lymphocytes. Activation of lymphocytes by cross-linking of the CD3 complex was detected in a 5-h ^{51}Cr-release cytotoxic assay at an effector:target cell (Daudi) ratio of 50:1. Specific ^{51}Cr release varied between 21% and 42% in the different tests. Lymphocytes were activated in the presence of rTGFβ (*dotted bars*), crude (*hatched bars*) or heated (*black bars*) tumour supernatants

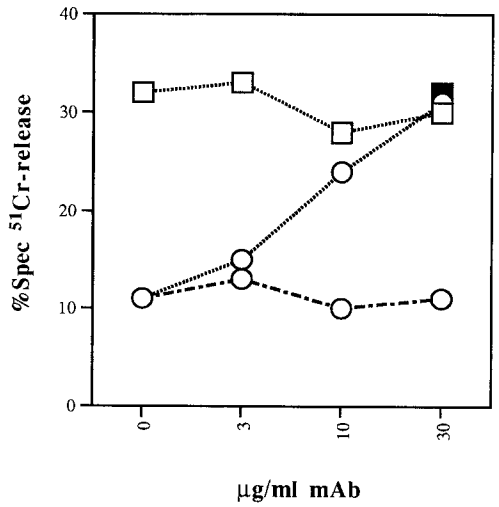


Fig. 4 Anti-TGFβ mAb abrogates the inhibition of lymphocyte activation by tumour supernatant 32. Activation of lymphocytes and the cytotoxicity test were performed as in Fig. 3. Lymphocytes (2×10^5) were suspended in 0.1 ml medium (□ · · · □). Anti-TGFβ mAb (○ · · · ○) or control mAb (○ – · – · ○) was added to 0.1 ml undiluted heated supernatant; TGFβ mAb was added only to the cytotoxicity assay (■)

According to a comparison with rTGFβ, the supernatants contained 0.03–0.4 ng/ml TGFβ, which was thus produced by approximately 10^6 tumour cells. After heat treatment, the inhibitory effect of the supernatants increased, indicating that they contained latent TGFβ as well. The heated supernatants were five- to tenfold more efficient than the crude samples and corresponded to 0.3–>1.0 ng/ml rTGFβ. The proportion of active and latent TGFβ varied in the supernatants collected from the different tumours.

The dose response of the growth inhibition mediated by rTGFβ and by the supernatant of ovarian carcinoma cells (no. 32) is given in Fig. 2A. It is important to note that the inhibitory effect of the supernatant was neutralised by the anti-TGFβ mAb (Fig. 2B).

The stronger growth inhibition of the heated supernatants, compared to the crude aliquots, and its abrogation by the TGFβ-specific mAb demonstrated that the tumour cells produced both active and latent TGFβ.

Inhibition of lymphocyte-mediated cytotoxicity

Eleven of the supernatant samples were also tested for their effect on the natural killer function of blood lymphocytes. Lysis of Daudi cells was reduced in a dose-dependent manner when the lymphocytes were preincubated with rTGFβ for 16 h. A 1 ng/ml rTGFβ solution abrogated the lytic potential. The supernatants were also inhibitory and their effect corresponded to that of 0.05–0.3 ng/ml rTGFβ. The heated supernatant aliquots had stronger inhibitory activity, corresponding to 0.2–0.5 ng/ml rTGFβ. When mAb against TGFβ was added to the supernatant used for preincubation of the effector lymphocytes, their lytic function did not decrease (these results are not shown).

Activation of lymphocytes by cross-linkage of the CD3 molecule complex

Activation of lymphocytes exposed to immobilised CD3 mAb was monitored by measuring the induction of DNA synthesis and the generation of cytotoxic activity. Induction of DNA synthesis was inhibited by the supernatants in all 12 experiments performed. The inhibition brought about by the supernatants (and by rTGFβ) was dose-dependent (these results are not shown). Similarly, when lymphocytes were activated in the presence of supernatants, they lysed Daudi cells with lower efficiency. According to these results, the TGFβ content in the crude and heated supernatants varied in the ranges 0.03–0.3 ng/ml and 0.2–1.0 ng/ml respectively (Fig. 3). TGFβ-specific mAb arrogated the supernatant-mediated inhibition in a dose-dependent manner (Fig. 4).

Effect of tumour cells on the activation of lymphocytes

We have thus obtained evidence for the production of TGFβ by the tumour cells during in vitro incubation. In the next step we tested whether the response of lymphocytes to the activating stimulus can be suppressed by the presence of tumour cells. To this end, tumour cells were added to the lymphocytes when they were incubated in wells coated with CD3 mAb. Activation of the lymphocytes was monitored by the induction of cytotoxic function against Daudi and against the autologous tumour cells. Generally, the activated lymphocytes had an elevated lytic effect against Daudi cells. In the majority of experiments the lymphocytes did not lyse the autologous tumour cells, unless they were activated. In Fig. 5A and B, 5 experiments are shown in

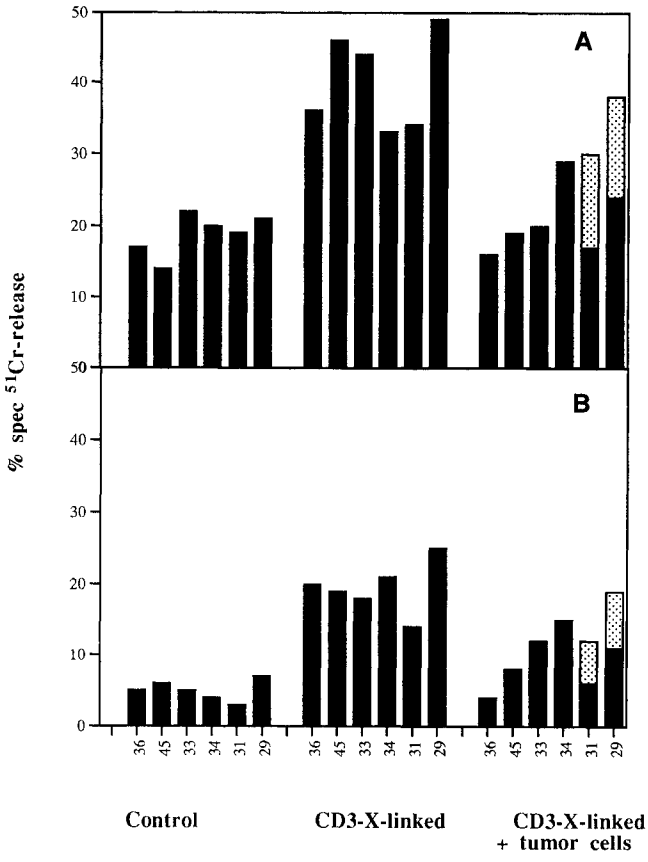


Fig. 5A, B Lymphocyte activation by CD3 cross-linkage is inhibited by tumour cells. Activation of lymphocytes and the cytotoxicity assay were performed as in Fig. 3. Tumour cells (identified by the patient number) were added to the activation step at a lymphocyte:tumour cell ratio of 10:1. Target: (**A**) Daudi, (**B**) identical tumour. *Dotted bars* TGFβ mAb was present during lymphocyte activation at 30 μg/ml

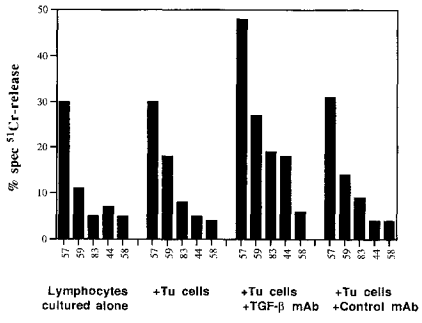


Fig. 6 Anti-TGFβ mAb potentiates the induction of cytotoxic activity against autologous tumour cells in 5-day MLTC. Mixed lymphocyte-tumour cell cultures (MLTC) at a lymphocyte:tumour cell ratio of 10:1. Cytotoxicity was detected in a 5-h ^{51}Cr-release assay at an effector: target cell ratio of 50:1. Anti-TGFβ, or control mAb (30 μg/ml each) was present in the 5-day MLTC

which cytotoxicity was not induced or the effect was weaker if tumour cells were present during activation, In 2 of these experiments (tumours 31 and 29) TGFβ mAb was added to the samples during activation and this measure partially restored the induction of lytic function.

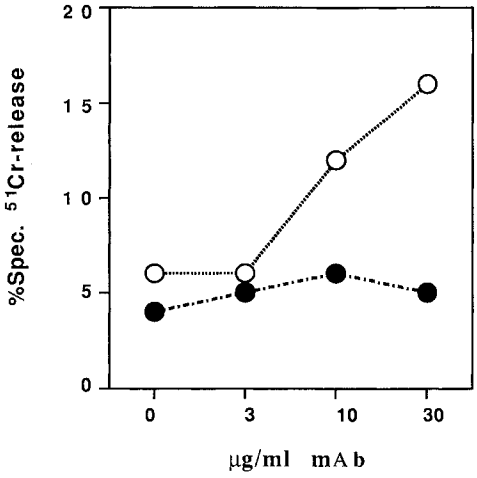


Fig. 7 Induction of cytotoxic activity against autologous tumour cells. Dose dependence of the potentiating effect of TGFβ mAb. The MLTC and the cytotoxicity assays were performed as in Fig. 6. Lymphocytes were cultured with autologous melanoma (04) cells in the presence of TGFβ mAb (○ · · · ○) or control mAb (● – · – ·)

Generation of cytotoxic effectors in 5-day mixed lymphocyte/tumour cell cultures

Lymphocytes of five patients were cultured with the autologous tumour cells for 5 days. One aliquot was cultured alone and three aliquots contained tumour cells. To one of these three mixed cultures TGFβ mAb, and to another the control mAb was added. After 5 days the cytotoxicity of lymphocytes against tumour cells was tested (Fig. 6). In 4/5 experiments the lymphocytes of the MLTC containing the anti-TGFβ mAb had stronger cytotoxic activity.

Figure 7 shows the dose-dependent effect of TGFβ mAb. It is, therefore, likely that TGFβ is produced in the culture and inhibits the lymphocyte response.

Discussion

Cytokines belonging to the TGFβ family are produced by almost all cell types, both normal and malignant ones [26, 27]. They can regulate a wide variety of cellular functions [20]. In experimental systems the results depend on the test conditions, on the functional state of the cells, and on the presence of other cytokines [32]. Therefore the results can be discordant. Usually TGFβ inhibits cell proliferation, but growth-stimulation effects have also been reported [1, 15, 19].

The growth-modifying effect of TGFβ on a tumour may be direct, but it may also act indirectly by influencing the environment through inducing the production of various cytokines, fibronectins, interstitial collagens, small secretory proteoglucans, thrombospondin, tenascin and laminin (reviewed in [20]), and the expression of adhesion molecules. The outcome of the influence on tumour growth is thus the result of these composite effects.

Previously tumour cell lines were shown to produce TGFβ [4,10, 18]. Here we demonstrated that ex vivo carcinoma cells can produce both active and inactive TGFβ and we obtained evidence that, because of this property, they can suppress lymphocyte functions.

Activated lymphocytes and macrophages can also produce TGFβ [2, 39], therefore we were careful to control the purity of the tumour cell suspensions. We used samples that contained fewer than 3% normal cells, as judged by reactivity with mAb specific for T cell, B cell and monocyte markers.

The participation of TGFβ in tumour growth regulation was shown earlier in experimental systems. The growth of stable transfectants of a fibrosarcoma cell line (K-BALB) for dexamethasone-inducible expression of antisense RNA to p65 was inhibited both in vitro and in vivo. Dexamethasone treatment of the nude mice induced regression of the growing tumours [12]. The effect of the antisense p65 was attributed to inhibition of TGFβ production. In other experiments, a murine fibrosarcoma with strong immunogenicity, was manipulated for over-production of TGFβ. These cells inhibited lymphocyte responses in vitro and did not induce cytotoxic T lymphocytes when inoculated into syngeneic mice, while the parental cell line did so [33].

The role of TGFβ in the suppression of the host defence was also demonstrated with a human breast cancer cell line inoculated into nude mice. The cells did not grow in hosts treated with TGFβ mAb [3].

Suggestive evidence for TGFβ production by tumour cells was obtained in patients with advanced hepatocellular carcinoma. The patients' sera contained elevated TGFβ levels, which returned to normal values after surgery [30].

Tumour cells are usually resistant to the growth-inhibitory effect of TGFβ. Resistance can coincide with the acquisition of metastasising properties. In one experiment, the growth of a metastasising subline of the H-*ras*-transformed murine fibroblasts was even stimulated by TGFβ [29].

While most cell types produce latent TGFβ, several malignant lines e.g. glioblastoma, osteosarcoma and some Burkitt lymphoma lines were shown to secret the active form as well [4, 10, 18].

We obtained evidence for the presence of active and latent TGFβ in the supernatants of ex vivo ovarian and lung carcinoma and malignant melanoma cells when they were incubated in vitro for 16 h. The secreted TGFβ could inhibit the cytotoxic function and the activation of lymphocytes. These results thus substantiate the assumption that TGFβ production may contribute to the escape of tumour cells from a potential immune response.

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