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Transforming growth factor-beta 1 (TGF-β1)- and β2-like activities in malignant pleural effusions caused by malignant mesothelioma or primary lung cancer

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SUMMARY

We investigated the levels of TGF-β in malignant pleural effusions (MPE) caused by malignant mesothelioma (MESO) or primary lung cancer. TGF-β levels in MPE caused by MESO were 283·9 ± 219·2 pM (mean ± s.d.) and were three to six times higher than those due to primary lung cancers ($P < 0{\cdot}01$ or $P < 0{\cdot}05$). We also evaluated TGF-β1- and β2-like activities in MPE using specific polyclonal antibodies. Although TGF-β1-like activity could be detected in all cases, TGF-β2-like activities were detected in five of seven in MESO and in a few cases with primary lung cancer. These results demonstrate that the levels of total TGF-β and TGF-β2-like activity may be clinically useful to differentiate MESO from primary lung cancer. Our data also suggest that TGF-β may help further characterize the clinical features of MESO.

Keywords TGF-β1 TGF-β2 malignant pleural effusion malignant mesothelioma primary lung cancer

INTRODUCTION

Malignant mesothelioma (MESO) is a neoplasm which originates from the mesothelium or peritoneum which, in turn, is derived from the mesoderm in embryogenesis [1]. Asbestos fibres are well known to be one of the causes of MESO. Clinically, MESO manifests as pleural thickening on plain chest roentgenogram and chest computed tomography (CT). In malignant pleural effusion (MPE) caused by MESO, the concentration of hyaluronic acid is higher than in MPE caused by adenocarcinoma (AD). This finding is useful for differentiating between MESO and AD [2].

TGF-β is a 25-kD hetero- or homodimeric polypeptide. TGF-β, *in vivo*, modulates immune reactions [3] and promotes fibrogenesis [4]. Major sources of TGF-β, *in vivo*, are the many kinds of malignant cells as well as platelets and bone [5]. They produce TGF-β in latent and/or active forms [5]. It has been reported that at least three types of TGF-β isoforms exist *in vivo* (TGF-β1, β2 and β3) [6]. TGF-β, in general, inhibits the growth of epithelial cells [7]. TGF-β is unique in its ability to stimulate the proliferation of normal human mesothelial cells, epithelial cells which originate from mesoderm [8]. Moreover, TGF-β is chemotactic for fibroblasts [9] and stimulates hyaluronic acid production by these cells [10]. Previous studies showed that normal human mesothelial cells and MESO produce TGF-β [11,12]. In addition, TGF-β augments hyaluronic acid production by normal human mesothelial cells [13]. Although it is suggested that TGF-β is not an autocrine cytokine for MESO, it may promote the proliferation of normal human mesothelial cells and mesenchymal cells in a paracrine fashion, or in combination with other cytokines [11,12]. In embryogenesis, TGF-β2 mRNA and its protein are found mainly in mesoderm-derived tissues, and promote the development of such tissues [6]. In this context, we hypothesize that TGF-β greatly contributes to pleural thickening, which is one of the clinically characteristic features of MESO. In the present study, we measured the levels of TGF-β and activities of TGF-β isoforms in MPE by MESO and primary lung cancer.

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PATIENTS AND METHODS

Malignant pleural effusions

MPE were obtained by thoracocentesis from seven patients with small cell lung carcinoma (SM), 10 with AD, six with squamous cell carcinoma (SQ) and seven with MESO. Benign pleural effusions were obtained from 15 patients with tuberculous pleurisy. Every patient had been diagnosed by bronchoscopy, pleural biopsies, cytology and tumour marker analysis of pleural effusions. Pleural effusion was centrifuged at 1300 *g* for 15 min at 4°C. In general, TGF-β exists in active and inactive forms *in vivo* and *in vitro* [5], so that we had to evaluate each form of TGF-β.

The supernatant supplemented with 10% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) was dialysed against 1 M acetic acid for evaluation of total (both active and inactive) TGF-β, or against 2 mM HEPES pH 7·0 (Wako Chemicals, Osaka, Japan) for only active TGF-β. The samples were centrifuged at 25 000 g for 15 min at 4°C, and these supernatants were stored at −70°C until assay.

Assay of TGF-β

An assay of the growth inhibition of Mv1Lu mink lung epithelial cell (CCL64; American Tissue Culture Collection, Rockville, MD) by TGF-β was modified as follows [14]. Mv1Lu cells were cultured at 1×10^4 cells/well in a 24-well culture plate (Corning, NY) in 0·5 ml Dulbecco's modified Eagle's medium (DMEM; Flow Labs, Irvine, UK) supplemented with 5% fetal calf serum (FCS), streptomycin 100 μg/ml and penicillin 100 U/ml. Aliquots of recombinant TGF-β1 (rTGF-β1; King Brewery, Kakogawa, Japan), porcine TGF-β2 (pTGF-β2; R&D, Minneapolis, MN) or samples were added at this time. As a control, an aliquot of DMEM was added. After 72 h of incubation at 37°C in 5% CO_2 25 μl of 20 μCi/ml of ^{125}I-deoxyuridine (^{125}I-UdR; Amersham Co., Arlington Heights, IL) were added and cultured for 24 h. The cells were washed with cold PBS with 1% BSA and fixed with 10% ice-cold trichloroacetic acid (TCA) and dissolved in 1 N NaOH. ^{125}I-UdR activity was measured with a gamma counter (Aroca, Tokyo, Japan). In a preliminary study, IL-1, IL-6, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) did not affect the growth of Mv1Lu cells.

Evaluation of TGF-β1- and β2-like activities

To evaluate further the activities of TGF-β isoforms (TGF-β1 and β2), neutralization was performed with the specific polyclonal rabbit antibody against TGF-β1 and β2, TGF-β2 (R&D), or with the specific polyclonal chicken antibody against TGF-β1 (King Brewery). In preliminary studies, Mv1Lu cell growth inhibition by 160 pM rTGF-β1 or pTGF-β2 was blocked completely with each antibody at a dose of 100 μg/ml. Non-immune rabbit IgG was used as a control. The specific polyclonal antibody against TGF-β2 used in this study may very weakly cross-react with TGF-β3 [15]. In mouse embryogenesis, TGF-β2 and TGF-β3 mRNA and proteins were found in both pleura and mesentery [16]. Therefore, it is possible that a little TGF-β3-like activity may exist in MPE caused by MESO. Based on this, TGF-β1- and TGF-β2-like activities were shown not as molarity, but as percentage growth inhibition percentage.

Measurement of platelet factor-4

To evaluate the TGF-β level in MPE, the influence of platelets must be considered, because platelets are, *in vivo*, the major source of TGF-β. Both TGF-β and platelet factor-4 (PF-4) are stored in α-granules in platelets. PF-4 indicates the release of α-granules from platelets [17]. The levels of PF-4 in MPE were measured with commercially available ELISA kits (Diagnostica Stago, France), as previously described [18]. Briefly, 96-well microtitration plates were coated with anti-PF-4 rabbit MoAb (10 μg/ml) in 100 μl/well of PBS pH 7·4. Following overnight incubation at 4°C, the wells were blocked with a 1% BSA in PBS solution for at least 1 h at room temperature, and washed three times with PBS containing 0·05% Tween 20 (Tween–PBS; washing was always performed with this buffer). Two hundred microlitres of samples or standard in PBS containing 0·1% BSA (BSA–PBS) were added to the wells and the plate incubated at room temperature for 1 h. Subsequently, the wells were washed five times, supplemented with 100 μl of peroxidase-labelled anti-PF-4 MoAb (diluted to 1 : 1000 with BSA–PBS) and incubated at room temperature for 1 h. Finally, 100 μl of enzyme substrate (1 mg/ml o-phenylenediamine in 0·1 M sodium citrate buffer pH 5·0) were added to each well and the plates incubated at room temperature for 3 min. The reaction was stopped by adding 50 μl of 3 M H_2SO_4 to each well and the absorbance at 492 nm was determined, using a EIA Reader (Richmond, CA).

Statistical analysis

The data obtained from the five groups were independent of one another. They were non-parametrically compared using the Mann–Whitney U-test.

RESULTS

Quantification of TGF-β

The level of TGF-β was determined by the Mv1Lu cell growth inhibition assay. The levels of TGF-β were calculated from a dose-dependent standard curve of rTGF-β1 (Fig. 1).

The total TGF-β level in MPE caused by SM was 48·3 ± 22·6 pM, 89·3 ± 47·7 pM by AD, 74·7 ± 49·9 pM by SQ, and 283·9 ± 219·2 pM by MESO (mean ± s.d.) (Fig. 2). In pleural effusions caused by tuberculous pleurisy, the TGF-β level was 46·1 ± 35·2 pM (Fig. 2). TGF-β levels in MPE caused by MESO were three to six times higher than those in MPE caused by primary lung cancer or TB ($P < 0{\cdot}01$ or $P < 0{\cdot}05$). On the other hand, there was no significant difference in the level of TGF-β among MPE caused by SM, AD, SQ or tuberculous pleurisy. The active TGF-β level in MPE was less than 5% of the total TGF-β in every sample studied (data not shown).

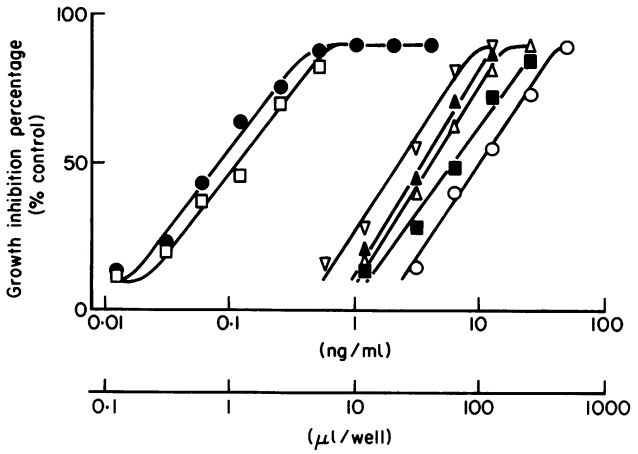

Fig. 1. Dose-dependent curves of recombinant (r)TGF-β1, porcine (p)TGF-β2 (ng/ml) and acidified samples of malignant pleural effusion (MPE; μl/well) by the growth inhibition assay with CCL64 cells (see Patients and Methods). Acidified samples were prepared for measurement of total (active and inactive) TGF-β activity. The results represent the mean of triplicate experiments. Neutralized samples for evaluation of active TGF-β activity are not shown. ●, rTGF-β1; □, pTGF-β2; ○, tuberculous pleurisy; ■, small cell carcinoma; △, adenocarcinoma; ▲, squamous cell carcinoma; ▽, malignant mesothelioma.

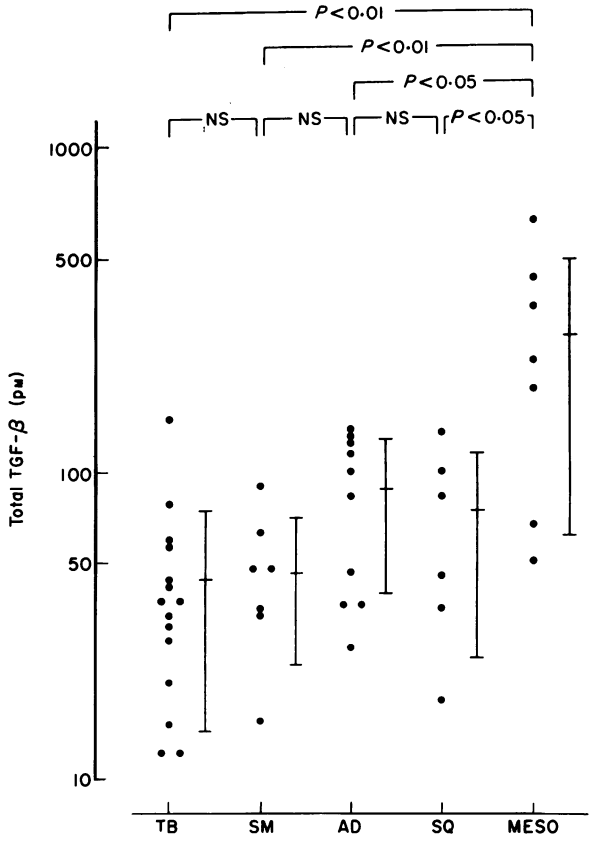

Fig. 2. The levels of total TGF-β in each malignant pleural effision (MPE) and tuberculous pleural effusions (mean ± s.d.). TGF-β levels in MPE caused by malignant mesothelioma (MESO) were significantly higher than those caused by primary lung cancer and tuberculous pleurisy (TB; $P < 0{\cdot}01$ or $P < 0{\cdot}05$). There was no significant difference in the TGF-β levels among adenocarcinoma (AD), squamous cell carcinoma (SQ), small cell carcinoma (SM), and TB. NS, Not significant. Bar shows mean ± s.d.

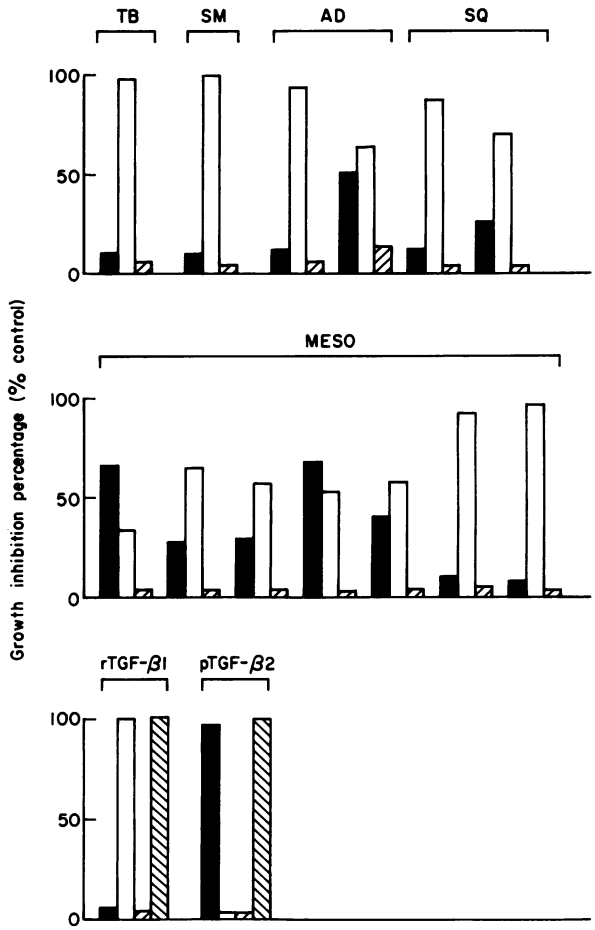

Fig. 3. Blocking of TGF-β activity in malignant pleural effusion (MPE) by the specific anti-TGF-β antibodies. CCL64 cells were cultured with each sample of MPE, recombinant (r)TGF-β1 or porcine (p)TGF-β2 (4 ng/ml) in the presence of the specific antibody against TGF-β1, TGF-β2 or TGF-β1 and β2 at 100 μg/ml. Non-immune rabbit IgG at 100 μg/ml was used as a control. The results show the mean of triplicate experiments. ■, Anti-TGF-β1; □, anti-TGF-β2; ▨, anti-TGF-β1 and β2; ▧, rabbit IgG.

The PF-4 level in each MPE was less than 20 ng/ml (data not shown), which was less than that in normal human plasma [18]. Although MPE is frequently haemorrhagic, these results indicate that the TGF-β level derived from activated platelets by haemorrhage is negligible in pleural effusions.

TGF-β1- and TGF-β2-like activities in MPE (Fig. 3)

In each case, all TGF-β activity was completely neutralized by the specific polyclonal rabbit antibody against TGF-β1 and β2. Moreover, to evaluate the activities of TGF-β isoforms, TGF-β activity in MPE was neutralized by the specific polyclonal antibody against TGF-β1 or TGF-β2. The full extent of TGF-β1- and TGF-β2-like activities is shown by non-immune rabbit IgG in Fig. 3. In five out of seven cases of MESO, one of 10 cases of AD, and one of six cases of SQ, TGF-β2-like activity was 30–50% of the total TGF-β activity in MPE. In every other case of MPE and in every case of tuberculous pleural effusion, only TGF-β1-like activity was detected.

DISCUSSION

We report that the level of TGF-β in MPE caused by MESO is higher than that in MPE caused by primary lung cancer. TGF-β2-like activity was very low or absent in many samples, but significantly high in five out of seven cases of MESO. The procedures used to differentiate between MESO and AD include the hyaluronic acid level in the pleural effusion, and pathological examinations of pleural effusion and pleura [2]. In addition to these procedures, our findings may be used to differentiate between MESO and AD.

In this study we did not determine how much of the TGF-β in MPE was produced by malignant cells. In pleural effusion caused by tuberculous pleurisy, we believe the sources of TGF-β to be fibroblasts and normal mesothelial cells [19]. In MPE caused by SM, TGF-β may be produced by non-malignant cells in the pleura, because SM has no TGF-β mRNA [19]. MESO, AD and SQ express TGF-β mRNA and secrete TGF-β protein, in contrast to SM [11,20]. Therefore, some of the TGF-β in MPE caused by these malignancies is thought to be derived from these malignant cells. Miyazono and colleagues reported that a malignant cell produces small latent TGF-β complexes, which have no latent TGF-β binding protein [21]. We did not

study this binding protein in MPE. In our study more than 95% of TGF-β in MPE was of the latent form. Latent TGF-β, a stable form *in vivo*, has biological functions [22]. In previous reports, TGF-β produced by AD and SQ promoted stroma formation, and mediated their invasion and metastasis [23,24]. Therefore, latent TGF-β in MPE may help to promote tumour development *in vivo*.

Although TGF-β, in general, inhibits the proliferation of epithelial cells [7], TGF-β stimulates some epithelial cells, such as normal human mesothelial cells [8]. Moreover, TGF-β augments hyaluronic acid production by normal human mesothelial cells and fibroblasts [10,13]. These findings and our data suggest that TGF-β may greatly contribute to the formation of clinical features of MESO, characterized by thickening pleura on chest films and large amounts of hyaluronic acid in MPE in MESO.

Many kinds of malignant cells produce TGF-β1 and TGF-β2, at various rates [25]. In the mouse embryo, TGF-β2 mRNA and its protein were found primarily in mesoderm-derived organs [6]. Rosa *et al.* reported that TGF-β2-like activity augments mesoderm induction [26]. TGF-β1 is also important for the development of mesoderm-derived organs [27]. Previously obtained data suggest that TGF-β1- and TGF-β2-like activities may greatly contribute to the proliferation of the pleura derived from mesoderm. Because MESO is a neoplasm of the pleura, higher amounts of TGF-β2-like activity may be a characteristic result in MESO. Therefore, our results may be clinically useful for the differentiation of pleural effusion between MESO and primary lung cancer.

In conclusion, members of the TGF-β superfamily may be important determinants of the clinical features and pathogenesis of MESO. Further *in vivo* and *in vitro* studies are required to test our hypothesis.

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