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Transforming growth factor β production by spontaneous malignant mesothelioma cell lines derived from Fisher 344 rats

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Abstract We investigated whether transforming growth factor β (TGF-β) is involved in the growth of malignant mesothelioma (MM) cells in culture. TGF-β production was examined in two mesothelioma cell lines (MeET-4 and -6) that were established from rat spontaneous MM in our laboratory. TGF-β bioactivity in conditioned medium of these cell lines was analyzed using a CCL64 mink lung epithelial cell growth inhibition assay and found to be 30–70 times higher than that of normal rat mesothelial cells (MCs). The MM cell lines also showed considerably higher levels of TGF-β mRNA expression when compared with MCs. The bioactivity and mRNA expression level were greater in MeET-4 than MeET-6. When MeET-4 was treated with antisense TGF-β1 oligonucleotide (ODN), a significant decrease in both anchorage-dependent and -independent growth was observed. Treatment with exogenous TGF-β resulted in no effects on the growth pattern of the MM cell lines, while proliferation of the MCs was slightly induced. It is considered that TGF-β appears to be produced by rat spontaneous MM cells through an autocrine mechanism and could modulate the malignant growth of the tumor cells.

Keywords Transforming growth factor-β · Rat · Spontaneous malignant mesothelioma

Introduction

Malignant mesothelioma (MM) is an aggressive tumor that arises from the thoracic and abdominal serosal membrane [11]. Most cases of MM can be linked to asbestos exposure in humans, although the pathogenesis of the lesion has still been a target of future studies. It is proposed that a possible trigger of cell transformation can be owed to DNA damage of the mesothelial cells (MCs) caused by the generation of oxygen free radicals by macrophages laden with asbestos fibers, with the subsequent growth stimulation by growth factors elaborated by the macrophages or the MCs themselves [8, 26, 28]. A number of studies on the growth of MM have been conducted to explore the primary growth stimulants, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor, and transforming growth factor-α [8, 9, 29, 33, 35, 36, 37]. Similarly, it has been reported that a significant level of transforming growth factor (TGF)-β is produced by some human MM cells [22, 27, 32]. In addition, previous studies on asbestos-induced mouse MM, using antisense TGF-β oligonucleotide (ODN) have suggested that TGF-β may function as a direct growth factor in the development of MM [6, 25].

In Fisher 344 (F344) rats, abdominal MM is a rather rare spontaneous tumor with an aged male predominance [11, 12, 23, 24]. The morphological features of the F344 rat MM are similar to those of human or asbestos-induced murine MM, but they are more uniform and, typically, show a simple papillary growth of epithelial-type cells [10, 23, 24, 31]. Previously, we established cell lines from rat spontaneous mesothelioma [18]. These cell lines shared the same growth characteristics with asbestos-induced human and murine MM cell lines in such disposition as serum independent proliferation, tumorigenicity, and soft agar colony formation. It was suggested that altered secretion of some growth factors could potentiate the modulation of growth property in F344 rat MM cells [18]. As it is conceivable that some types of growth factors may play a common role in the promotion of a critical step of MM development in both humans and rats, clarification of altered profiles of various growth factors in the rat MM cell lines would be helpful to understand the mechanism(s) for the development of

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asbestos-related MM. Among the growth factors, IGF, PDGF, and TGF-β, which have been suggested to be involved in this tumor growth, TGF-β was selected for the present study with rat MM cell lines because IGF and PDGF are reported to have a significant species difference in their production between human and rat MCs [29, 37]. The present report describes the bioactivity of TGF-β and its mRNA expression in the two cell lines derived from the F344 rat spontaneous MM, and the role of TGF-β in the growth of MM cells is discussed. The effects of exogenous TGF-β on the growth of MM cells and normal MCs are also described for comparison.

Materials and methods

In the present study, we used two cell lines, MeET-4 and -6, from the F344 rat spontaneous MM [18] that were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku, Tokyo, Japan) with 10% fetal bovine serum (FBS, Gibco, N.Y.), 2 mM L-glutamine, and antibiotics (kanamycin, 5 ng/ml; ICN Biomedicals, UK) at 37°C, 95%O_2/5%CO_2. Normal rat MCs were obtained from the retroperitoneal cavity of male adult F344 rats. The rats were anesthetized with ether and were immediately killed by exsanguination from the axillary artery. These animals were handled according to the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [14]. The scrotum wall was removed under sterile conditions, washed with phosphate-buffered saline (PBS), then bathed in DMEM with 10% FBS. The inner surface of the scrotum was scraped repeatedly with a cell scraper (Coster, Cambridge, Mass.). The cells obtained were seeded onto 90 mm culture dishes. Subsequently, the MC cultures were maintained up to ten passages in DMEM with additives used for MM cells. Immunohistochemistry revealed positive reactions against keratin and vimentin in these cells.

For preparation of conditioned media, MCs and MM cells were seeded onto 90-mm culture dishes and grown for 24–48 h until reaching approximately 80% confluency. The media was then replaced three times over an 8-h period with serum-free DMEM. Thereafter, serum-free DMEM was added and conditioned for 48 h before it was concentrated 200 times by Vivapore 2 (Vivascience Ltd., Binbrook Hill, England) and frozen at –80°C until assayed. Cells from the conditioning dishes were trypsinized and counted for determination of the amount of TGF-β per cell.

TGF-β bioactivity was determined using the CCL64 mink lung epithelial cell growth inhibition assay [4]. Briefly, CCL64 cells (Mv.1.Lu, Riken Cell Bank, Wako, Saitama, Japan) grown at low density were trypsinized, washed twice in assay medium [DMEM supplemented with 10 mM HEPES (hydroxyethylpiperazine ethanesulfonic acid), 0.05 mM 2-mercaptoethanol, and 0.5% bovine serum albumin (Roche Diagnostics, Mannheim, Germany)], seeded into 96-well flat bottom plates at 5×10^4 cells/well, and incubated at 37°C for 2 h. To detect latent TGF-β, half of each conditioned media sample was acidicly activated by adding HCl to a final concentration of 0.045 N, incubated for 20 min at room temperature, and then neutralized with NaOH/HEPES to a final concentration of 0.045 N NaOH/7.5 mM HEPES. Samples and standards (recombinant human TGF-β1, Roche Diagnostics) were diluted in assay medium and added in triplicate to the plates of CCL64 cells. Following a 48-h incubation, a viable cell number was assessed using a cell counting kit (Dojindo Laboratories, Kumamoto, Japan). Optical density at 450 nm was determined with a plate reader NJ-2300 (InterMed Japan Co. Ltd., Tokyo, Japan). TGF-β levels were calculated from the standard curve and corrected for concentration and dilution factors, the conditioning period, and the cell numbers in the conditioning culture.

Total RNA was prepared from mesothelioma or MCs using the Isogen RNA extraction kit (Nippon Gene, Toyama, Japan). To prevent from contamination of genomic DNA, the RNA was treated with deoxyribonuclease I (DNase I; Takara Biomedicals, Shiga, Japan). For the polymerase chain reaction (PCR) analysis of RNA, a RNA PCR kit (Takara Biomedicals) and the following primers were used:

1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH):
   sense, "5′-ACCACAGTCCATGCCATCAC-3′"
   (GeneBank no. X02231),
   antisense, 5′-TCCACCACCCTGTTGCTGTA-3′
2. TGF-β1: sense, "5′-GCCCTGGATACCAACTACTGCTTC-3′"
   (GeneBank no. X52498),
   antisense, 5′-TCAGCTGCACTTGCAGGAGCGCACGATCAT-3′

These reagents and primers were used according to the manufacturer's recommendations.

The conditions for amplification were as follows: GAPDH, 94°C for 2 min for one cycle and 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min for 28 cycles; and TGF-β1, 94°C for 2 min for one cycle and 94°C for 1 min, 63°C for 1 min, and 72°C for 2 min for 28 cycles. Cycle curve studies confirmed that the reactions had not reached the plateau of the amplification curve for any primer pair. PCR products were subjected to electrophoresis, and the gel was stained with Vistra Green (American Pharmacia Biotech, Tokyo, Japan). Stained PCR products were analyzed with a Chemifluorescence imager (American Pharmacia Biotech), and normalized values (TGF-β1/GAPDH) were evaluated.

Antisense TGF-β1 and control ODNs purchased from Bionostick (Göttingen, Germany) were used in this study according to the manufacturer's recommendation (2 μM). For the anchorage-dependent cell proliferation assay, 100 μl MeET-4 cell suspension (5×10^4 cells/ml) were seeded onto 96-well microtiter plates and incubated for 12 h in DMEM containing 10% FBS to allow cell adhesion. Thereafter, antisense TGF-β1 or control ODNs were added to each well and incubated at 371 for 48, 72, and 96 h. Each treatment was performed on at least five wells, and wells without any ODNs were also settled. Plates were assessed for relative cell number using the cell counting kit.

Before the anchorage-independent cell proliferation assay, subconfluent MeET-4 cells grown on 90-mm culture dishes were preincubated in DMEM containing 10% FBS and antisense TGF-β1, with or without control ODNs, for 48 h. Then, cells were harvested and suspended in 1.5 ml DMEM containing 10% FBS and 0.5% bactoagar with antisense TGF-β1 or with or without control ODNs. The cells were seeded onto a 60-mm dish with a solid layer of 7 ml DMEM containing 0.5% bactoagar and 10% FBS. Dishes were incubated for 2 weeks at 37°C, and soft agar colonies were counted with a minimum size cut-off of 20 cells per colony.

To examine the effects of exogenous TGF-β on proliferation of MM cells or MCs, 10^4 cells per well were seeded onto a 96-well microtiter plate and incubated with DMEM containing 10% FBS for 12 h. The medium was replaced with serum-free DMEM and incubated for an additional 12 h. TGF-β (human recombinant TGF-β1, Roche Diagnostics) was added to each well in triplicate at the concentrations of 0, 0.1, 1, and 10 ng/ml. After 48 h of incubation, cell number was obtained using the cell counting kit. Bioactivity of TGF-β was statistically analyzed using the Student's *t*-test. The data of anchorage-dependent and anchorage-independent cell proliferation and the effect of exogenous TGF-β on cell growth were evaluated using one-way layout analysis of variance (ANOVA)

Results

Bioactivity and mRNA expression of TGF-β

Both rat MM cell lines and MCs expressed detectable TGF-β bioactivity (Table 1). MeET-6 produced a relatively lower level of TGF-β bioactivity than did MeET-4, but the total (active+latent) amount of TGF-β expression

Table 1 Transforming growth factor (TGF)-β1 mRNA expression and TGF-β protein secretion in mesothelial cells (MCs) and malignant mesothelioma (MM) cell lines. *GAPDH* glyceraldehyde-3-phosphate dehydrogenase; *NV* normalized value (TGF-β1/GAPDH)

Cell type	Cell line	mRNA (NV) $\times 10^{-2}$	Protein secretion		
				Sample treatment	
				Acid-activation (Latent+active)	Untreated (Active)
Normal mesothelial cells		18	Mean	5.1[a]	
			SD	0.40	1.40
Mesothelioma cell lines	MeET-4	95	Mean	345.3[b]	83.9[b]
			SD	23.19	2.24
	MeET-6	79	Mean	265.8[b]	79.2[b]
			SD	2.30	0.48

[a] TGF-β secretion measured by the CCL64 cell growth inhibition assay in pmol/10^6 cells/24 h. The results are representative of three separate determinations
[b] Significant difference from the value of normal mesothelial cells at $P<0.01$ of probability

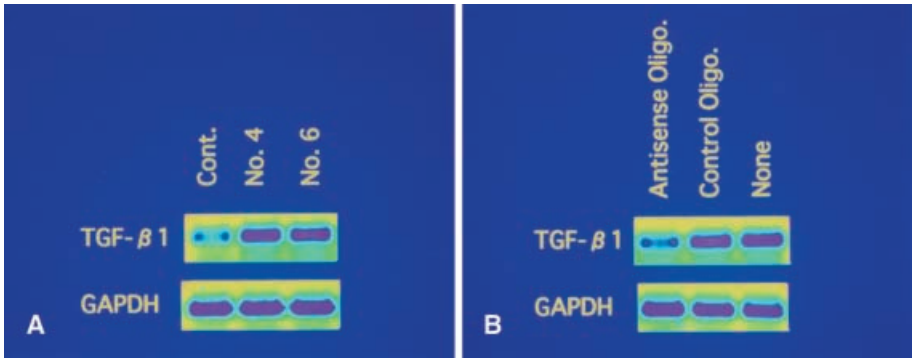

Fig. 1 **A** Polymerase chain reaction (PCR) assay for transforming growth factor (TGF)-β1 expression in mesothelial cells (MCs) and malignant mesothelioma (MM) cells. Samples are identified as follows: *Cont* MC; *no.4* MeET-4; *no.6* MeET-6. TGF-β1 expression values normalized by GAPDH (glyceraldehyde-3-phosphate dehydrogenase) contents are presented in Table 1. **B** PCR assay for antisense oligonucleotide (ODN)-mediated inhibition of TGF-β1 RNA expression in MeET-4. Samples are identified as follows: *Antisense Oligo.* MeET-4 treated with antisense TGF-β1 ODN; *Control Oligo.* MeET-4 treated with control ODN; *None* untreated MeET-4

in MeET-6 was still 30 times higher than that in MCs. Reverse transcriptase-PCR analysis was conducted for TGF-β1, the major member of the TGF-β family. The results confirmed the expression of TGF-β1 mRNA in both MM cell lines and MCs (Fig. 1A) and revealed a high level of expression of TGF-β1 mRNA in MeET-4. In MeET-6, the expression of TGF-β1 mRNA was relatively lower than that in MeET-4, and the result was consistent with the lower level of TGF-β secretion of the cell line. In MCs, the expression level of TGF-β1 mRNA was very low, about 23% of the level expressed in MeET-4 (Table 1).

Effect of antisense TGF-β1 ODN on cell growth

MeET-4 showed rapid growth and the highest colony formation efficiency and tumorigenicity among the three rat MM cell lines established in our laboratory [13]. Furthermore, the largest amount of TGF-β protein secretion and TGF-β1 mRNA expression of the cell line were detected in the present study as described above. Therefore, MeET-4 was selected to examine the effects of anti-

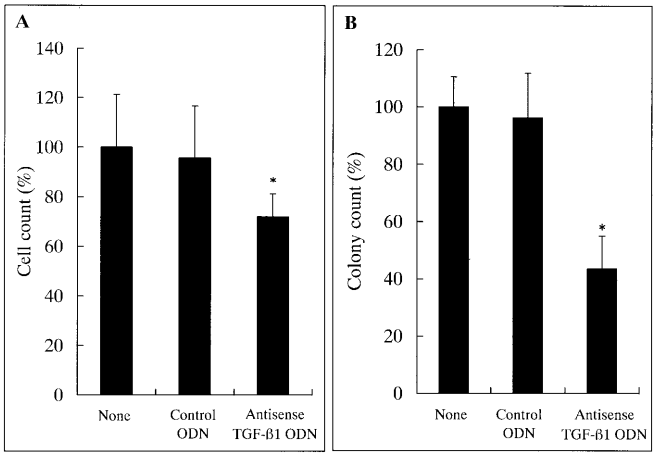

Fig. 2 Effects of antisense TGF-β1 ODN on anchorage-dependent (**A**) and anchorage-independent (**B**) proliferation of MeET-4. **A** Anchorage-dependent cell proliferation 48 h after treatment with oligonucleotides (ODNs). **B** Soft agar colony formation after 7 days of treatment with ODNs. Cell and colony counts are presented as a percentage of those of untreated control (*None*) and expressed as the average±SD of three separate determinations. *Significant difference from the value of untreated control at $P<0.05$% of probability

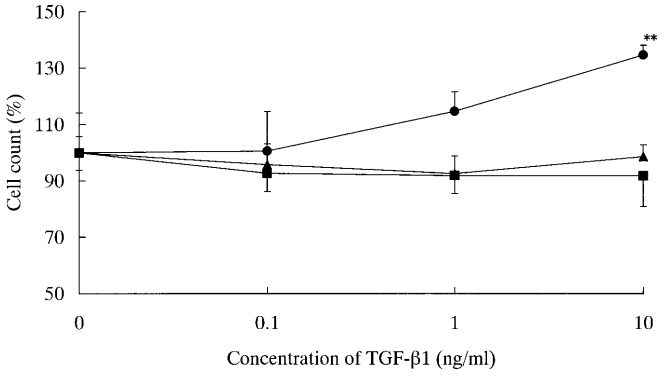

Fig. 3 Responsiveness of mesothelial cell (MC; ●) and malignant mesothelioma (MM) cell lines, MeET-4 (■) and MeET-6 (▲), to exogenous transforming growth factor (TGF)-β. Cells were exposed to a concentration of TGF-β ranging from 0.1 ng/ml to 10 ng/ml, and relative cell counts (percentage to the control) were determined after 48 h. Data are expressed as the average±SD of three separate determinations. **Significant difference from the value of untreated control at $P<0.01\%$ of probability

sense TGF-β1 ODN on the growth of the rat MM cell line.

As shown in Fig. 2, both the anchorage-dependent and anchorage-independent proliferative capacity of MeET-4 was significantly reduced by treatment with antisense TGF-β1 ODN in the media. Antisense TGF-β1 depressed the cell population on tissue culture plates by 65% in the untreated control and 71% in the specificity control at 78 h after the treatment. Colony formation during the 7 days following pretreatment with antisense TGF-β1 ODN was also significantly decreased, and the colony count was less than half of those untreated (40%) and treated with control ODN (45%). To determine whether the inhibition of cell proliferation of MeET-4 corresponded to a reduction in the amount of TGF-β produced, TGF-β1 mRNA was assessed using RT-PCR. TGF-β1 mRNA was reduced by 76% at 48 h after the addition of antisense TGF-β1 ODN (Fig. 1B).

Effect of exogenous TGF-β on cell growth

When recombinant human TGF-β1 was added to the culture medium of the rat MM cell lines and MCs grown on culture dishes under serum-free conditions, the rat MCs showed a dose-dependent increase in proliferation. Relative cell count was increased significantly, and the value was up to 130% of the control value when treated with 10 ng/ml of exogenous TGF-β for 48 h. On the contrary, the MM cell lines did not react against exogenous TGF-β (Fig. 3).

Discussion

In this study, we examined TGF-β1 synthesis in recently established spontaneous rat MM cell lines. Tumorigenicity in syngeneic rats and colony formation in soft agar are characteristics in these cell lines. A significant amount of TGF-β production was noted at the levels of both mRNA and protein production. RNA studies were conducted on TGF-β1, the major member of TGF-β family. In MCs, the expression of mRNA and protein production of TGF-β were also observed, but the levels were significantly lower than those expressed in MM cell lines. Antisense TGF-β1 ODN inhibited both anchorage-dependent and anchorage-independent proliferation of MeET-4. Exogenous TGF-β failed to stimulate the growth of MM cells under serum-free conditions, while quiescent MCs responded mitogenically to TGF-β.

The expression of mRNA and protein production of TGF-β in MM cell lines were as high as those of the previous studies with human and mouse MM cell lines [5] and activated human macrophages [1]. There was a concordance between the level of mRNA expression and protein amount. MeET-4, which showed a better growth in soft agar and higher potency of tumorigenicity [18], was more efficient to produce TGF-β than MeET-6. Rat normal MCs, however, secreted only a basal level of TGF-β. The large difference in TGF-β production between MCs and MM cells was unexpected. It was reported that the expression of TGF-β mRNA was similar between MM cells and MCs in human [8], while the production of TGF-β protein in the present cell lines derived from rat MM and MCs was higher in the former than the latter. There have been only limited data on TGF-β in MCs of rats available enough for comparison with the present results. As to the expression of TGF-β1 mRNA in MCs in rats, Bermudez et al. reported considerably big differences among established cell lines [3]. It is quite possible that there is a species difference in TGF-β production between MM cells and MCs. However, at least under the conditions of the present study using MCs derived from rats, overexpression of the growth factor in asbestos-free malignant cells was indicated.

In the investigation of the effect of antisense TGF-β1 ODN on cell growth, MeET-4 was selected for its higher productivity of TGF-β protein and more growth potency. The antisense TGF-β1 ODN effectively inhibited TGF-β1 mRNA expression in MeET-4 and caused a 35% depression in cell proliferation on culture dishes. Because only a 9% reduction of growth was recorded for control ODN, mitogenic activity of TGF-β to spontaneous rat MM cells was clearly indicated. Similar results were described in asbestos-related mouse MM cells after suppression of TGF-β (β1 and β2) production using specific antisense ODNs in vitro [25].

Because the TGF-β family is a multifunctional polypeptide, it is suggested that they have many key roles in the tumor development process. They showed abilities to modulate tumor cell extracellular matrix adhesion, invasion and spread of tumor cells, vascularization, and immunological anti-tumor defense mechanisms [2, 8, 15, 16, 17, 19, 30]. It was reported that antisense TGF-β1 ODN treatment to asbestos-related mouse MM cells inhibited their colony formation in soft agar and tumorigenicity in syngeneic mice, while anchorage-dependent

proliferation was not affected [6]. Proliferation of MeET-4 cells was depressed in both anchorage-dependent and anchorage-independent conditions. A significant decrease in colony formation (less than 50% of untreated MeET-4) might suggest that indirect effects of TGF-β1, other than mitogenic potential, would influence anchorage-independent growth of spontaneous rat MM cells. In asbestos-related MM, inhibition of anti-tumor immunoresponse, which was mainly modulated by T-cell lymphocytes, was also shown as a function of TGF-β [5, 6]. The same potency can be expected for the present MM cell lines, although more studies should be needed to support this speculation.

Antisense TGF-β1 ODN markedly reduced MM cell growth. Therefore, a further study was addressed to see whether exogenous TGF-β1 could potentiate mitogenic responses of rat MM cells and MCs. Monolayer cultures of human MCs made quiescent by serum deprivation are promoted to go through one round of DNA synthesis with a supplement of TGF-β [7, 21]. In the present study, quiescent rat MCs responded to growth stimulation of exogenous TGF-β and increased by 30% in relative cell count 48 h after supplementation. Meanwhile, relative cell counts of rat spontaneous MM cell lines were comparable at any concentration of TGF-β added. It was considered that serum deprivation could not make MM cells arrest in growth because MM cells had established an autocrine loop of growth factors, including TGF-β. In the previous study, it was demonstrated that our MM cell lines could grow with a low concentration of FBS [18]. As described before, retarded growth was observed when TGF-β production in MM cells was suppressed by antisense ODN. Taken together, it was surmised that TGF-β in rat MM cells was supplied through an autocrine mechanism. The autocrine loop of TGF-β has been also reported in asbestos-related human [21] and rat [20] mesotheliomas. Considering that an autocrine loop had been established in MM cells, it can be quite reasonable that MM cells produced a larger amount of TGF-β than normal MCs.

In the present study, we examined only TGF-β1 at the mRNA level. Human MM cells were reported to express TGF-β2 predominantly, while TGF-β1 expression was located in the stroma of tumor tissue [13]. The effects of TGF-β2 on the growth of human MM cells and the synthesis of the extracellular matrix by MM cells are more effective than TGF-β1 [25, 34]. Fitzpatrick et al. reported that human MM cells produce both isoforms of TGF-β, and antisense TGF-β1 and β2 equally inhibit the malignant growth property of MM cells [6]. A further study is ongoing to elucidate the effects of other isomers of TGF-β, especially TGF-β2, on the growth of MM cell line obtained in the present study.

In conclusion, a distinct overexpression of TGF-β was demonstrated in the rat spontaneous MM cells at the levels of mRNA expression and protein secretion, and these results were consistent with those reported in asbestos-related human and murine MMs. It has been suggested that TGF-β may function as a promoter of aggressive growth of MM cells through an autocrine mechanism, but the expression of TGF-β is not related to the stimulation of asbestos.

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