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Suppression of TNF-Stimulated Proliferation of Diploid Fibroblasts and TNF-Induced Cytotoxicity Against Transformed Fibroblasts by TGF-β

Ryutaro Kamijo[1,2], Ken Takeda[1], Masao Nagumo[2] and Kunio Konno[1]

[1]1st Department of Biochemistry, School of Medicine and
[2]2nd Department of Oral and Maxillofacial Surgery, School of Dentistry, Showa University, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142, Japan

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Human transforming growth factor-β (TGF-β) dose-dependently inhibited proliferation of WI-38 cells, normal human diploid fibroblasts, stimulated by tumor necrosis factor (TNF). Inhibition occurred at 1 ng/ml concentration of TGF-β. Also, TGF-β dose-dependently suppressed cytotoxicity of TNF against L-929 cells, murine transformed fibroblasts. The concentration of TNF required for 50% cytolysis of L-929 cells was changed from 30 ng/ml to 350 ng/ml by 10 ng/ml TGF-β. This suppression was abolished when L-929 cells were treated with actinomycin D or cycloheximide, suggesting that TGF-β might inhibit the action of TNF via de novo protein synthesis. This response was not due to down regulation of TNF receptors nor to alteration of the affinity of TNF for its receptor.

Tumor necrosis factor (TNF) is a monocyte/macrophage-derived protein originally identified by its ability to induce hemorrhagic necrosis of some tumors in vivo and cytotoxicity against certain tumor cells in vitro (1,2). Despite its original definition, TNF is now recognized as a regulatory cytokine with multiple biological activity. For example, TNF is known to have mitogenic effects on fibroblasts (3,4).

Transforming growth factor-β (TGF-β) is a 25-KDa peptide produced by inflammatory or immunoregulatory cells such as platelets (5), lymphocytes (6) and macrophages (7). Originally identified by its ability to cause phenotypical transformation

Abbreviations: TNF, tumor necrosis factor; TGF-β, transforming growth factor-β.

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of rat fibroblasts (8); TGF-β is now known to regulate numerous cellular actions, particularly in cells of immune surveillance and in connective tissue as well as epithelial cells (9,10,11). Recent studies indicate that TGF-β is a bifunctionl regulator of cellular growth, and that the effects of TGF-β on cells are not functions of the peptide itself, but rather of the total set of growth factors and their receptors that operate in the cells at any given time (12). Growth factors have been reported to interact both synergistically and antagonistically (13,14). It thus seems important to investigate whether there is an interaction between TGF-β and TNF that is related to proliferation of fibroblasts.

In the present study, we examined whether TGF-β suppressed TNF-induced enhancement of fibroblast proliferation. We also investigated the effects of TGF-β on TNF-induced cytotoxicity against transformed fibroblasts.

MATERIALS AND METHODS

Reagents: RPMI1640 medium was purchased from Grand Island Biological Co., Grand Island, New York and fetal bovine serum (FBS) was obtained from Filtron Pty. Ltd., Victoria, Australia. Recombinant human TNF (rHuTNF) was kindly donated by Dainippon Pharmaceutical Co., Osaka, Japan. Human TGF-β (HuTGF-β) was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. rHuTNF and HuTGF-β was highly purified and determined to be homogeneous by SDS-PAGE. Actinomycin D and cycloheximide were purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Bolton-Hunter reagent was purchased from ICN Radiochemicals, Irvine, California, U.S.A.

Cell lines and cell culture: WI-38 cells, diploid fibroblasts established from fetal human lung, was purchased from Dainippon Pharmaceutical Co., Osaka, Japan. L-929 cells, murine transformed fibroblasts, was provided by Green Cross Corp., Osaka, Japan. Both cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS. Incubation was carried out at 37°C in a humidified 5% CO_2 incubator.

Cell proliferation assay: Experiments of fibroblast proliferation were done with WI-38 cells at passage level 25-30 (with each passage corresponding to approximately two cell generations). Cells were seeded in a 96-well microtiter plate at a density of 8,000 cells/well in 0.1 ml RPMI1640 medium supplemented with 1% FBS. Highly purified TGF-β was added after 18 h incubation. TNF was added after an additional 30 min incubation. After 4 days, the medium was removed, and cells

were washed twice with saline, fixed and stained for 30 min with 2% ethanol containing 0.2% crystal violet. Bound dye was eluted with 0.1 ml of 1% sodium dodecyl sulfate. The absorbance was measured at 590 nm using spectrophotometer (Immunoreader NJ-2000, Inter Med, Tokyo).

Assay of TNF-induced cytotoxicity: L-929 cells were seeded into a 96-well microtiter plate at a density of 3×10^4 cells/well in 0.1 ml of RPMI1640 medium supplemented with 10% FBS and allowed to adhere overnight. Varions concentrations of TNF and/or TGF-β were then added. In certain experiments, actinomycin D or cycloheximide was added simultaneously with TNF and/or TGF-β. After 18 h incubation, the viable cell density was determined by the same methods as those used for proliferation assay.

Receptor binding assay: TNF was radiolabeled without significant loss of cytotoxicity against L-929 cells by the method described by Bolton and Hunter (15). L-929 cells were cultured in 24-well microtiter plates at a density of 5×10^5 cells/well in 1 ml of RPMI1640 medium supplemented with 10% heat inactivated FBS. WI-38 cells were also cultured in 12-well plates at a density of 3×10^5 cells/well in 1 ml of RPMI1640 medium supplemented with 1% heat inactivated FBS. Both cell types were allowed to adhere overnight. The cells were then washed with fresh medium and incubated at 37°C with various concentrations of TGF-β for 15 h. The cells were then washed with cold fresh medium and incubated at 0°C for 3 h with various amount of ^{125}I-labeled TNF (188 Ci/nmol). After incubation, cells were washed 4 times with cold medium, solubilized with 2 ml of 1% sodium dodecyl sulfate and counted to determine bound radioactivity. Data were corrected for nonspecific binding in the presence of a 100-fold excess of unlabeled TNF, and TNF specific binding sites were expressed as the number of recepors per cell.

RESULTS

Effect of TGF-β on TNF-stimulated fibroblast proliferation: The growth enhancing activity of TNF on WI-38 cells was dose-dependently suppressed in the presence of more than 0.1 ng/ml TGF-β, and TNF-stimulated proliferation of WI-38 cells was completely inhibited by 10 ng/ml TGF-β (Fig 1). The proliferation of WI-38 cells was not suppressed by TGF-β alone.

Effect of TGF-β on TNF-induced cytotoxicity against L-929 cells: Murine L-929 cells are susceptible to the cytotoxic action of TNF. It has been shown that the presence of actinomycin D during TNF treatment enhances target cell susceptibility to TNF. First of all, the effect of TGF-β on TNF-induced cytotoxicity against L-929 cells was investigated in the absence of actinomycin D. In this condition, more than

1 ng/ml concentration was required for the appearance of cytotoxicity. This was 1,000-times that needed in the presence of actinomycin D. The cytotoxic action of TNF against L-929 cells was dose-dependently reduced by TGF-β at concentrations exceeding 0.1 ng/ml (Fig 2). The concentration of TNF required for 50% cytolysis of L-929 cells was changed from 30 ng/ml to 230 ng/ml by 1 ng/ml TGF-β, and to 350 ng/ml by 10 ng/ml TGF-β. TGF-β itself only marginally enhanced growth of L-929 cells. Subsequently, we studied the action of TGF-β on TNF-induced cytotoxicity against L-929 cells in the presence of actinomycin D or cycloheximide. It has been shown that 8×10^{-7} M actinomycin D blocked RNA synthesis and 8×10^{-6} M

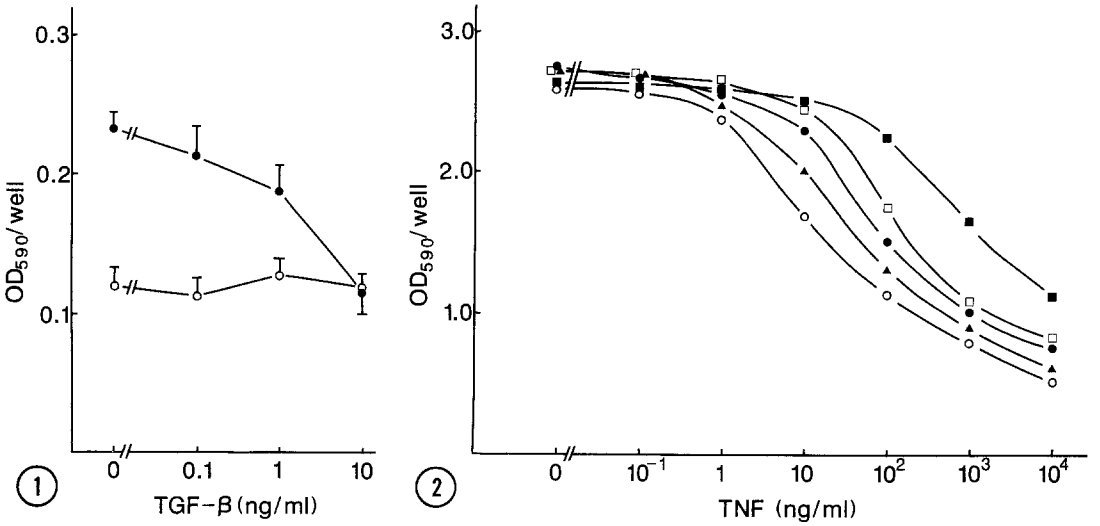

Figure 1. Antagonistic effect of TGF-β on TNF-stimulated proliferation of diploid fibroblasts. Human diploid fibroblast WI-38 cells were seeded in 96-well plates in 0.2 ml of RPMI1640 medium supplemented with 1% FBS. After attachment of the cells, TNF and TGF-β were added as indicated. Cell density was determined at day 4 as described in MATERIALS AND METHODS. Each point represents the mean ± S.E. of three determinations. ○, control; ●, 1 ng/ml TNF.

Figure 2. Effect of TGF-β on TNF-induced cytotoxicity against transformed fibroblasts. Murine transformed fibroblast L-929 cells were seeded in 96-well plate in 0.1 ml of RPMI1640 medium supplemented with 10% FBS. After attachment of cells, TNF and TGF-β were added as indicated. After 18 h incubation, cell lysis was detected as described in MATERIALS and METHODS. Each point represents the mean of three determinations; S.E. of each point was within 5%. TGF-β concentrations: ○, control; ▲, 0.1 ng/ml; ●, 1 ng/ml; □, 10 ng/ml; ■, 100 ng/ml.

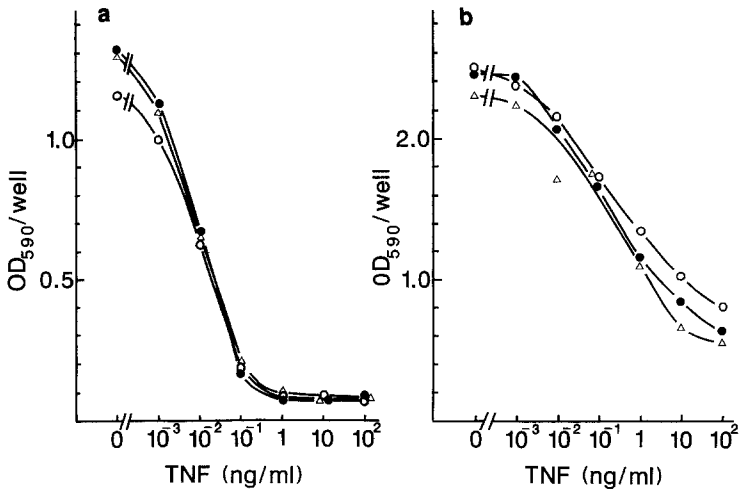

Figure 3. Effect of actinomycin D and cycloheximide on suppression of TNF-induced cytotoxicity by TGF-β. Effect of TGF-β on TNF-induced cytotoxicity was assayed in the presence of actionomycin D (a) and cycloheximide (b). Experimental conditions as in legend of Fig. 2 except that actinomycin D (8×10^{-7}M) or cycloheximide (8×10^{-6}M) were added simultaneously with TNF and TGF-β. Each point represents the mean of three determinations; S.E. of each point was within 5%. TGF-β concentrations: ○, control; △, 1 ng/ml; ●, 10ng/ml.

cycloheximide inhibited protein synthesis in L-929 cells (16). The suppression of TNF-induced cytotoxicity by TGF-β was abolished when L-929 cells were treated with actinomycin D or cycloheximide at these concentrations (Fig 3, a.b.).

Changes in TNF receptor binding by TGF-β: We first examined whether TGF-β competes with TNF for binding to the same cellular receptor. The binding of ^{125}I-TNF to L-929 and WI-38 cells was inhibited by unlabeled TNF but not by TGF-β (data not shown). The results indicate that TGF-β does not block TNF binding. Subsequently, we investigated whether TGF-β could interfere with TNF activity by either down regulating TNF receptors or changing the affinity of the TNF receptors. Binding sites on L-929 and WI-38 cells were 377/cell and 1490/cell, respectively, and these numbers were not changed by 0.1 ng/ml to 10 ng/ml TGF-β (Table 1). The dissociation constants of TNF for its receptors,

Table 1. Comparison of binding activity of TNF to WI-38 and L-929 cells

Cell line	Kd(nM)		Number of binding sites per cell	
	Treated with		Treated with	
	None	TGF-β 10 ng/ml	None	TGF-β 10 ng/ml
WI-38	2.89	3.63	1,490	1,735
L-929	0.75	0.68	377	395

The TNF binding assay is described in MATERIALS AND METHODS. Data were corrected for nonspecific binding in the presence of 100-fold excess of unlabeled TNF. Each value represents the mean of duplicate experiments.

0.75 nM for L-929 cells and 2.89 nM for WI-38 cells, were also not changed by TGF-β (Table 1). Therefore, TGF-β did not interfere with the cytotoxic action of TNF by blocking TNF binding nor by altering the affinity of TNF for its receptor.

DISCUSSION

We have examined the effects of TGF-β on TNF-stimulated fibroblast proliferation and TNF-induced cytotoxicity against transformed fibroblasts. Our results show that TGF-β suppresses these TNF actions. In the fibroblast proliferation experiment using WI-38 cells, serum concentration of culture medium was 1% to eliminate the influence of growth factors contained in FBS as much as possible. Culture in serum-free medium seems appropriate to eliminate the influence of FBS completely, but stimulation of fibroblast proliferation by TNF could not be observed when cells were cultured in serum-free medium. In contrast, TNF remarkably stimulated cell proliferation and TGF-β dose-dependently suppressed stimulation by TNF in the presence of 1% FBS. TGF-β alone had no cytotoxic effect on WI-38 cells. These results suggest that suppression of

the proliferation of WI-38 cells by TGF-β may be caused by TGF-β interference with TNF activity.

Previous studies showed that the actions of TGF-β on cells were bifunctional and TGF-β could act either synergistically or antagonistically with other growth factors (12,13,14). In Fischer rat 3T3 fibroblasts transfected with a cellular myc gene, TGF-β synergizes with platelet-derived growth factor to stimulate colony formation but inhibit the colony formation induced by epidermal growth factor (12). On the other hand, TGF-β inhibits anchorage independent growth of NIH 3T3 cells induced by platelet-derived growth factor (13). In monolayer culture, TGF-β suppresses the growth of primary rat embryo cells induced by platelet-derived growth factor and epidermal growth factor (13).

Our data also demonstrate that TGF-β suppressed TNF-induced cytotoxicity against transformed fibroblasts, L-929 cells. This suppressive effect of TGF-β was abolished when L-929 cells were treated with actinomycin D or cycloheximide, suggesting that TGF-β might inhibit the action of TNF via de novo protein synthesis. The suppression of TNF-induced cytotoxicity by TGF-β is only partial, whereas the suppression of fibroblast proliferation by TGF-β is complete. These results suggest that there may be more than two intracellular pathways to induce cytotoxicity by TNF, and one of these is not identical to that needed for cell proliferation.

As the number of specific binding sites or the binding affinity of TNF were not significantly changed in L-929 or WI-38 cells by treatment with TGF-β, the suppressive effect of TGF-β on TNF action may not be due to the interference of TNF binding to its receptors.

Our results suggested that TGF-β may influence inflammatory reactions or the development of tumor cells through modification of TNF activity.

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