Effects of TGF- β and TGF- β -neutralizing Antibody on Normal Skin Fibroblasts and Scar-derived Fibroblasts

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Summary: Transforming growth factor-beta (TGF- β) is known as an important cytokine for scar formation in wound healing. The purpose of the present study was to investigate the effects of TGF- β and its neutralizing antibody on normal skin fibroblasts and scar-derived fibroblasts in culture. Endogenous TGF- β levels were similar in all fibroblasts. Cell proliferation increased when TGF- β 1 or β 2 was added to the cultures, and the increase was higher and started at a lower level in the scar-derived fibroblasts ($p < 0.05$). The increase of fibroblasts was suppressed by the addition of TGF- β -neutralizing antibody to the cultures, and the suppression rate was higher in the scar-derived cells ($p < 0.05$). Percentages of the cells in the growth phases of cell cycle decreased in the normal skin fibroblasts ($p < 0.05$) when TGF- β -neutralizing antibody was added. Our findings showed that scar-derived fibroblasts and normal skin fibroblasts have a different sensitivity to TGF- β . Further study is needed on the effect of the neutralizing antibody to the cell counts and cell cycle of scar-derived fibroblasts.

Key words TGF- β , TGF- β -neutralizing antibody, scar-derived fibroblasts, normal skin fibroblasts, cell cycle

INTRODUCTION

Transforming growth factor-beta (TGF- β) is involved in various cell activities such as proliferation, division, and growth suppression [1-7]. In wound healing, TGF- β accelerates the proliferation of fibroblasts [8] as well as the synthesis of such extracellular matrix as fibronectin and collagen [7,9] and of collagenase [10]. It also suppresses proliferation of epidermal cells [11]. In vivo studies demonstrated that TGF- β takes an important role in scar formation [12-14].

The authors hypothesized that fibroblasts that are derived from normal human skin and those derived from human scar might have different responses to TGF- β . The present study therefore examined (i) the levels of endogenous active-TGF- β contained in normal skin fibroblasts and scar-derived fibroblasts, (ii) the dose-dependence of these reactions of each type of fibroblasts when TGF- β 1 or β 2 was added to the cultures, and the changes according to the dosage,

(iii) the reaction of each type of fibroblasts when TGF- β -neutralizing antibody, that suppresses both TGF- β 1 and β 2, was added to the cultures, and (iv) the effects of the TGF- β -neutralizing antibody on the cell cycle of those fibroblasts.

MATERIALS AND METHODS

Cell cultures

Scar tissues and normal skin for the primary culture of fibroblasts were obtained from surgically resected hypertrophic scar and skin of 6 patients (2 males and 4 females, age range between 18 and 55 years, mean age 34 years) who had received skin graft after having thermal burn. These tissues were tested for antibodies to HIV, HBV, HCV, and ATL before use, and they were found to be negative. For sterilization, each skin piece was soaked in phosphate-buffered saline supplemented with penicillin G

potassium (100 U/ml, Meiji Seika K. K., Tokyo) and streptomycin sulfate (0.1 mg/ml, Meiji Seika). The PBS was Mg and Ca -free[PBS(-)]. The tissue was cut into pieces, treated with 0.02% EDTA·2 Na/PBS(-) for 10 min, and seeded on a flask with 75 cm² base area (Becton Dickinson, Tokyo) by using the explant culture method. Culture medium was Dulbecco's modified Eagle's medium (GMEM, Gibco, USA) supplemented with 10% fetal calf serum (FCS, Hyclone, USA). The cells were incubated in 10% CO₂ at 37 °C, and the culture medium was changed every 3 days. When the cells became confluent, the cells were transferred to another flask with 175 cm² of base area, and they were defined as the first passage (p-1). The third passage of these cells (p-3) was used in the present study.

Preparation of experimental model

5×10³ p-3 cells were seeded on a culture plate with 1.8 cm² of base area (Becton Dickinson), and cultured for 3 days with DMEM supplemented with 10% FCS. In order to avoid having the effects of cytokines contained in the culture medium, the cells were cultured one more day by changing the medium to DMEM supplemented with 1% bovine serum albumin (BSA, Wako Jun Yaku Kogyo, Osaka, Japan), and then used for the experiments.

Measurement of endogenous TGF-β

Endogenous TGF-β1 level was measured in the culture supernatant collected before the addition of TGF-β or its neutralizing antibody by using the human TGF-β ELISA kit (R&D Systems, Minneapolis, USA). In order to activate latent type TGF-β1 in the supernatant, 50 μl of 1N HCl was added, followed 10 min later, by 50 μl of 1.2 N NaOH for neutralization. 200 μl of the treated supernatant was poured into each of the 96-wells of an antibody plate (solid-phase plate for recombinant human TGF-β soluble receptor Type II), and reacted for 3 hrs at room temperature. Then peroxidase-labeled human anti-TGF-β antibody was added, and the supernatant was reacted for another 1.5 hrs. After washing the plate, substrate was added for staining with enzyme reaction, and absorbance was measured with a micro-plate reader (measurement wave length: 450 nm, reference length: 540 nm). Measured cytokine levels were converted in terms of 10³ cells.

Addition of TGF-β1, TGF-β2, or neutralizing antibody

Recombinant human TGF-β1 or TGF-β2 (Austral

Biologicals, CA) was added to each culture plate at a dose of 0.5, 5, 10, or 20 ng/ml. Human TGF-β-neutralizing antibody to TGF-β (includes TGF-β1, β2, and β3) was added to each culture plate at a dose of 10, 20, or 40 μg/ml. TGF-β and the neutralizing antibody was diluted into DMEM supplemented with 1% BSA. The controls received only the DMEM supplemented with 1% BSA. Twenty-four hours later, culture supernatant was removed, the medium was washed with PBS(-), and then the cells were dissociated by adding 0.1% trypsin/0.02% EDTA·PBS(-). The cells were collected and centrifuged for 5 min at 1000 rpm. The supernatant was removed, and a cell suspension was prepared by adding 1.0 ml of DMEM. 100 μl of the cell suspension was poured into a 96-well microplate, 10 μl of WST-1 coloring agent (cell counting kit, Wako Jun Yaku, Osaka, Japan) was added, mixed well, incubated with 10% CO₂ at 37 °C for 4 hrs for staining, and absorbance was measured by using a micro-plate reader (Model 550, Japan Biorad Laboratories, Tokyo, Japan; measurement wave length: 450 nm, reference length: 650 nm). The data was converted into cell counts.

Cell cycle analysis

The cells incubated with the TGF-β neutralizing antibody were dissociated by treatment with 0.1% trypsin. After stopping the reaction to trypsin by adding 20 μl of FBS, the cells were centrifuged for 5 min at 1000 rpm. The supernatant was removed, and the cells were washed with PBS (pH 7.2). The cells were treated with 0.2% tryton X (500 μl), and the final concentration of RNase was adjusted to be 0.5%. Then, 500 μl of propidium iodide (PI, Sigma, USA) was added, and the cells were left for 10 min at room temperature in a dark room for staining. Flow cytometry (FACS Calibur, Becton Dickinson) was performed, and DNA cell cycle was analyzed [15,16].

Statistical analysis

Unpaired Student's t-test was performed, and *P* values less than 0.05 were considered statistically significant.

RESULTS

Endogenous TGF-β level per 10³ cells was 14±2.2 pg/ml in the scar-derived fibroblasts, and 16±1.6 pg/ml in the normal skin fibroblasts (no significant differences).

When TGF-β1 was added to the cultures, cell

counts of both the normal skin fibroblasts and scar-derived fibroblasts increased significantly ($p < 0.05$, vs control). In both types of the fibroblasts, the highest increase was achieved at 5 ng/ml, and the increase was not dose-dependent. There were no significant increases in the cultures of normal skin fibroblasts with 10 ng/ml or more of TGF- β 1, and of scar-derived fibroblasts with 20 ng/ml (Fig. 1).

When TGF- β 2 was added to the cultures, cell counts of both fibroblasts increased significantly

($p < 0.05$, vs control), and the highest increase was achieved at 5 ng/ml for both. The increase was more obvious and started at a lower dose in the scar-derived fibroblasts. When 10 or 20 ng/ml of TGF- β 2 was added, cell counts tended to decrease from the level obtained at 5 ng/ml. The increases became non-significant at 10 ng/ml or more in the normal skin cells, whereas the increase was still significant even at 20 ng/ml in the scar-derived cells (Fig. 2).

Addition of TGF- β -neutralizing antibody (0, 10,

Fig. 1. Number of fibroblasts after adding TGF- β 1 to the cultures. Left: normal skin fibroblasts. Right: scar-derived fibroblasts.

Fig. 2. Number of fibroblasts after adding TGF- β 2 to the cultures. Left: normal skin fibroblasts. Right: scar-derived fibroblasts.

Fig. 3. Number of fibroblasts after adding TGF- β -neutralizing antibody to the cultures. Left: normal skin fibroblasts. Right: scar-derived fibroblasts.

Fig. 4. Percentages of fibroblasts in each phase of cell cycle. Upper: normal skin fibroblasts. Lower: scar-derived fibroblasts.

20, or 40 $\mu\text{g/ml}$) resulted in a significant decrease of cell counts in both cell types ($p < 0.05$, vs control), and the decrease was more obvious and started at a lower dose in the scar-derived cells (Fig. 3).

Addition of TGF- β -neutralizing antibody (0, 5, or

50 $\mu\text{g/ml}$) significantly suppressed the growth phases (S phase and G2-M phase) in the normal skin fibroblasts ($p < 0.05$, vs control), while addition of the antibody did not affect the cell cycle of scar-derived fibroblasts (Fig. 4).

DISCUSSION

Previous in vivo experiments showed that TGF- β accelerates wound healing and angiogenesis [3,5]. Shah et al. [14] administered TGF- β -neutralizing antibody to the wounds prepared on the back of adult rats and demonstrated that scar formation was reduced in comparison to the controls. This findings proved the involvement of endogenous TGF- β in scar formation. Scott et al. [12] showed the presence of TGF- β within hypertrophic scar and mature scar, but not in normal skin, of thermal burn patients. These studies provided evidences of TGF- β involvement in scar formation during wound healing.

In the in vitro experiments of Younai et al. [17], collagen synthesis in normal skin fibroblasts and scar-derived fibroblasts was modulated when TGF- β or TGF- β -neutralizing antibody was used. Bettinger et al. [18] reported direct effects of TGF- β 1 on the proliferation of normal skin fibroblasts and scar-derived fibroblasts.

In our present study, we at first examined endogenous TGF- β 1 level in normal skin fibroblasts and scar-derived fibroblasts. Yang et al. [19] also tried to measure the TGF- β 1, β 2, and total TGF- β levels in 1996, but obtained data only for TGF- β 2, and reported that there were no significant differences between normal skin fibroblasts and scar-derived fibroblasts. Our present findings are consistent with Yang et al.'s precious findings in showing that TGF- β 1 and β 2 levels are not significantly different between normal skin fibroblasts and scar-derived fibroblasts.

TGF- β in mammal has 3 major isoforms, i.e., β 1, β 2 and β 3. Among them, β 1 and β 2 augment, and β 3 suppresses, scar formation [13]. In relation to the mechanism of these actions, the present study examined the effects of TGF- β 1 and β 2 on cultured fibroblasts. Gunji et al. examined direct effects of TGF- β 1 on the proliferation of human normal skin fibroblasts and human scar-derived fibroblasts in cultures. They showed that cell proliferation was accelerated dose-dependently, and the increase was lower in scar-derived fibroblasts [20]. Bettinger et al. also added TGF- β 1 to the cultures of normal skin fibroblasts and keloid-derived fibroblasts, and observed an acceleration of growth and collagen synthesis in only the keloid-derived fibroblasts [18]. On the other hand, other studies showed that TGF- β alone had no acceleration effects on DNA synthesis in fibroblasts [21], and there was no significant differences in the effects of TGF- β on cell proliferation between nor-

mal skin fibroblasts and keloid-derived fibroblasts [22,23]. Though the effects of TGF- β on cell proliferation varied in those studies including ours, the common finding was that TGF- β accelerates the proliferation of both normal skin fibroblasts and scar-derived fibroblasts. The variation could be attributable to individual difference of patients and of scar severity in each study.

In regards to TGF- β -neutralizing antibody, Younai et al. added the antibody to the cultures of fibroblasts that were derived from keloid, hypertrophic scar, or normal skin, then measured collagen level, and reported that the antibody suppressed the collagen production in all the 3 fibroblast cultures [17]. Our present study measured cell counts, and found a similar suppressive tendency.

Proliferation of normal skin fibroblasts and scar-derived fibroblasts was accelerated when TGF- β 1 or β 2 was added to the cultures, while the proliferation was suppressed by the addition of TGF- β -neutralizing antibody. This shows that the proliferation of fibroblasts is regulated, at least a part, by TGF- β . In the present study, endogenous TGF- β levels were similar in both types of fibroblasts, but in comparison to the normal skin fibroblasts, accelerated proliferation in the scar-derived fibroblasts started at a lower dose of TGF- β , and they also had more suppression due to the addition of the neutralizing antibody. These findings indicate that scar-derived fibroblasts are more reactive to exogenous TGF- β .

In the experiment on cell cycle, addition of TGF- β -neutralizing antibody suppressed the cycle of normal skin fibroblasts on the growth phase (S phase and G2-M phase), whereas no suppression occurred on the scar-derived fibroblasts. This finding seems to be contradictory to our another finding, i.e., cell counts of scar-derived fibroblasts after adding the neutralizing antibody decreased more than that of normal skin fibroblasts. By considering the result on cell cycle, the decrease of cell counts in scar-derived fibroblasts could be a temporal phenomena and the scar-derived cells might maintain latent proliferation capacity even after the addition of the antibody. This point needs to be examined in future experiments utilizing various conditions.

Our present findings showed that scar-derived fibroblasts possess sensitivity different from that of normal skin fibroblasts, and indicates that TGF- β itself takes an important role in wound healing process as a scar-formation mediator. In future clinical practice, TGF- β -neutralizing antibody could be utilized as a remedy for hypertrophic scar, but as

indicated in our cell cycle experiment, cell cycle in scar-derived fibroblasts is rarely affected by TGF- β -neutralizing antibody, and therefore their latent proliferation capability would be maintained. Continuous administration of TGF- β -neutralizing antibody may be needed in order to achieve the desired goal in its clinical application.

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