

ORIGINAL ARTICLE

## TGF- $\beta$ 1 INDUCES FIBROBLAST PROLIFERATION PARTLY THROUGH ACTIVATION OF RaF/MEK/ERK SIGNALING PATHWAY BY INHIBITION OF RAF-KINASE INHIBITOR PROTEIN EXPRESSION

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**Abstract** The Raf-kinase inhibitor protein (RKIP) physically binds to Raf and MEK, and inhibits the Raf/MEK/ERK signaling pathway. Transforming growth factor (TGF)- $\beta$ 1 is a multifunctional cytokine that has a proliferative effect on fibroblasts. A considerable effort has been applied to studies of cell growth inhibition by TGF- $\beta$ 1, whereas far less attention has been given to the elucidation of the mechanism of TGF- $\beta$ 1-induced growth stimulation. In this study, we revealed that TGF- $\beta$ 1 significantly enhanced the proliferation of normal human dermal fibroblasts (NHDFs). In addition, we showed that TGF- $\beta$ 1 inhibited the expression of RKIP mRNA and protein. Moreover, a transfection experiment demonstrated that overexpression of RKIP significantly inhibited NHDF cell growth. These data point to a relationship between enhanced NHDF cell growth and reduction of RKIP expression by TGF- $\beta$ 1, suggesting that TGF- $\beta$ 1 may induce fibroblast proliferation partly through the inhibition of RKIP. Furthermore, we revealed that EGF and PDGF-BB, known activators of the Raf/MEK/ERK signal transduction pathway, failed to modulate RKIP expression. Finally, TGF- $\beta$ 1 induced a transient phosphorylation of ERK1/2. Although detailed mechanisms of NHDF proliferation via RKIP are yet unknown, it will be important to investigate the molecular status of RKIP in certain proliferative diseases, such as keloids, in which TGF- $\beta$ 1 has been reported to play a pathogenic role by inducing hyperproliferation of dermal fibroblasts.

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**Key words:** Raf-kinase inhibitor protein; transforming growth factor  $\beta$ ; fibroblast; proliferation; signal transduction.

### Introduction

The phosphatidylethanolamine-binding protein (PEBP) is a 21-to-23-kDa basic protein originally purified from bovine brain that shows a preferential affinity for phosphatidylethanolamine. PEBP is expressed in a wide variety of mammalian tissues including the spleen, testis, ovary, muscle, stomach, and brain<sup>1)</sup>. Using the yeast two-hybrid system, Yeung *et al.* (1999) have identified a novel Raf-kinase inhibitor, Raf-kinase inhibitor protein (RKIP), and demonstrated that RKIP is identical to PEBP<sup>2,3)</sup>. They revealed that RKIP physically binds to Raf and

MEK and causes inhibition of the Raf/MEK/ERK signaling pathway.

The transforming growth factor (TGF)- $\beta$ 1 is a multifunctional cytokine that has a wide variety of physiological effects including the regulation of cell proliferation and differentiation and modulation of extracellular matrix production<sup>4)</sup>. Generally, TGF- $\beta$ 1 inhibits the proliferation of epithelial cells, endothelial cells, and lymphocytes, whereas some fibroblast cell lines proliferate in response to TGF- $\beta$ 1. The proliferative effect of TGF- $\beta$ 1 has been proposed to be important for wound healing and development of fibroproliferative diseases.

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However, while there have been many studies that explored cell growth inhibition by TGF- $\beta$ 1, far less attention has been paid to elucidating the mechanism of growth stimulation by this cytokine.

The Raf/MEK/ERK signal transduction pathway plays a pivotal role in cell proliferation and differentiation in a variety of cells<sup>5</sup>. This pathway transduces signals from the extracellular compartment through intracellular modules composed of the serine/threonine kinase Raf, tyrosine/threonine kinase MEK, and serine/threonine kinase ERK. Ligands, such as growth factors, bind to their respective receptors on the cell membrane and activate the small GTPase Ras. Activated Ras phosphorylates Raf and then activates the MAP kinase kinase MEK. In turn, MEK phosphorylates ERK. Activated ERK translocates into the nucleus and regulates the target gene expression. Similarly to many growth factors such as EGF and PDGF, it has been shown that TGF- $\beta$ 1 can activate the Raf/MEK/ERK signaling pathway<sup>6,7</sup>.

The purpose of this study was to investigate the involvement of RKIP in cell proliferation of cultured normal human dermal fibroblasts (NHDFs) stimulated by TGF- $\beta$ 1. We revealed that overexpression of RKIP significantly inhibited NHDF growth. We also found that RKIP mRNA and protein expression was specifically downregulated by TGF- $\beta$ 1. In addition, the reduction of RKIP expression by TGF- $\beta$ 1 was associated with the enhancement of NHDF cell proliferation. These findings suggest that downregulation of RKIP may be a part of the mechanism mediating TGF- $\beta$ 1-induced proliferation of dermal fibroblasts in our experimental conditions.

## Subjects and Methods

### 1. Cell culture and growth factors

NHDF cell cultures were obtained from Kurabo (Osaka, Japan) and maintained in the Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). Prior to the growth factor treatment, cells were serum-starved with MEM containing 1% FBS for 24 h. Transforming growth factor-beta 1 (TGF- $\beta$ 1) was purchased from Roche Diagnostic (Basel, Switzerland). Platelet-derived growth factor-BB (PDGF-BB) and epidermal growth factor (EGF) were from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

### 2. RKIP expression vector

Plasmid construction for the RKIP cDNA expression vector comprising the entire open reading frame was carried out by RT-PCR using primer sets designed to contain XhoI and NotI linkers. Amplified cDNA was digested with XhoI and NotI and the digest was fractionated on a 1% agarose gel. The 585-bp fragment containing the entire RKIP open reading frame was ligated downstream of the cytomegalovirus enhancer and  $\beta$ -actin promoter in the pCY4B plasmid (kind gift from Dr Kaneda, Osaka University), which is modified from the pCAGGS expression vector. The integrity of the nucleotide sequence of RKIP cDNA was confirmed by DNA sequencing.

### 3. Transfection experiment

Subconfluent NHDF were transiently transfected in 100-mm dishes with the LipofectAMINE 2000 transfection reagent. Prior to the transfection, cells were fed with 5 mL of the Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. pCY4B-RKIP cDNA or pCY4B vectors (8  $\mu$ g each) were mixed with 20  $\mu$ L of LipofectAMINE 2000 in 0.5 mL of the Opti-MEM medium and incubated for 20 min at room temperature. The DNA- LipofectAMINE mixture was directly added to the culture medium and cells were incubated with the mixture for another 3 h. Transfected cells were then harvested using trypsin and plated

into 96-well plates for subsequent evaluation of the cell proliferation. Transfection efficiency was assessed by the microscopic examination of NHDFs co-transfected with an expression vector for enhanced green fluorescence protein (eGFP).

#### 4. Cell proliferation assay

Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQueous One solution Reagent, Promega Corp., Madison, WI), according to the manufacturer's instruction. NHDFs were plated on 96-well plates in MEM containing 1% FBS for the time periods indicated. Cells were stained with the MTS reagent at various time points following treatment of plates with TGF- $\beta$ 1 at different concentrations. The optical density was read by a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA) at 490 nm wavelength. Averaged blank values (no cells, no cytokine) at the same time point were subtracted from sample values for correction.  $A_{490}$  values were calculated as percentages of the cells. The results were expressed as the mean  $\pm$  standard deviation (SD). The Student's *t*-test was used for statistical analysis and data were considered to be significantly different if  $P < 0.05$ .

#### 5. Western blot analysis

NHDFs were incubated with various concentrations of TGF- $\beta$ 1 in MEM containing 1% FBS and harvested at the indicated time points. Cell lysates were prepared using 5  $\times$  Reporter Lysis Buffer (Promega Corp., Madison, WI) with a Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich, Inc., St. Louis, MO) according to the manufacturer's instructions. Proteins (40  $\mu$ g/lane) were separated by SDS-polyacrylamide gel electrophoresis on 12.5 % (w/v) polyacrylamide gels and electroblotted to nitrocellulose membranes. For detection of RKIP protein expression, a rabbit anti-RKIP primary antibody was used<sup>8,9)</sup>. Horseradish

peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibody. Enhanced chemiluminescence was used for detection. The expression level of  $\beta$ -actin was determined by an anti- $\beta$ -actin antibody for normalization of the total protein quantity in each lane. Activated MAPK was detected by incubating blots with an anti-ACTIVE<sup>®</sup> MAPK pAb (1:3000, v/v; Promega Corp., Madison, WI) as the primary antibody, followed by incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG (1/2000, v/v). An anti-ERK2 antibody (1/2000, v/v; BD Transduction Labo., Franklin Lakes, NJ) was used to monitor the protein load.

#### 6. Northern blotting analysis

Total RNA was extracted from NHDFs using an RNeasy mini kit (Qiagen, Hilden, Germany). Fifteen micrograms of total RNA was separated by electrophoresis and blotted onto the blotting membranes. RKIP cDNA was generated by RT-PCR and labeled by the random primer method. Primers for RT-PCR were designed from the published RKIP mRNA sequence (Moore et al, 1996). Hybridization signals were quantified by densitometry and normalized to *GAPDH* mRNA levels.

## Results

### 1. Induction of fibroblast proliferation by TGF- $\beta$ 1

It is known that while TGF- $\beta$ 1 mainly exerts an inhibitory influence on the proliferation of epithelial cells, endothelial cells and lymphocytes, in fibroblasts, TGF- $\beta$ 1 could have both stimulatory and inhibitory effects, depending on the stage of differentiation and culture conditions. Indeed, an upregulation of proliferation has been documented in some fibroblast cell lines in response to TGF- $\beta$ 1 treatment. Therefore, we initially examined how TGF- $\beta$ 1 affected NHDF proliferation. In our experimental setting, 1 and 10 ng/mL TGF- $\beta$ 1 significantly enhanced NHDF

proliferation at the 72-h time point after its application, as assessed by an MTS assay (Fig. 1).

### **2. Inhibitory effect of TGF- $\beta$ 1 on RKIP expression**

Next, we investigated modulation of RKIP expression in proliferating NHDFs by TGF- $\beta$ 1. We extracted samples from NHDFs following the treatment with 0.1 or 1 ng/mL TGF- $\beta$ 1 and performed western blot analysis. As shown in Fig. 2a, RKIP protein levels were clearly decreased in 48 h after the treatment. Also, we carried out northern blot analysis of RKIP mRNA. NHDFs were incubated with 0.01, 5, and 10 ng/mL TGF- $\beta$ 1 for 24 h. Total cellular RNA was isolated and subjected to northern blot analysis. TGF- $\beta$ 1 suppressed RKIP mRNA expression in a dose-dependent manner (Fig. 2b). For the time-course experiment, cells were incubated with 1 ng/mL TGF- $\beta$ 1 for the indicated time periods and total RNA was isolated by the same method. As shown in Fig. 2c, RKIP mRNA levels started to decrease in 30 min and became maximally suppressed at 24 and 48 h following the start of incubation with TGF- $\beta$ 1.

### **3. Inhibition of NHDF proliferation by overexpressed RKIP**

In order to determine the effect of RKIP on NHDF proliferation, the cells were transfected with an RKIP-expressing or a control vector and subsequently incubated in MEM containing 10% FBS. Exogenous RKIP expression in NHDFs was validated by immunoblotting (data not shown). As shown in Fig. 3, RKIP overexpression suppressed NHDF proliferation in 24 and 48 h after transfection.

### **4. Lack of effect of PDGF-BB and EGF on RKIP protein synthesis**

We examined the effects of other growth factors that activate the Ras/Raf-1/MEK-ERK signaling pathway, such as EGF and PDGF-BB, on RKIP protein synthesis under the same experimental conditions. We did not reveal

any significant changes in RKIP protein levels within 72 h after respective treatments (Fig. 4).

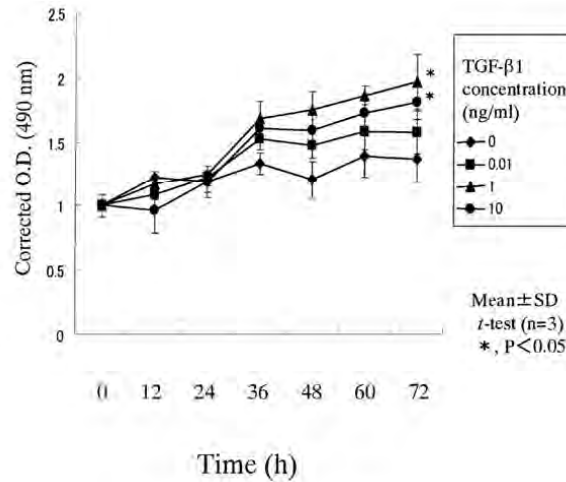
### **5. Activation of ERK1/2 by TGF- $\beta$ 1 in NHDFs**

To examine the modulatory effects of TGF- $\beta$ 1 on MAPK in NHDFs, western blot analysis was performed on cell lysates treated with 5 ng/mL TGF- $\beta$ 1 (Fig. 5). Phosphorylation of ERK1 and ERK2 was detected by a phosphor-MAPK antibody that specifically recognizes phosphorylated ERK1 and ERK2. TGF- $\beta$ 1 treatment of NHDFs upregulated ERK1/2 expression at the 20-min time point after cytokine treatment. ERK1/2 expression gradually declined thereafter, indicating that TGF- $\beta$ 1 causes only a transient activation of MAPK. No increases in ERK1/2 phosphorylation were observed in 24 and 48 h after TGF- $\beta$ 1 application (data not shown).

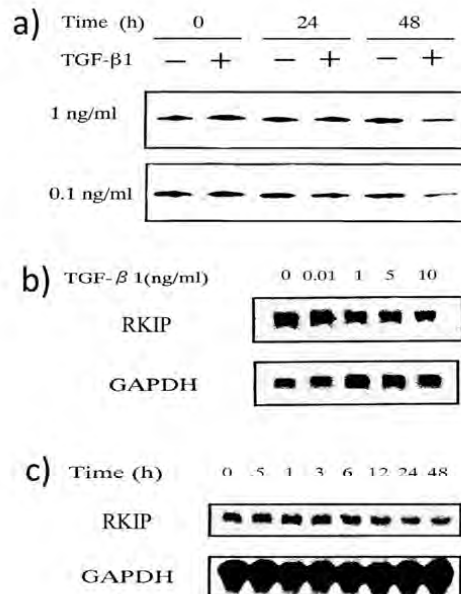
## **Discussion**

The purpose of this study was to investigate the involvement of RKIP in cell proliferation of cultured NHDFs following treatment with TGF- $\beta$ 1. TGF- $\beta$ 1 can either up- or downregulate fibroblast proliferation depending on culturing conditions. In our experimental setting, TGF- $\beta$ 1 significantly promoted the proliferation of cultured NHDFs (Fig. 1), while in the same culturing conditions, RKIP protein expression was significantly reduced (Fig. 2a). Furthermore, the decrease in RKIP expression was observed on the mRNA level (Fig. 2b and 2c). These results indicate a close correlation between the enhancement of NHDF cell growth and reduction of RKIP protein expression caused by TGF- $\beta$ 1.

Our transfection study also demonstrated that overexpression of RKIP significantly inhibited NHDF growth (Fig. 3), which is consistent with previously published observations in NIH3T3 cells. Collectively, these data suggest that RKIP can act as an inhibitor of cell proliferation in dermal fibroblasts<sup>2,3</sup>. However, overexpression



**Fig.1** Effect of TGF- $\beta$ 1 on NHDF proliferation. NHDF cells were incubated with 0, 0.01, 1, or 10 ng/mL TGF- $\beta$ 1 for the time periods indicated. Cell proliferation was assessed by MTS assay. Data are presented as the mean  $\pm$  S.D. of experiments performed in triplicate.

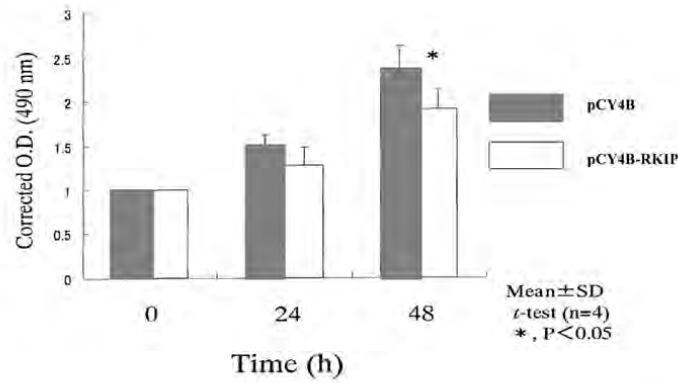


**Fig.2** Effects of TGF- $\beta$ 1 on RKIP expression in NHDFs. a) NHDF cells were incubated with 0.1 or 1 ng/mL TGF- $\beta$ 1 for the indicated time periods. RKIP was detected by western blotting with anti-RKIP antibodies. Results are representative of three independent experiments. b) NHDF cells were incubated with 0, 0.001, 1, 5, or 10 ng/mL TGF- $\beta$ 1 for 24 h. Total cellular RNA was isolated and subjected to northern blot analysis. *GAPDH* mRNA was used as a loading control. Similar results were obtained in three other independent experiments. c) NHDF cells were incubated with 1 ng/mL TGF- $\beta$ 1 for the indicated periods. Then, northern blot analysis was performed as described above.

of RKIP in NHDFs did not result in a complete suppression of cell proliferation (Fig. 3). Although the efficiency of our transient transfection experiment using LipofectAMINE 2000 reagent was as high as 70%, as determined

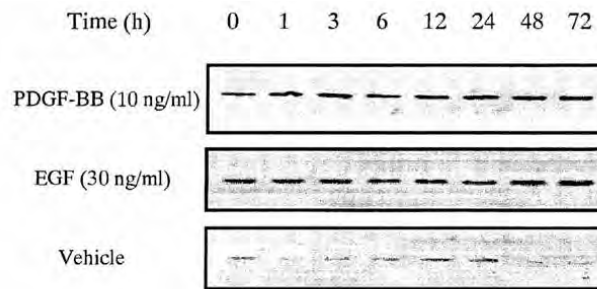
by parallel transfection experiments using a GFP expression vector (data not shown), the inhibitory effect of the overexpressed RKIP might be partially masked by a substantial proliferation of untransfected fibroblasts.





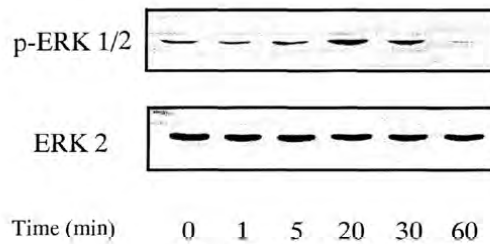
**Fig.3** Effects of transfection with pCY4B-RKIP.

NHDF cells were grown in 100-mm diameter dishes and transfected with pCY 4B-RKIP or pCY4B vectors. Three hours after transfection, the cells were plated into 96-well plates in MEM containing 1% FBS and further incubated for the time periods indicated. The medium was then changed to that containing 10% FBS. The number of viable cells was measured by the MTS assay at the time points indicated. The result is representative of three independent experiments.



**Fig.4** Effects of PDGF-BB and EGF.

The cells were incubated with 10 ng/mL PDGF-BB, 30 ng/ml EGF, or control vehicle for the indicated time periods. RKIP was detected by western blotting with anti-RKIP antibodies. The result is representative of three independent experiments.



**Fig.5** Effect of TGF- $\beta$ 1 on ERK1/2 activation in NHDFs.

The cells were incubated with 5 ng/mL TGF- $\beta$ 1 in MEM containing 1% FBS for the indicated time periods. Activation of ERK1/2 was detected by western blotting with p-ERK1/2 antibodies. ERK2 was used as a loading control. Similar results were obtained in three other independent experiments.

Activators of the Raf/MEK/ERK signal transduction pathway EGF and PDGF-BB failed to modulate RKIP expression at concentrations sufficient for exerting biological effects (Fig. 4). These findings indicate that the modulation of

RKIP expression by TGF- $\beta$ 1 may be specific. TGF- $\beta$ 1 induced only a transient ERK1/2 phosphorylation (Fig. 5), as activation of ERK1/2 was not observed at later time points. Taking into account that the reduction of RKIP

protein expression by TGF- $\beta$ 1 was associated with enhanced proliferation of dermal fibroblasts, we initially hypothesized that reduced RKIP expression would result in a hyperactivation of the Raf/MEK/ERK signaling pathway and expected to confirm this by demonstrating the increased phosphorylation of ERK1/2. However, we observed a significant delay between the time points of maximal suppression of RKIP mRNA expression and peak values of ERK1/2 phosphorylation induced by TGF- $\beta$ 1 (Fig. 2c and Fig. 5). However, we could not explain the detailed molecular mechanisms of this discrepancy.

In this study, we revealed that TGF- $\beta$ 1 reduces the expression of RKIP mRNA. Modulation of RKIP production by growth factors or cytokines has not been properly investigated. Further experiments are required to determine the detailed pathway from induction of TGF- $\beta$ 1 to the decrease in the level of RKIP mRNA. What could be the biological significance of the decreased RKIP expression in proliferating dermal fibroblasts stimulated by TGF- $\beta$ 1? It will be important to investigate the molecular status of RKIP in various proliferative diseases, such as keloids, in which the increased local expression of TGF- $\beta$ 1 has been reported to play a pathogenic role by inducing hyperproliferation of dermal fibroblasts<sup>10)</sup>.

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