Tongue epithelial KT-1 cell-cycle arrest by TGF- β associated with induction of p21^{Cip1} and p15^{Ink4b}

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Abstract Tongue epithelium continuously turns over in adults. Our previous study showed that epidermal growth factor and fibroblast growth factor-2 stimulated proliferation of KT-1 cells derived from tongue epithelium, suggesting that these signals serve as positive regulators for tongue epithelial proliferation. To investigate a negative regulation of tongue epithelial cell proliferation, we studied effects of transforming growth factor- β (TGF- β) on KT-1 cells. Proliferation assays showed that TGF- β inhibited proliferation of KT-1 cells in a dose dependent manner. Cell-cycle analysis showed that TGF- β induced G₀/G₁ cell cycle arrest in KT-1 cells. We also examined expressions of Ink4 and Cip/Kip family mRNA by quantitative reverse transcription-polymerase chain reaction. We found that TGF- β induced p15^{Ink4b} and p21^{Cip1} mRNA expressions. These results strongly suggest that G₀/G₁ cell cycle arrest is associated with increased p15^{Ink4b} and

p21^{Cip1} expressions. Moreover, p21^{Cip1} mRNA was localized in suprabasal cells of tongue epithelium, suggesting that p21^{Cip1} play a role in cell-cycle exit along with tongue epithelial differentiation. Taken together, our results suggest that TGF- β signaling serves as negative regulator of tongue epithelial cell proliferation, and may control tongue epithelial cell differentiation through modulating expression of p21^{Cip1}.

Keywords Tongue epithelium · Growth inhibition · Transforming growth factor- β · Differentiation · p15^{Ink4b} · p21^{Cip1} · Serum-free · Cell culture

Introduction

The tongue epithelium is a multilayered squamous epithelium, which originates from basal cells. Epithelial layers of the tongue are composed of at least three types: keratinocytes, secretory cells, and lingual papillae. These cells are continuously renewed even in adults by undergoing proliferation, maturation, and death processes that appear to be controlled by growth factors. It was reported that many kinds of growth factors and their receptors are expressed in tongue epithelial cells, suggesting that these growth factors affect diverse cellular processes of tongue epithelial cells (Miura et al. 2001; Sun and Oakley

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2002; Fan et al. 2004; Suzuki et al. 2007), but the mechanisms of controlling their proliferation and commitment to differentiation into different types of functional epithelial cells are little understood.

Transforming growth factor- β s (TGF- β s) are widely expressed in both adult and embryonic tissues and play important roles in tissue homeostasis through modulating cell proliferation, cell differentiation, cell death, and cell motility. TGF- β signaling is mediated through ligand-initiated heteromeric complex formation of ligand-binding type II receptor (T β R-II) and signal-transducing type I receptor (T β R-I) with serine or threonine kinase activity (Piek et al. 1999; Massague and Gomis 2006). The activated receptor complex then phosphorylates Smad2 and Smad3, which form complexes with Smad4 to modulate expression of their target genes.

The mammalian cell cycle is regulated by the sequential activation and inactivation of a highly conserved family of cyclin-dependent kinases (CDKs). In TGF- β signaling, two families of cyclin-dependent kinase inhibitors (CDKIs) play pivotal roles in G₀/G₁ cell-cycle progression. The INK4 family consists of p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c}, and p19^{Ink4d} that inhibit kinase activity of the cyclinD-CDK4/6 complex. The Cip/Kip family is composed of p21^{Cip1}, p27^{Kip1}, and p57^{Kip2} that inhibit kinase activity of the cyclinE-CDK2 complex (Sherr and Roberts 1999; Massague et al. 2000). In primary limbal cells, TGF- β induces mRNA of p57^{Kip2} and p15^{Ink4b} (Chen et al. 2006). In human intestinal cell lines Caco-2 and tsFHI, p21^{Cip1} was markedly increased within 3 days (Caco-2) and 8 h (tsFHI) after TGF- β treatment (Ding et al. 1998; Tian and Quaroni 1999). These results suggest that the varieties and time course of CDKIs induction depend on cell types and lineage.

In our previous study, we isolated and cultured integrin β_1 -positive cells from mouse-tongue epithelium, and established cell line KT-1 (Ookura et al. 2002). We found that EGF and FGF-2 stimulated proliferation of KT-1 cells and that calcium ion (1 mM) promoted multilayered cell differentiation, suggesting that this cell line is useful for analyzing the growth and differentiation of tongue epithelial cells. In this study, we used a defined serum-free medium for KT-1 cell culture and examined whether TGF- β affects cell proliferation and cell-cycle progression of KT-1 cells.

Materials and methods

Cell culture

KT-1 cells were cultured and passaged as previously described with some modifications (Ookura et al. 2002). Chelated fetal bovine serum was replaced with a cell-culture-grade bovine serum albumin (Proliant Biologicals, Ankeny, IA). The culture medium used in this study was a cocktail of D-MEM low-glucose medium (Gibco, Grand Island, NY) and MCDB153 HAA medium (Research Institute for the Functional Peptides Co., Yamagata, Japan) containing 10 μ g/mL transferrin (Wako Pure Chemicals, Osaka, Japan), 5 μ g/mL insulin (Wako Pure Chemicals), 5 ng/mL EGF (Sigma, St. Louis, MO), 0.2 μ M hydrocortisone (Sigma), 0.5 μ M ethanolamine (Sigma), 0.5 μ M phosphoryl-ethanolamine (Sigma), 20 μ g/mL heparin, 0.1% (wt/v) BSA, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 0.5 μ g/mL FungizoneTM. The ratio of D-MEM low-glucose medium to MCDB 153 HAA medium was 1:3.

Reverse transcription-polymerase chain reaction and quantitative reverse transcription-polymerase chain reaction

The tongue epithelium was prepared as described below. Ringer's solution (150 mM NaCl, 4.7 mM KCl, 3.3 mM CaCl₂, 0.1 mM MgCl₂, 2 mM HEPES, and 7.8 mM glucose) containing 2.5 mg/mL collagenase type IV (Worthington Biochemical, Lakewood, NJ) and 2 mg/mL elastase (Worthington Biochemical) were injected beneath epithelial layers of a dissected tongue. After incubation for 20 min at 31 °C, the tongue epithelia were gently peeled off the underlying muscle layers.

Total RNAs from tongue epithelia and KT-1 cells were extracted with an RNeasy mini kit (Qiagen, Tokyo, Japan). Reverse transcription was performed with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA was amplified by PCR using TaKaRa Ex Taq (Takara Bio Inc., Shiga, Japan). Primers used in this study are listed in Table 1. The PCR products were separated on 2% agarose gels and visualized by ethidium-bromide staining.

Real-time PCR reactions were performed with an ABI PRISM 7000 Sequence Detection System

Table 1 Primer sequences used for RT-PCR and qRT-PCR analysis

Gene name	Primer sequence (strand)	Product size (bp)	Annealing temperature (°C)
TβR-I	5'-GCACCATCTTCAAAAACAGGGG-3' (+) 5'-GCCAAACTTCTCCAAACCGACC-3' (-)	415	58
TβR-II	5'-GGATGTGGAAATGGAAGCCC-3' (+) 5'-ATGAAGAAAAGTCTCGCCCGC-3' (-)	387	58
Smad2	5'-AGAGTTGAGACCCAGTCTTGC-3' (+) 5'-CGCACTCCCCTTCCTATATGTC-3' (-)	506	58
Smad3	5'-GGGGAGCAGAGTACAGGAGACA-3' (+) 5'-CCTCACCCAGGACACATACTCC-3' (-)	765	58
Smad4	5'-CACTATGAGCGGGTTGTCTCAC-3' (+) 5'-CAATGGAGCACCAGTACTCAGG-3' (-)	583	58
Gapdh	5'-TGACAACCTTGGCATTGTGG-3' (+) 5'-CCCTGTTGCTGTAGCCGTAT-3' (-)	476	58
p15 ^{Ink4b} (qPCR)	5'-CCCTGCCACCCTTACCAGA-3' (+) 5'-CAGATACCTCGCAATGTCACG-3' (-)	169	60
p16 ^{Ink4a} (qPCR)	5'-CCCGAACTCTTTCGGTCGTA-3' (+) 5'-GTTCGAATCTGCACCGTAGTTG-3' (-)	94	60
p18 ^{Ink4c} (qPCR)	5'-GTCCGTTAGTTGGTTGGCTGT-3' (+) 5'-TTCCACTTAGGCAGTTTGAAGG-3' (-)	114	60
p19 ^{Ink4d} (qPCR)	5'-CCTACAGGCAACAGCCATCTAA-3' (+) 5'-CTCACTCTGAGGCTAGGGCTTT-3' (-)	90	60
p21 ^{Cip1} (qPCR)	5'-CTTGCCTCTGGTGTCTGAGC-3' (+) 5'-TGCGCTTGGAGTGATAGAAATC-3' (-)	111	60
p27 ^{Kip1} (qPCR)	5'-CTGGGTTAGCGGAGCAGTGT-3' (+) 5'-CTTCTGTTCTGTTGGCCCTTTT-3' (-)	90	60
p57 ^{Kip2} (qPCR)	5'-CGCAAACGTCTGAGATGAGTTA-3' (+) 5'-AGCCCAGAGTTCTTCCATCGT-3' (-)	92	60
Gapdh (qPCR)	5'-TGTGTCCGTCGTGGATCTGA-3' (+) 5'-TTGCTGTTGAAGTCGCAGGAG-3' (-)	150	60

(Applied Biosystems, Foster City, CA) using a SYBR Green Real-Time PCR Master Mix-Plus- (Toyobo, Osaka, Japan). The reaction conditions were an initial denaturation for 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Melting curve analysis was performed at the end of the program. Quantification of each product was analyzed with ABI Prism 7000 SDS Software (Applied Biosystems). Expression levels of target genes were normalized with the amounts of GAPDH mRNA.

Cell-proliferation assay

Cell proliferation was determined by WST-8 and BrdU incorporation assay. WST-8 assay was performed

using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. KT-1 cells were seeded at 1×10^4 cells/well in a 96-well plate for 16 h and then treated with 0, 0.01, 0.1, 1 and 10 ng/mL of human TGF- β 3 (R&D Systems, Minneapolis, MN) for 72 h. Culture medium was replaced every day with each concentration of TGF- β 3.

BrdU-incorporation assay was performed as previously described (Nakamura et al. 2010). Briefly, we cultured cells on coverslips for 16 h and treated the cells with 1 ng/mL of human TGF- β 3 for 24 h. One h before fixation, BrdU (Roche Applied Science, Mannheim, Germany) was added to the culture medium to a final concentration of 10 μ M. The cells

were immunostained using BrdU labeling and detection Kit I (Roche Applied Science) and detected with an Alexa 488-anti mouse IgG (Molecular Probes, Eugene, OR). After immunostaining, the cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI; Sigma). The percentages of BrdU-labeled cells were determined by counting DAPI-stained and BrdU-labeled cells from five random fields; over 500 DAPI-stained cells were counted for each sample.

Cell-cycle measurement

We cultured cells and treated them with 1 ng/mL of TGF- β 3 for 24 h as described above. The cells were harvested by trypsinization and resuspended in staining solution (0.2% Triton X-100, 100 μ g/mL RNaseA, 50 μ g/mL Propidium iodide). For each cell population, 10,000 cells were analyzed by FACSCanto II (BD Biosciences, Bedford, MA), and the proportion in G₀/G₁, S and G₂/M phases was estimated using Modfit 3.0 LT software (Verity, Topsham, ME).

In situ hybridization

Dissected tongue was embedded in OCT compound (Sakura FineTech, Tokyo, Japan) and frozen in a liquid-nitrogen bath. Tissues were sectioned at 10 μ m and stored at -80°C until use. Complementary DNA fragments of p15^{Ink4b} and p21^{Cip1} were amplified with the following primers: p15^{Ink4b} forward, 5'-CTCACCGAAGCTACTGGGTCTC-3'; p15^{Ink4b} reverse, 5'-GTCAGAATCCAGGCATCAAGG-3'; p21^{Cip1} forward, 5'-CATGTCCAATCCTGGTGATGTC-3'; and p21^{Cip1} reverse, 5'-GTGTGAGGACTCGGACAATG-3'. The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI). The generated plasmid was verified by DNA sequencing and used as a template for synthesizing an RNA probe. An antisense probe was synthesized with digoxigenin-UTP using an RNA transcription kit (Roche Applied Science). In situ hybridization was performed as previously described (Miura et al. 2001). The detection reaction was performed by nitro blue tetrazolium (NBT, Roche Applied Science) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche Applied Science).

Statistical analysis

All data are averages of at least three independent experiments, and bars represent standard deviations of the means. Statistical analysis of BrdU incorporation assay and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed by the Student *T*-test. Statistical analysis was carried out using the statistical programming language R (www.r-project.org). The results were considered statistically significant when the *p*-value was less than 0.05.

Results

KT-1 cells express TGF- β signaling component mRNA

We first performed reverse transcription-polymerase chain reaction (RT-PCR) analysis to examine whether the mRNAs of the TGF- β signaling component were expressed in tongue-epithelial model KT-1 cells and tongue epithelia. We found that tongue epithelia and KT-1 cells expressed the TGF- β signaling component mRNAs (Fig. 1).

TGF- β 3 inhibits KT-1 cell proliferation and induces G₀/G₁ cell-cycle arrest

Characterization of TGF- β 1, TGF- β 2, and TGF- β 3 revealed that they have highly similar biological activities in vitro, but that TGF- β 2 is much less potent than TGF- β 1 and TGF- β 3 as a growth inhibitor of some epithelial cell lines (Cheifetz et al. 1990). In addition, in our preliminary study TGF- β 3 tended to

Fig. 1 Expression of TGF- β signaling component mRNA. RT-PCR analysis was performed using tongue epithelia (TE) and KT-1 cells. Gapdh was used as a loading control. H₂O was used as a negative control

give more reproducible results than TGF- β 1 on KT-1 cells (data not shown). In this study, we thus investigated the effect of TGF- β on KT-1 cells with TGF- β 3.

We performed proliferation assay and cell-cycle measurement to study the effects of TGF- β 3 on the cellular function of KT-1 cells in a serum-free condition. Cell proliferation was assessed by WST-8 assay and BrdU incorporation assay. These assays reflect cell numbers (WST-8) and proliferation activity (BrdU). The WST-8 assay indicated that treatment with TGF- β 3 inhibited KT-1 cell growth in a dose-dependent manner (Fig. 2a). Compared with a vehicle treatment (0 ng/mL), treatment with 0.01, 0.1, 1, and 10 ng/mL of TGF- β 3 suppressed KT-1

cell growth by 8.5, 35, 55, and 50%, respectively. To confirm the effect of TGF- β on inhibiting KT-1 cell proliferation, the BrdU incorporation assay was also carried out. The BrdU incorporation assay demonstrated that TGF- β 3 significantly reduced BrdU-incorporated cells (Fig. 2b). The percentages of BrdU-labeled cells were $41.9 \pm 6.9\%$ (0 ng/mL of TGF- β 3) and $13.2 \pm 3.9\%$ (1 ng/mL of TGF- β 3).

We next measured the cell cycle distribution of KT-1 cells by flow-cytometric analysis to study the growth-inhibition mechanism of TGF- β . Compared with a vehicle treatment (0 ng/mL; 47.3%), treatment with 1 ng/mL of TGF- β 3 increased the ratio of G₀/G₁ phase (80.2%). Moreover, the ratio of S phase was reduced from 37.5 to 14.0% after TGF- β 3 treatment

Fig. 2 Effects of TGF- β on KT-1 cell proliferation and cell-cycle profile. **a** Cell growth was measured with the WST-8 assay in the presence of TGF- β 3. Growth of KT-1 cells was inhibited in a dose-dependent manner by treatment with TGF- β 3. The absorbance at 590 nm was normalized to the cell treatment with 0 ng/mL of TGF- β 3. **b** Percentage of BrdU-labeled cells (BrdU⁺). TGF- β 3 (1 ng/mL) reduced the

percentage of BrdU⁺ cells. This experiment was repeated three times. The Asterisk indicates $p < 0.01$, compared with 0 ng/mL of TGF- β 3 (control). **c** Cell-cycle distribution of KT-1 cells with 0 ng/mL (left) or 1 ng/mL of TGF- β 3 (right). TGF- β 3 increased the ratio of G₀/G₁ phase and reduced the ratio of S phase. This experiment was repeated three times with similar results

(Fig. 2c). The reduced percentage of S phase agreed with the result of the BrdU-incorporation assay.

TGF- β 3 induces expression of p15^{Ink4b} and p21^{Cip1} in KT-1 cells

Ink4 and Cip/Kip families are known to regulate the G₁/S phase transition by inhibiting kinase activity of G₁ cyclin-CDK complexes (Sherr and Roberts 1999). In TGF- β mediated growth inhibition, Ink4 and Cip/Kip families play important roles in G₁ cell-cycle arrest. We accordingly examined the expression change of Ink4 and Cip/Kip family mRNAs by qRT-PCR to characterize the mechanisms of the KT-1 cell-growth inhibition by TGF- β . We detected all CDKIs mRNA in KT-1 cells with qRT-PCR and found that TGF- β 3 treatment significantly increased p15^{Ink4b} and p21^{Cip1} mRNA expressions in KT-1 cells (1.93 ± 0.14 , 1.94 ± 0.09 , respectively), whereas expressions of other members of Ink4 and

Cip/Kip families were unaffected (Fig. 3a). The ratio of copy number p15^{Ink4b}/GAPDH of TGF- β 3 treated samples was 0.019 ± 0.001 and that of p21^{Cip1}/GAPDH was 0.28 ± 0.01 . The changes of p15^{Ink4b} and p21^{Cip1} mRNA levels were also examined at the indicated time points. After treatment with TGF- β 3, the increase of p15^{Ink4b} mRNA was rapid and sustained, whereas the increase of p21^{Cip1} mRNA was rapid and transient (Fig. 3b).

p21^{Cip1} mRNA are expressed in suprabasal cells of tongue epithelium

We performed in situ hybridization for p15^{Ink4b} and p21^{Cip1} mRNA in tongue epithelium to determine their localization. Having examined in situ hybridization for p15^{Ink4b} mRNA, we were unable to get signals for p15^{Ink4b} mRNA in tongue epithelium (data not shown). The localization of p21^{Cip1} mRNA was mainly observed in suprabasal cells, not in terminally

Fig. 3 TGF- β 3 regulates CDKIs mRNA expression in KT-1 cells. **a** CDKIs mRNA levels in KT-1 cells after TGF- β 3 treatment. KT-1 cells were treated with 0 ng/mL (Control) or 1 ng/mL of TGF- β 3 for 6 h. Total RNA from each sample was used in qRT-PCR analysis. Expression levels of each gene were normalized with control samples. Asterisks indicate

$p < 0.01$, compared with control samples. **b** Time course of p15^{Ink4b} and p21^{Cip1} mRNA levels. RNA was obtained from 0 h (untreated), 2 h, 6 h, and 24 h after TGF- β 3 treatment. Expression levels of each time point were normalized with 0 h samples

differentiated cells of tongue epithelia (Fig. 4). Basal cells of tongue epithelium exhibited weak signals or no signals at all for p21^{Cip1} mRNA.

Discussion

In this study, we demonstrated that TGF- β 3 inhibited proliferation of KT-1 cells derived from tongue epithelium. Moreover, we showed that TGF- β 3 increased the expression of p15^{Ink4b} and p21^{Cip1} mRNA on KT-1 cells.

The WST-8 assay and the BrdU incorporation assay indicated that TGF- β 3 suppressed proliferation of KT-1 cells in a dose-dependent manner (Fig. 2a, b). Cell-cycle analysis revealed that TGF- β 3 increased the ratio of G₀/G₁ phase and reduced that of S phase (Fig. 2c). These results strongly suggest that TGF- β 3 inhibited proliferation of KT-1 cells by inducing G₀/G₁ cell-cycle arrest. CDKs play an important role in G₁ cell-cycle arrest by inhibiting kinase activity of G₁ cyclin-CDK complexes (Sherr and Roberts 1999). In TGF- β mediated growth inhibition, TGF- β s cause G₀/G₁ cell-cycle arrest by inducing the expression of CDKIs, whereas the CDKIs increased by TGF- β appear to vary among cell types. In this study, we found that TGF- β 3 increased the expression of p15^{Ink4b} and p21^{Cip1} mRNA in KT-1 cells, while TGF- β 3 did not affect the expression of the other CDKIs mRNA (Fig. 3a). In TGF- β mediated growth inhibition in KT-1 cells, this result suggests that p15^{Ink4b} and p21^{Cip1}, but not the others, could possibly inhibit kinase activity of G₁ cyclin-CDK complexes and then lead to G₀/G₁ cell-cycle arrest.

CDKIs not only affect G₀/G₁ cell-cycle arrest but also play an important role in cell-cycle exit (Siegenthaler and Miller 2005). Cell-cycle withdrawal is believed to be coupled to cellular differentiation. For example, it was reported that expression of p21^{Cip1} led to cell-cycle exit and differentiation of primary keratinocytes (Missero et al. 1996; Rangarajan et al. 2001). In tongue epithelium, a BrdU injection experiment demonstrated that basal cells undergo cell-cycle progression, whereas suprabasal cells exit from the cell cycle (Hirota et al. 2001; Hamamichi et al. 2006). Hirota et al. (2001) also reported that p21^{Cip1} protein was expressed in

suprabasal cells of tongue epithelium, suggesting that p21^{Cip1} plays a role in cell-cycle exit associated with tongue epithelial cell differentiation. Similarly, our in situ hybridization result indicated that p21^{Cip1} mRNA was mainly located in suprabasal cells, not in terminally differentiated cells of tongue epithelium (Fig. 4). In the qRT-PCR assay, we demonstrated that treatment with TGF- β 3 induced rapid and transient p21^{Cip1} mRNA expression (Fig. 3b). A previous study revealed that transit expression of p21^{Cip1} permitted progression of keratinocyte to the late stages of differentiation (Di Cunto et al. 1998). Taken together, these results suggest that TGF- β signaling also controls the tongue epithelial cell cycle and differentiation through modulating the expression of p21^{Cip1}.

In conclusion, we demonstrated that TGF- β 3 inhibited KT-1 cell proliferation in a dose-dependent manner. Furthermore, TGF- β 3 increased expression levels of p15^{Ink4b} and p21^{Cip1} mRNA and induced G₀/G₁ cell-cycle arrest in KT-1 cells. These results suggest that TGF- β signaling negatively regulates proliferation of tongue epithelial cells.

Fig. 4 Expression of p21^{Cip1} mRNA in tongue epithelium. In situ hybridization was performed with digoxigenin-labeled anti-sense RNA probes. The signals for p21^{Cip1} mRNA were observed in suprabasal cells of tongue epithelium. Dotted lines indicate the epithelial-mesenchymal boundary, and dashed lines, the basal-suprabasal cell layer boundary. Scale bars indicate 20 μ m

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