Neural stem cells lose telomerase activity upon differentiating into astrocytes

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Abstract

Serum-free mouse embryo (SFME) cells were established by D. Barnes et al., and are known to be a neural stem cell line, which differentiate into astrocytes upon treatment with TGF- β . Therefore, SFME cells is thought to be a model well suited to analyze the differentiation mechanism of neural stem cells. Until now, we have investigated the regulation mechanisms of telomerase activity and telomere length in human cancer and normal cells. Telomerase is the enzyme responsible for the synthesis and maintenance of telomere repeats located at chromosomal ends and is normally expressed in embryonic and germline cells, but not in most normal cells. Here, using SFME cells, we attempted to analyze the regulation mechanism of telomerase activity in neural stem cells and to detect a change upon differentiation into astrocytes. When SFME cells were cultured in the presence of TGF- β , cells showed an elongated morphology and decreased its growth to 50% of control culture. Cells also expressed the glial fibrillary acidic protein (GFAP), a marker for astrocytes, indicating that TGF- β induced differentiation in SFME cells from neural stem cells into astrocytes. At the same time, TGF- β also inhibited telomerase activity and repressed the expression of the mouse telomerase reverse transcriptase (mTERT), demonstrating that SFME cells was vested with a finite replicative life span upon treatment with TGF- β . To understand the mechanisms regulating *mTERT* levels during differentiation into astrocytes, we have estimated the expression level of *c-myc*, which is known to be a key molecule in activating the *TERT* promoter. As a result, TGF- β -treated SFME cells were shown to repress the expression of *c-myc*. Furthermore, promoter analysis, using the 5'-region of the *mTERT* gene, which possess two E-box elements bound to c-Myc/Max, demonstrated that *mTERT* promoter activity greatly decreased in TGF- β -treated SFME cells as compared to non-treated SFME cells. These suggest that *c-myc* might play a critical role in the expression of *mTERT*, and that down-regulation of *c-myc* dependent upon the astrocytic differentiation in SFME cells might cause the repression of *mTERT* in TGF- β -treated SFME cells.

Abbreviations: hTERT – human telomerase reverse transcriptase; mTERT – mouse telomerase reverse transcriptase; BMP2 – bone morphogenetic protein 2; CNS – central nervous system; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; GFAP – glial fibrillary acidic protein; LIF – leukemia inhibitory factor; RT-PCR – reverse transcription-polymerase chain reaction; SFME – serum-free mouse embryo; TGF- β – transforming growth factor- β ; TRAP – telomeric repeat amplification protocol.

Introduction

Telomeres are highly conserved sequences at the ends of eukaryotic chromosome. In most mammalian cells,

telomeric DNA consists of tandem repeats of the sequence TTAGGG. Telomeres are widely thought to play a role in the maintenance of chromosome structure and stability (Greider, 1996). Telomerase

is a ribonucleoprotein enzyme that synthesizes the telomeric DNA sequence onto the 3' ends of chromosomes. Embryonic cells and germline cells have been shown to express telomerase activity. In contrast, most normal somatic cells express low or undetectable levels of telomerase, and as a consequence their telomeres shorten with each cell division cycle (Harley et al., 1990; Hastie et al., 1990), and eventually reach the threshold length that leads to replicative senescence as a result of genomic instability. On the other hand, it has been suggested that multipotent cells, such as embryonic and germline cells, acquire their immortality by maintaining telomerase activity (Thomson et al., 1998).

Multipotent stem cells would be an invaluable source for regeneration studies of the injured or damaged cells and organs. Recently, to regenerate and/or rescue the damaged central nervous system (CNS), a worldwide attention has been focused on the studies of neural stem cells. Neural stem cells exist not only in the developing mammalian nervous system but also within the adult nervous system of all mammalian organisms (McKay, 1997; Alvarez-Buylla et al., 1998). Mouse embryonic neuroepithelial cells are known to contain cells that have the potential to differentiate into both neurons and glial cells like astrocytes (Reynolds et al., 1992; Johe et al., 1996; Rajan and McKay, 1998). Serum-free mouse embryo (SFME) cells have been characterized as a multipotent neural stem cell line. SFME cells cultured in the serum-free medium continues to grow without reaching the crisis stage or showing gross genomic alteration. Furthermore, SFME cells do not show tumorigenicity in syngeneic and athymic mice (Loo et al., 1987). In addition, SFME cells differentiate into astrocytes in the presence of TGF- β or serum (Sakai et al., 1990). Therefore, SFME cells are thought to be valuable for investigating the differentiation mechanism of neural stem cells.

As mentioned above, SFME cells can be maintained for extended period, thus is thought to be immortal, which suggests that these cells maintain its immortality by telomerase. Previously, the total brain of a murine embryo has been shown to express significant level of telomerase activity (Greenberg et al., 1998; Martin-Rivera et al., 1998). However, little is known about the regulation of telomerase regulation during brain development and neural differentiation. In this report, we attempted to study the regulation mechanisms of telomerase activity during the differen-

Figure 1. The effect of TGF- β on SFME cell proliferation. SFME cells were cultured for 9 days in the presence of 10 ng ml⁻¹ TGF- β (○) or the normal growth medium (●, control). Cells were counted using a cell counter (Sysmex F-300, TOA, Kobe, Japan) every day.

tiation of neural stem cells into astrocytes using SFME cells.

Materials and methods

Cells culture and reagents

SFME cells were cultured in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Gaithersburg, MD) supplemented with 100 μ g ml⁻¹ insulin (Sigma, St. Louis, MO), 10 μ g ml⁻¹ transferrin (Sigma), 10 μ g ml⁻¹ high-density lipoprotein (Biomedical Technologies Inc., Stoughton, MA), 50 ng ml⁻¹ EGF (Sigma), 25 μ g ml⁻¹ ampicillin (WAKO, Osaka, Japan), 200 U ml⁻¹ penicillin (Meiji, Tokyo, Japan), 200 μ g ml⁻¹ streptomycin (Meiji) and 10 nM sodium selenite (Sigma) in a humidified atmosphere with 5% CO₂ at 37 °C. SFME cells were treated for the indicated periods of time with 10 ng ml⁻¹ TGF- β (AUSTRAL Biologicals, San Ramon, CA).

RT-PCR

Total RNA was prepared by using TRIzol reagent (Life Technologies). 2.5 μ g of total RNA was annealed with 500 ng of oligo (dT)₂₀ at 70 °C for 10 min, followed by reverse transcription at 42 °C for 50 min using SuperScript II reverse transcriptase (Life Technologies). PCR was performed by using 1 μ l of the RT-reaction mixture for the amplification of either *mTERT* (mouse telomerase reverse transcriptase), *c-myc*, *GFAP* (glial fibrillary acidic protein) or *mGAPDH* in a total volume of 10 μ l containing 0.25 units of *Taq* polymerase (Roche Diagnostics,

Indianapolis, IN) and 10 pmol of primers specific for *mTERT* (5'-TAGAGGATTGCCACTGGCTC-3' and 5'-TCCAATGCTCTGCAGCTTGC-3'), or for *c-myc* (5'-TTCTCTCCTTCCTCGGACTC-3' and 5'-TGGCAGCTGGATAGTCCTTC-3'), or for *GFAP* (5'-ATGATGGAGCTCAATGACCG-3' and 5'-GGTTTCATCTTGAGCTTCT-3') or for *mGAPDH* (5'-CCGTAGACAAAATGGTGAAGGT-3' and 5'-GTG-GTGCAGGAGGCATTGCTGA-3'). Amplifications were performed 31 cycles (30 sec at 94 °C, 30 sec at 60 °C and 1 min at 72 °C) for *mTERT*, 27 cycles (30 sec at 94 °C, 30 sec at 58 °C and 1 min at 72 °C) for *c-myc*, 27 cycles (30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C) for *GFAP* and *mGAPDH*. All PCR reactions were done under hot-start conditions and analyzed by electrophoresis on 3% agarose gel.

Measurement of telomerase activity

Telomerase activity was measured by the PCR-based TRAP (Telomeric Repeat Amplification Protocol) assay (Kim et al., 1994) with some modifications (Katakura et al., 1997).

Primers and conditions for PCR were as previously described (Katakura et al., 1997). Quantification of telomerase activity was performed by the Kodak EDAS system (Eastman Kodak Co., Rochester, NY).

Measurement of *mTERT* promoter activity

Genomic DNA was extracted from SFME cells using the DNA Extractor WB Kit (WAKO). Using this mouse genomic DNA as template, the *mTERT* promoter region (−1560 to −27) was isolated by PCR using specific primers (5'-CCCCTCCCTCCTCTTCTTGTG-3' and 5'-CACGTGCGGGAACCAAGATG-3') as shown in Figure 5. This fragment containing the *mTERT* promoter was inserted into *Nhe* I site of the pGL3-Basic luciferase (firefly luciferase) plasmid (Promega, Madison, WI). As a positive control, pGL3-TK, which has the firefly gene under the transcription control of TK promoter, was used. For promoter assays, approximately 1×10^5 cells/well in 24-well plates were co-transfected with the *mTERT* promoter-luciferase construct (300 ng/well) and the pRL-TK (150 ng/well, Promega) which expresses the *Renilla* luciferase and was used as an internal control for transfection efficiency. All transfections were performed using the LipofectAMINE PLUS Reagent (Life Technologies). In TGF- β treated cells, cells were treated with TGF- β (10 ng ml^{−1}) after transfection. The cells

were harvested at 48 hr after transfection and the luciferase assay was done using the Dual-Luciferase Reporter Assay System (Promega). Samples were counted for 10 sec in a LUMINESCENCE READER BRL-301 (ALOKA, Tokyo, Japan), and the data is represented as the relative ratio of firefly luciferase/*Renilla* luciferase light unit.

Results and discussions

TGF- β induced differentiation of neural stem cells into astrocytes

We used SFME cells, which is a cell line established from 16-day-old Balb/c mouse embryo cells, to investigate the change in telomerase activity during the differentiation of neural stem cells. SFME cells is known to retain neural stem cell phenotypes when cultured in serum free media, and >80% of SFME cells differentiate into astrocytes upon treatment with TGF- β (Sakai et al., 1990). Here, we confirmed that differentiation of SFME cells into astrocytes does indeed occur upon treatment with TGF- β . TGF- β weakly inhibited the growth of SFME cells (Figure 1), which was observed from day 4 after treatment with TGF- β .

Furthermore, TGF- β -treated SFME cells showed extended cell morphology characteristic to astrocytes (Figure 2). RT-PCR analysis demonstrated that *GFAP* was induced in TGF- β -treated SFME cells, but not in non-treated SFME cells (Figure 2). These results suggest that TGF- β induced cell differentiation in SFME cells to become astrocytes.

It has previously been shown that TGF- β inhibited the growth of primary astrocytes (Toru-Delbauffe et al., 1990; Vergelli et al., 1995), although TGF- β induced differentiated SFME cells continued to grow at the reduced growth rate. It is unclear at present why astrocytes differentiated from SFME cells lacks the mechanisms of growth inhibition by TGF- β . On the other hand, SFME cells were maintained in serum-free medium supplemented with EGF and other growth factors (Ernst et al., 1991). SFME cells arrested growth at the G1 phase of cell cycle and subsequently underwent apoptosis upon the withdrawal of EGF (Rawson et al., 1990). Slinsky et al. (1999) has reported that p53, p21 and MDM-2 are not required for this G1 arrest and apoptosis in SFME cells by the withdrawal of EGF. According to these studies, p53 pathway in SFME cells does not appear to mediate G1 arrest and apoptosis. It has been previously shown that TGF- β increases the expression of

Figure 2. Effect of TGF- β on SFME cell differentiation. Photomicrographs of SFME cells grown for 2 days in the absence (A) or presence of TGF- β (10 ng ml $^{-1}$) (B). (C) Expression of *GFAP* mRNA during TGF- β -induced astrocytic differentiation. SFME cells were treated with TGF- β (10 ng ml $^{-1}$) for 2 days, and RT-PCR assays were performed to detect *GFAP* and *GAPDH* mRNA.

p21^{WAF1/CIP1}, and thereby TGF- β induces growth suppression of several cell types (Pardali et al., 2000). Sun et al. (1998) has reported that the overexpression of MDM-2 in culture cells could overcome the anti-proliferative effect of TGF- β . Therefore, incomplete growth inhibition in response to TGF- β observed in differentiated SFME cells may be caused by defects in p53 signaling pathway. Further studies are needed to clarify the signaling pathway of TGF- β relating to the differentiation and proliferation of SFME cells.

Differentiated SFME cells into astrocytes lost its telomerase activity

Several reports indicate that neuronal differentiation leads to the down-regulation of telomerase activity (Kurk et al., 1996; Chen et al., 1997; Fu et al., 1999, 2000; Tian et al., 1999; Haik et al., 2000). Here, we also measured the telomerase activity of the TGF- β induced differentiated SFME cells with the TRAP assay. Treatment of SFME cells with TGF- β at 10 ng

ml $^{-1}$ resulted in a remarkable reduction of telomerase activity, while non-treated SFME cells retained strong telomerase activity (Figure 3), indicating that SFME cells are immortal from the standpoint of a replicative life-span, and that SFME cells are vested with a finite replicative life-span upon differentiation. Therefore, the telomere length in differentiated SFME cells would shorten during long-term culture because of the loss of telomerase activity. These differentiated cells would then enter into the senescence and crisis stage. It has been demonstrated that some cell lines suppressed telomerase activity during terminal differentiation (Sharma et al., 1995; Albanell et al., 1996; Bestilny et al., 1996; Savovsky et al., 1996), which is distinct from our case in that SFME cells maintains its growth potential even after differentiation. We can rephrase these results as that the suppression of telomerase activity in SFME cells is dependent upon differentiation into astrocytes, but not upon cell cycle arrest. Suppression of telomerase activity independent

Figure 3. The effect of TGF- β on the telomerase activity of SFME cells. SFME cells were subcultured in the absence or presence of TGF- β (10 ng ml^{-1}). To measure telomerase activity semi-quantitatively, 1×10^6 cells were harvested, washed and lysed. Telomerase activity was determined in each cell extract using the TRAP assay method. The TRAP assay products were visualized by SYBR Green I staining.

Figure 4. Expression of *mTERT* and *c-myc* mRNA during TGF- β -induced astrocytic differentiation. SFME cells were treated with TGF- β (10 ng ml^{-1}) for 2 days, and RT-PCR assays were performed to detect *mTERT*, *c-myc* and *GAPDH* mRNA.

of cell cycle arrest was also observed in our previous study showing that TGF- β decreased telomerase activity in human cancer cells with maintaining its growth potential (Katakura et al., 1999). Both cases suggests that TGF- β transmits specific signals to suppress telomerase activity both in cancer cells and neural stem cells.

Undifferentiated SFME cells expressed high level of telomerase activity (Figure 3, lane 1). Telomerase activity has been shown to be responsible for the replicative immortality of human cells, thus exists at high

level in germ lines, embryonic tissues and embryonic stem (ES) cells (Wright et al., 1996; Thomson et al., 1998). Therefore, the high level of telomerase activity observed in SFME cells means that they have an infinite replicative life-span. In fact, it has been observed that SFME cells have been continuously passaged without evidence of growth crisis or senescence for over 200 doublings when cultured in serum-free medium (Loo et al., 1987). Here, we conclude that SFME cells are neuronal stem cells and might be a useful model cell line for identifying the pathways or regulation mechanisms of telomerase activity during neuronal cell differentiation.

*TGF- β repressed the expression of *mTERT* and *c-myc* mRNA*

To elucidate the detailed mechanism for the down-regulated telomerase activity in the differentiated SFME cells, we next measured the expression level of *mTERT* mRNA in the TGF- β treated-SFME cells using the RT-PCR analysis. *mTERT* expression was shown to be repressed in SFME cells exposed to TGF- β at 10 ng ml^{-1} for 2 days when compared with the expression of the internal control gene *GAPDH* (Figure 4). This result is consistent with the report that expression of *TERT* was tightly linked to the enzymatic activity of telomerase (Greenberg et al., 1998; Martin-Rivera et al., 1998). Moreover, it has been reported that *mTERT* mRNA levels parallel a marked decrease in telomerase activity during terminal differentiation of the mouse erythroleukemia cells (Greenberg et al., 1998). Therefore, we conclude that the down-regulated telomerase activity as shown in Figure 3 might be caused by the transcriptional repression of *mTERT* mRNA. The regulation mechanisms of *mTERT* expression must be elucidated to understand the molecular events that lead to neural differentiation. These findings suggest that the expression of *mTERT* is also tightly associated with the differentiation of SFME cells into astrocytes. However, very little is known about the expression and regulation of the *mTERT* gene during neural differentiation. It has recently been reported that the repression of *TERT* expression during differentiation was correlated with the loss of *c-myc* expression (Armstrong et al., 2000). In agreement with their report, the expression of *c-myc* in SFME cells was also shown to decrease upon differentiation by treatment with TGF- β (Figure 4). These results suggested that the repression of *c-myc* may trigger the repression of *mTERT* expression dur-

Figure 5. The effect of TGF- β on the transcriptional activity of *mTERT* promoter in SFME cells. A diagram of the *mTERT* promoter is shown by fixing the ATG codon as +1 (A). Moreover, two E-box elements are shown in box (Greenberg et al., 1999). (B) The reporter constructs were transfected into SFME cells, and then treatment with TGF- β was done for 2 days. Two days after transfection, luciferase expression levels for each reporter construct were assessed using the Dual-Luciferase Reporter Assay System (Promega). pGL3-Basic is a negative control, and pGL3-TK is a positive control. Data for each reporter construct is shown as the mean and standard deviation for samples done in triplicate.

ing the neural differentiation process. As shown in Figure 5A, the *mTERT* promoter possesses two E-box elements bound to c-Myc/Max which are located at -837 and -32 bp from the translation start point (+1). Thus, we isolated *mTERT* promoter by PCR using mouse genomic DNA as the template, then measured the *mTERT* promoter activity in differentiated SFME cells by luciferase assay. Figure 5B clearly demonstrated that the *mTERT* promoter activity in TGF- β induced differentiated SFME cells greatly decreased as compared to non-differentiated SFME cells. Considering together with the fact that *c-myc* is known to be a strong transcriptional activator for *hTERT* via E-box elements (Horikawa et al., 1999; Takakura et al., 1999; Wu et al., 1999), our results suggest that down-regulation of *c-myc* may lead to the repression of *mTERT* promoter activity. Down-regulation mechanisms of *c-myc* may be dependent upon neural differentiation and/or be a response to the TGF- β signaling. These questions must be clarified to elucidate the mechanisms for *mTERT* repression dependent upon neural differentiation.

Furthermore, *mTERT* expression in SFME cells was not completely repressed when treated with TGF- β for 2 days (Figure 4). In addition, the growth

potential partially remained in TGF- β treated-SFME cells (Figure 1).

These results suggested that the entry of SFME cells into a state of terminal differentiation might need other various molecules than TGF- β , or the continuously treatment of TGF- β . In fact, it has been reported that there are multiple routes for differentiation of CNS stem cells into astrocytes (Rajan and McKay, 1998). Nakashima et al. have demonstrated that LIF and BMP2 function in synergy to induce differentiation into astrocyte, but BMP2 or LIF alone did not induce astrocyte development (Nakashima et al., 1999). Thus, different growth factors may be necessary for the complete neuronal differentiation of SFME cells. Further studies on neural differentiation of SFME cells may provide valuable information about the neuronal aging, neuronal dysfunction and neuronal death, such as Alzheimer's disease and Parkinson's disease.

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