Adenovirus-Mediated Antisense Vector-Induced Inhibition of Human Telomerase RNA May Induce Differentiation of CD34+ Cells

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Background: Background Telomerase activation and human telomerase RNA (hTR) expression are known to be related to the preservation of the "stemness" of stem cells. In this study, we have inhibited the expression of hTR to find the relationship between the telomerase activity and differentiation of normal hematopoietic stem cells.

Methods: We used cord blood collected from 10 full term pregnant women. We classified the CD34+ hematopoietic stem cells from the same donor into three groups: the Ad-OA group was treated with the recombinant adenoviral (Ad) vector Ad-OA using telomerase antisense, the Ad-M6 group was treated with a mutant version of the Ad-OA without telomerase antisense, and a control group without any treatment.

Results: The mean number of colony-forming cells (CFCs) were 110 ± 38 for the Ad-OA groups, 540 ± 56 for the Ad-M6 groups, and 650 ± 72 for the control groups. Thus, CFCs in the Ad-OA group were lower than in the Ad-M6 group ($P < 0.01$). The myeloid portion of the CFCs in the Ad-OA group was higher than the Ad-M6 and control groups ($P < 0.01$). The Ad-OA group showed a higher percentage of granulocytes suggesting more of a tendency for myeloid differentiation than the Ad-M6 and control groups ($P < 0.01$). We found that the suppression of telomerase activity by the antisense telomerase adenovirus induced the differentiation of hematopoietic stem cells confirmed by differential cell count and cytochemical staining.

Conclusion: These findings suggest that the activity of the telomerase may play a role in the differentiation of normal CD34+ hematopoietic stem cells into mature cells. *(Korean J Hematol 2006;41: 172-178.)*

Key Words: Telomerase, Antisense adenovirus, Hematopoietic stem cell, Differentiation

INTRODUCTION

The RNA component (hTR) and telomerase catalytic subunit (hTERT) are the major components of telomerase. The hTR is known to act as a template for telomere synthesis.[1] Telomerase activation via hTR expression has been regarded as being associated with persistent survival of cells. Thus, immortalizing cells are considered to have elevated activity of telomerase. Cancer cells and stem cells are well-known immortalizing cells having characteristics of dividing continuously. Stem cells have two peculiar characteristics, self-renewal and pluripotency. However, it is still not clear which signal direct stem cells self-renewing into differentiating to a certain lineage. Previous reports suggest that the differentiation process from immortalized cells might be associated with down-regulated telomerase activity.[2,3] These results imply that the elevated telomerase activity of stem cells may be associated with controlling the balance between self-renewal and differentiation. Therefore, the modulation of telomerase activity in stem cells may be a possible way to differentiate human stem cells into a certain type of cells that we want. There has been some studies reporting a direct link between decreased telomerase activity and terminal differentiation in leukemic cells and normal hematopoietic stem cells.[4,5] However, it is still not determined whether the decreased telomerase activity causes differentiation of stem cells or differentiated stem cells have decreased telomerase activity. To answer this question, the effects of telomerase inhibition on hematopoietic stem cells was investigated in the only one previous study.[6] In this study, we have tried to verify the effect of the telomerase antisense sequence expressing adenoviral vector on the proliferation and differentiation as well as apoptosis of CD34+ cells harvested from cord blood.

MATERIALS AND METHODS

1. CD34+ cell isolation

Cord blood was collected from ten full term pregnant women during delivery after the acquirement of informed consents, and was studied according to the procedures approved by the institutional review board of Korea University Medical Center. CD34+ cells were purified by positive selection using a magnetic cell sorting (MACS) progenitor enrichment kit according to the manufacturer's protocol (MiltenyiBiotec, Bergisch Gladbach, Germany). The purity of enriched progenitor cells was $92\pm4\%$. Testing the CD34+ hematopoietic stem cells from the same donor was classified into three groups: the Ad-OA group was treated with the recombinant adenoviral (Ad) vector Ad-OA with telomerase antisense, the Ad-M6 group was treated with a mutant version of Ad-OA without telomerase antisense, and a control Mock group without any treatment. The Ad-M6 group was included in this study to evaluate the direct effect of adenovirus itself without telomerase suppression on CD34+ cells.

2. Adenoviral vector infection and long-term culture

Our recombinant adenoviral (Ad) vector Ad-OA is a E1a/E1b-deficient vector,[7] It contains residues 94 and 76 of the telomerase template sequence driven by the cytomegalovirus (CMV) immediate promoter, inserted into the E1 region (Fig. 1). The vector was amplified and titrated in a U293-cell line. The vector Ad-M6 contains six mismatched nucleotides in the antisense cassette, which reduces or prevents binding with the telomerase RNA.[6] The treatment of the adenovirus vector to CD34+ cells was performed in a similar manner as described by Song et al.[7] 5×10^4 cells were plated in a 35mm sized dish prior to infection, and adenovirus of Ad-OA or Ad-M6 were added in 1mL of media at a 50moi. Half of the medium was replaced with fresh adenoviral super-
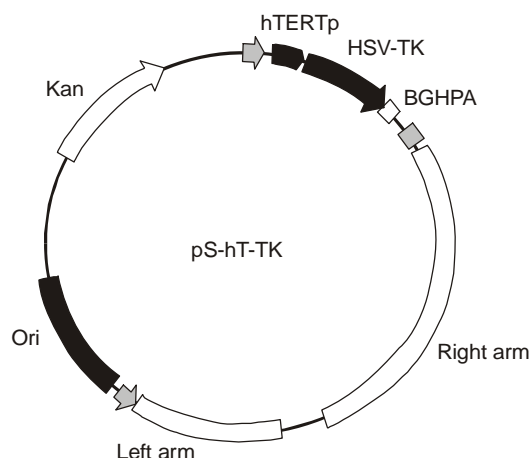
Fig. 1. Structure of recombinant adenoviral vector with deficient E1a/E1b. It contains residues 94 and 76 of the telomerase template sequence driven by the cytomegalo-virus immediate promoter, inserted into the E1 region.

natant of phoenix cells, which was repeated three times during 24 hours. The CD34+ cells of the control group were processed by the same method without adenoviral supernatant. Twenty-four hours after the last infection, transfected CD34+ cells (5×10^4) per a dish were co-cultured with irradiated human bone marrow stromal cells; the media was composed of IMDM (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% FBS, 12.5% horse serum, L-glutamine and 1% penicillin-streptomycin according to the protocol of long-term culture. Media were maintained without change until the 7th day of culture.

3. Evaluation

Infection efficiency of adenoviral vectors was determined by flow-cytometric assessment of a green fluorescent protein (GFP) expression, and the telomerase activity was examined by a telomeric repeat amplification protocol (TRAP) assay with 2,000 cells using a TeloTAGGG Telomerase PCR ELISA Kit in accordance with the manufacturer's instructions (Roche, Mannheim, Germany).[8]

To evaluate the proliferation potential of CD34+ cells processed by Ad-OA, Ad-M6, 0.1×

10^4 of CD34+ cells of each group were cultured in semi-solid Methocult® H4444 media (StemCell Technologies, BC, Canada) composed of 1% methylcellulose, 30% FBS, 1% bovine serum albumin (BSA), 50ng/mL human stem cell factor, 10ng/mL human granulocyte-macrophage colony-stimulating factor, 10ng/mL human interleukin-3, and 3units/mL erythropoietin. After 14 days of Methocult culture in a humidified incubator at 37°C and 5% CO_2, the plates were scored for colony-forming cells (CFCs) using an inverted microscope and gridded scoring dishes based on the standard criteria of the manufacturer's atlas.

After 3 and 7 days of long-term culture, total cell count with morphology and the degree of apoptosis were evaluated. Total cell counts were determined by counting in a hemocytometer; cellular morphology with differential counts of the WBC series was observed with Wright and cytochemical stains by microscopy. The apoptotic cells were detected by an Annexin V-FITC Kit (Clontech, USA). Briefly, the cells were rinsed and then suspended with 200μL of a binding buffer. After adding 5μL of Annexin V-FITC and 10μL of propidium iodide, the mixture was incubated at room temperature for 10minutes in the dark. The cells were then analyzed by a FACScalibur flow cytometer (Becton Dickinson, Mountain View, Calif) using CELLQuest software (Becton Dickinson, San Jose, Calif).

4. Statistical analysis

All data are presented as mean±SEM and statistical significance was determined using a one way ANOVA test. Results were considered significant when the P value was less than 0.05.

RESULTS

1. Infection efficiency and telomerase activity

The infection efficiency per a dish of Ad-OA group and Ad-M6 group was 36.8±8.2% and 41.5±12.7%, respectively without significant difference. After transfection, the telomerase activity

Table 1. Differential cell counts of Ad-OA, Ad-M6, and control group a3 and 7 day after long-term culture with CD34+ cells

	Day 3 (%, mean±SEM)			Day 7 (%, mean±SEM)		
	Ad-OA	Ad-M6	Control	Ad-OA	Ad-M6	Control
Blast	21±6	47±12	68±8	2±1	18±9	20±6
Lymphocyte	17±7	16±7	18±7	8±3	5±2	16±11
Granulocyte	45±5*	12±6	7±3	33±12*	9±3	16±5

*$P<0.01$. Abbreviations: Ad-OA, group treated with the recombinant adenoviral (Ad) vector Ad-OA with telomerase antisense; Ad-M6, group with a mutant version of Ad-OA without telomerase antisense; control, group without any treatment.
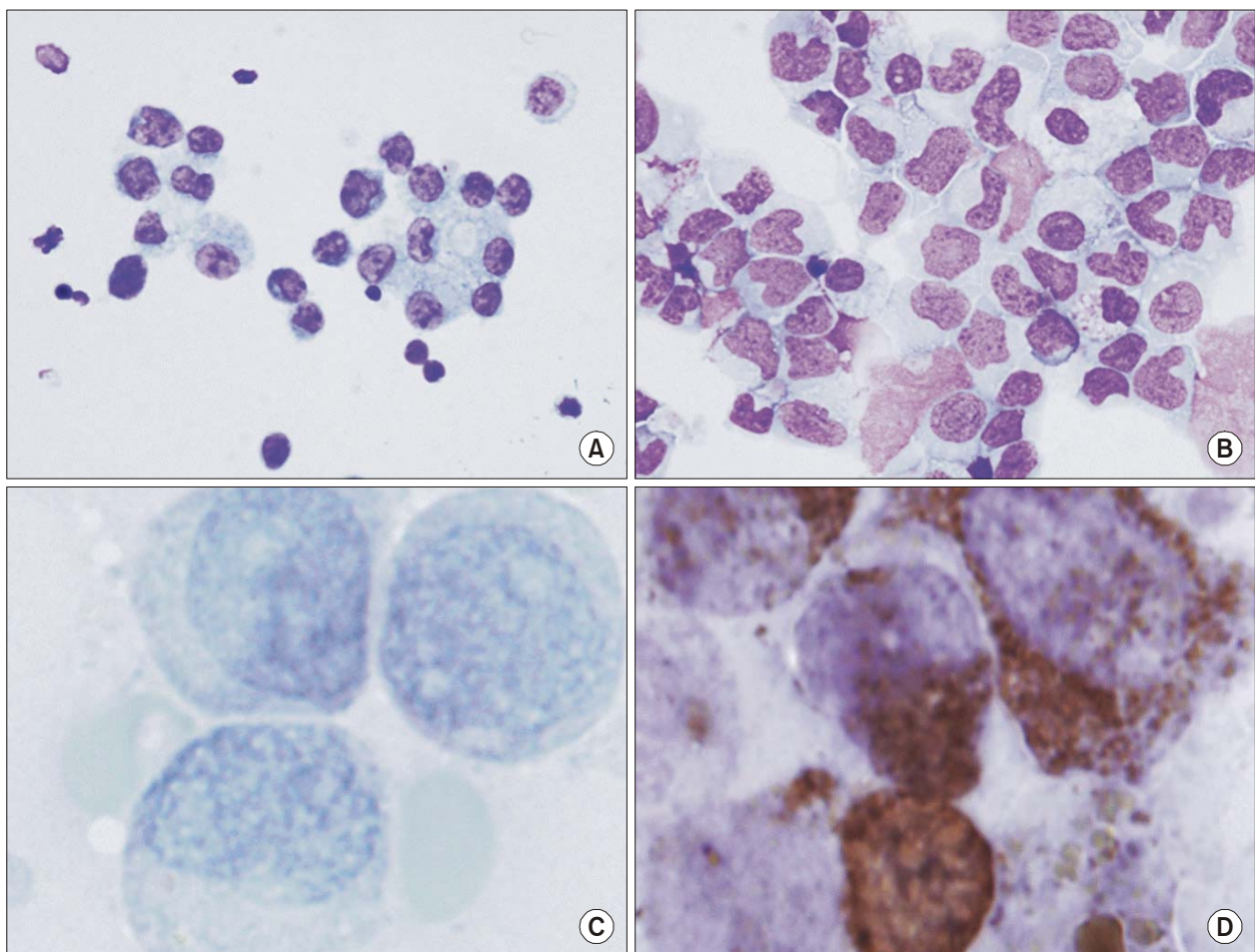

Fig. 2. Cell morphology in Ad-OA and Ad-M6 groups at day 7 of long-term culture with CD34+ cells. Top. Blood cell morphology with Wright Stain (×400): significantly higher percentage of granulocytes in the Ad-OA group compared to (B) those in the Ad-M6 group (A) this suggests a differentiation induction effect of telomerase antisense. Bottom. Granulocyte morphology of the myeloperoxidase stain (×1,000): There is a more dense myelopeoxidase stain pattern in the Ad-OA group when compared to (D) that in the Ad-M6 group (C) this suggests a maturation induction effect of telomerase antisense.

of CD34+ cells in Ad-OA group was 27±5%, which was significantly lower than those of the control group and the Ad-M6 group ($P<0.01$).

The telomerase activity of control and Ad-M6 group was 45±8% and 43±12%, respectively; without a significant difference.

2. Colony forming cells

The mean number of colony-forming cells (CFCs) per a dish in the Ad-OA, Ad-M6, and control groups was 110 ± 38, 540 ± 56, 650 ± 72, respectively. The number of CFCs in the Ad-OA group was significantly lower than those of the Ad-M6 and the control group ($P<0.01$). The percentile of erythroid portion including BFU-E in the Ad-OA, Ad-M6, and control groups was $13\pm18\%$, $28\pm14\%$, $31\pm8\%$. The percentile of myeloid portion including CFU-GM of the Ad-OA, Ad-M6, and control groups was $78\pm13\%$, $51\pm21\%$, $49\pm15\%$. The component myeloid portion of CFCs in the Ad-OA group was significantly higher than those of Ad-M6, and control group ($P<0.01$).

3. Cell count with morphology

The cell counts ($\times10^4$) per a dish at 3 days after long-term culture in the Ad-OA, Ad-M6, and control groups were 3.4 ± 1.7, 2.9 ± 2.1 and 3.7 ± 1.5, respectively, and there was no significant difference in the cell counts among the groups. The cell counts ($\times10^4$) at 7 days after long-term culture in the Ad-OA, Ad-M6, and control groups were 2.8 ± 0.9, 2.4 ± 1.5 and 4.3 ± 1.9, respectively. The cell count per a dish of the control group after 7 days of long-term culture was significantly higher than that of the Ad-OA and Ad-M6 groups ($P<0.01$). In addition, the differential cell counts of the Ad-OA group showed a significantly higher percentage of differentiated granulocytes; this suggests a greater tendency for myeloid differentiation than from the Ad-M6 and control groups. ($P<0.01$)(Table 1) Moreover, the granulocytes in the Ad-OA group showed a denser myeloperoxidase staining pattern, suggesting a greater maturation tendency compared to those of Ad-M6 and control groups. The findings of the Wright stain and the myeloperoxidase stain after 7 days of long-term culture in the Ad-OA group in comparison to the Ad-M6 group is presented in Fig. 2.
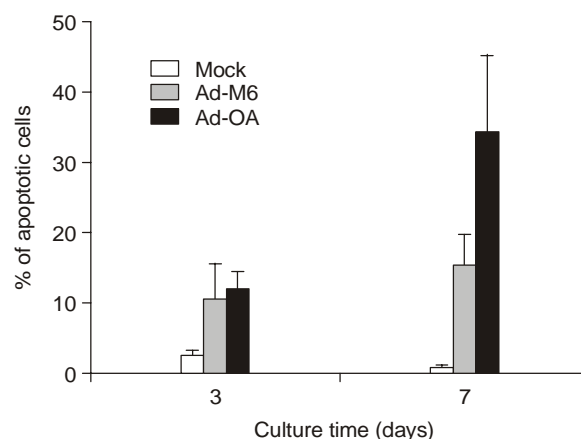

Fig. 3. The degree of apoptosis in CD34+ cells of Ad-OA, Ad-M6, and control Mock groups. The data represent mean values and standard deviation (S.D.) of three independent experiments.

4. Apoptosis

After 3 days of long-term culture, the degree of apoptosis per a dish of the Ad-OA group was $12.28\pm3.67\%$, which was comparable to those ($10.32\pm4.08\%$) of Ad-M6 group and significantly higher than those ($4.28\pm3.14\%$) of control group ($P<0.01$). After 7 days of long-term culture, the degree of apoptosis per a dish of the Ad-OA group was $34.40\pm5.94\%$. These results were significantly higher than those of the Ad-M6 ($13.36\pm6.82\%$) and control ($3.35\pm2.43\%$) groups ($P<0.01$, Fig. 3).

DISCUSSION

Sharma et al firstly reported that down regulation of telomerase activity is associated with the differentiation of immortal cells including cancer cells.[2] And then, there have been studies showing some evidences about the effect of telomerase suppression on the differentiation as well as apoptosis in cancer cells.[2-5] So, research to find new drugs that can induce differentiation and apoptosis of cancer cells by telomerase manipulation has become a central focus. For example, all-trans retinoic acid has recently been identified to induce apoptosis and differentiation in pro-

myelocytic leukemia cells by telomerase suppression.[5] By contrast, there have been a few reports on direct telomerase modulation in hematopoietic stem cells (HSCs). Uniquely, Zimmermann et al evaluated the effect of the augmentation and suppression of telomerase activity of HSCs using a retroviral vector containing a full-length hTERT cDNA; they found that telomerase function was directly associated with the proliferative capacity of HSCs, and suggested that it might have an additional role in lineage differentiation.[6] However, they didn't perform the study for differential count with blood cell morphology and the degree of apoptosis. In this study, we evaluated the effect of direct telomerase suppression, using antisense carrying adenovirus, on the differentiation by differential count and morphology as well as apoptosis by flowcytometry of CD34+ cells harvested from normal cord blood. As a result, we could observe the possibility for telomerase suppression of CD34+ cells to include the proliferation suppression by CFU findings and the induction of differentiation and maturation of WBC series by differential cell count and morphology. Of course, the apoptosis increment by telomerase suppression was observable. These findings suggest that telomerase suppression itself might induce differentiation of normal HSCs, and similar with the findings that all-trans retinoic acid induce apoptosis and differentiation of promyelocytic leukemia cells by telomerase suppression.[5] There has been some studies concerning to the association between telomerase activity and the proliferation and differentiation potential of cells. As a study for telomerase suppression to induce cellular differentiation, Liu et al reported that the loss of telomerase activity is closely associated with myofibroblast differentiation, and possibly functions as a potential trigger for myofibroblast differentiation and expression of telomerase suppresses myofibroblast differentiation.[9] Contrarily, there have been some studies reporting that telomerase augmentation is needed for the facilitation of differentiation. Liu et al

reported that telomerase deficiency impairs differentiation of mesenchymal stem cells and Foster et al observed for telomerase augmentation to facilitate osteoblast differentiation.[10,11] And Armstrong et al reported that overexpression of telomerase confers growth advantage, stress resistance, and enhanced differentiation of embryonic stem cells (ESCs) toward hematopoietic lineage.[12] But, Lee et al emphasized that ectopic mTERT expression in embryonic stem cells does not affect differentiation but confers resistance to differentiation- and stress- induced p53-dependent apoptosis.[13] Therefore, it seems that there is no established theory about the relationship between the changes of telomerase activity and the cellular differentiation, despite it is clear that telomerase suppression induces cellular apoptosis. Supposedly, the degree of telomerase modulation and the difference of physiologic telomerase activity of each stem cell seem to be the main factors to determine the result of telomerase modulation therapy on stem cells. For example, the telomerase augmentation mainly induces the differentiation in mesenchymal stem cells (MSCs), but proliferation of ESCs and telomerase suppression mainly induces cell deaths of MSCs but apotosis as well as differentiation of HSCs.[6,10-12]

In conclusion, the result of our study revealed that telomerase suppression developed not only the cellular apoptosis but also lineage differentiation of CD34+ cells. But there were some limitations in our study. The most important one was that we could not isolate the CD34+ cells infected with adenovirus among CD34+ cells due to technical problems. There might be a possibility that unknown reactions influencing cell biology in the adenovirus infected and non infected CD34+ HSCs could be present. The others were the relatively small sample size and short culture period due to the restricted cell number. Despite of these limitations, our study seems to a report showing that telomerase suppression might influence differentiation as well as apoptosis of CD34+ HSCs. Further study is now

warranted to clarify the role of telomerase suppression in HSCs.

요 약

배경: 텔로머라제 활성도와 텔로머라제 RNA의 발현은 줄기세포의 특성 유지에 관여하는 것으로 알려져 있다. 저자들은 텔로머라제 RNA 발현의 억제를 통해 조혈 줄기세포의 분화와 텔로머라제 활성도와의 관련성을 보고자 하였다.

방법: 10명의 임산부로부터 채취한 제대혈로부터 CD34+ 세포를 채집하여 다음과 같은 세 군으로 분류하였다. 텔로머라제 안티 센스를 포함한 재조합 아데노바이러스를 처리한 군(Ad-OA군), 텔로머라제 안티 센스를 포함하지 않은 아데노바이러스를 처리한 군 (Ad-M6군), 그리고 어떠한 처리를 하지 않은 대조군의 세 군을 나누어 집락 형성세포의 수, 세포 형태학적 특성 등을 비교 분석하였다.

결과: 평균 집락 형성 세포수(clony-forming cells)는 Ad-OA군에서 110±38로 Ad-M6군(540±56), 대조군(650±72)에 비해 유의있게 낮았다($P<0.01$). 그러나 Ad-OA군에서 중성구 형성군의 비율이 의미 있게 다른 두 군에 비해 높았으며 텔로머라제 억제를 통해 중성구로의 분화가 촉진되는 경향이 확인되었다. 세포 염색을 통한 형태학적 분석에서도 성숙한 중성구의 분획이 증가되어 조혈 줄기세포의 텔로머라제 억제를 통한 분화가 발생하였음을 확인하였다.

결론: 텔로머라제 활성도는 정상 CD34+ 조혈줄기세포의 성숙 세포로의 분화에 관련될 수 있으며 텔로머라제 활성도 조작을 통한 조혈줄기 세포의 분화기전에 대한 추가적인 연구가 요구된다.

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