

Evaluation of Energy Balance on Human Telomerase Reverse Transcriptase (hTERT) Alternative Splicing by Semi-quantitative RT-PCR in Human Umbilical Vein Endothelial Cells

Abstract

Background: Decreased high-energy phosphate level is involved in endothelial cell injury and dysfunction. Reduced telomerase activity in endothelial cells in parallel with reduced energy levels might be due to altered direction of alternative splicing machine as a complication of depleted energy during the process of atherosclerosis. **Materials and Methods:** Isolated human umbilical vein endothelial cells (HUVECs) were treated for 24 hours by oligomycin (OM) and 2-deoxy glucose (2-DG). After 24 hours, the effect of energy depletion on telomerase splicing pattern was evaluated using RT-PCR. Indeed, in both treated and untreated cells, nitric oxide (NO) and von Willebrand factor (vWF) were measured. **Results:** ATP was depleted in treated cells by 43.9% compared with control group. We observed a slight decrease in NO levels ($P = 0.09$) and vWF ($P = 0.395$) in the setting of 49.36% ATP depletion. In both groups, no telomerase gene expression was seen. Telomerase and housekeeping gene expression were found in positive control group (colon cancer tissue) and sample tissue. **Conclusions:** The absence of telomerase gene expression in HUVECs might be due to the mortality of these cells or the low level of telomerase gene expression in these cells under normal circumstances.

Keywords: ATP depletion, endothelial cell dysfunction, telomerase, telomere

Introduction

Telomerase is a reverse transcriptase enzyme, which is necessary for proper function of telomeres. It adds TTAGGG repeats to the ends of chromosomes. In this way, it protects terminal ends of chromosomes.^[1-4] Telomerase activity is controlled through various mechanisms, in which alternative splicing is one of them.^[5] Alternative splicing is a process in which various proteins are produced from single gene. Among eight alternatively spliced variants of telomerase gene, just full length variant is truly functional.^[6] Decreased telomerase activity in endothelial cells is seen in the process of atherosclerosis.^[7] This decreased telomerase activity is shown experimentally in regions susceptible to atherosclerosis.^[8] The cause of this decrease in telomere length is not well known, however, the theory of telomere shortening is vastly considered during the process of atherogenesis.^[9]

Endothelial cell dysfunction is one of the earliest events in the process of

atherosclerosis.^[10] Indeed, activated endothelial cells pour out UTP, ADP and ATP from themselves. ATP depletion and decrease in energy levels are in parallel with endothelial cell dysfunction.^[11-13] Reduction in adenosine nucleotide level plays an important role in the process of endothelial cell dysfunction.^[13,14] Diminished respiratory rate and ATP synthesis are due to the presence of different stimuli as free fatty acidosis, hypoxia, acidosis, etc.^[15,16] Decreased basic level of nitric oxide (NO) is associated with decreased level of ATP/ADP ratio, which is a marker of impaired recovery of cellular phosphorylation potential.^[13-18] Factors with the ability to maintain endothelial cell function, as 17-beta-hydroxy estrogen, increases efficiency of energy production in endothelial cells.^[19] Alternative splicing needs correct regulation, in which its mechanisms are not yet fully understood. Alternative splicing is an energy dependent process and in the setting of appropriate energy levels, incorrect splicing will occur.^[20,21]

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Decreased telomerase activity occurs in the process of endothelial cell dysfunction and atherosclerosis. Indeed, decreased ATP/ADP ratio is in parallel with endothelial cell dysfunction and atherosclerosis. Thus, it seems that this telomere dysfunction might be due to decreased energy levels, which could be attributed to impaired alternative splicing. Thus, in this study, we aimed to investigate the role of impaired energy balance on telomerase alternative splicing during the process of endothelial cell dysfunction and atherosclerosis.

Materials and Methods

Isolation of HVECs

Isolated placenta and cord were inserted in cold environment (4°C). Umbilical cord was inserted in a paper towel to get rid from blood and collection medium. A transverse incision was made at terminal end of placenta to determine vein and artery. Then, vein was cannulated after ligation of both terminal ends of vein. Internal lumen of cannula was filled with phosphate-buffer saline (PBS) (in both ends). After dilation of lumen, vein was transferred into incubator (37°C for 10 min). Then, umbilical cord was exited from incubator. Cells were isolated by manual massage and suction. Venous lumen was re-filled with prewarmed PBS. Internal lumen was filled by collagenase solution and cells were centrifugated for 5 min at 1000 rpm. Supernatant was transferred into tissue culture flask T25 with 4 ml of serum including culture medium without growth factor included. The next day, tissue culture medium was removed. Cells were washed using prewarmed serum-free culture medium. Then, 4 ml of tissue culture was added into cells. Cells were feeded with fresh serum plus culture medium. Cells were recultured after removing culture medium from confluent cells. Then, 1 ml of Trypsin-EDTA was added into flask. Flask was transferred into incubator for 5 min. After this stage, equal amounts of culture medium plus growth factor was added to cells. Cells were transferred into incubator and were feeded by changing culture medium every other day. In this study, cells were passaged for three times. Cultured cells were divided into test and control groups. Test cells were treated by oligomycine (OM) and 2-DG for 6 hours.^[1] Simultaneous administration of these agents leads to 50% reduction in ATP level without induction of apoptosis.^[1]

Endothelial cell characterization

Endothelial cells were characterized using observation of cobble-stone morphology under microscopic evaluation [Figure 1]. Endothelial cells were identified using flow cytometry by anti-CD34 (PECAM) monoclonal antibody.

Nucleotide measurement

ATP/ADP ratio was measured as a marker of endothelial cell dysfunction. Changes in ATP/ADP ratio were measured using measured by bioluminator detection. This kit works

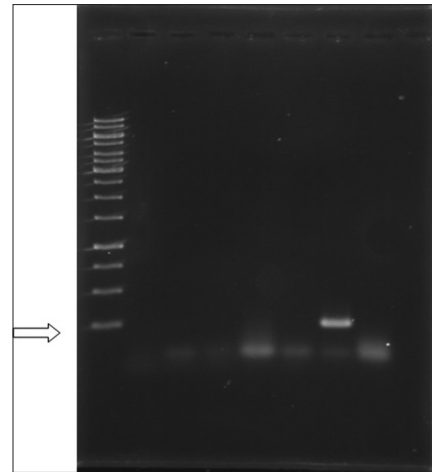


Figure 1: Photo indicates the presence of telomerase (4120 bp), 100 bp DNA ladder

based on luciferase enzyme. About 10000 cells were used. Nearly 10^3 - 10^4 (10 μ L) of cells were transferred into tissue culture palates after removing culture medium. Next, 100 μ L of nucleus releasing buffer was added into cells and cells were incubated in room temperature for 5 min. A total of 1 μ L of ATP monitoring enzyme was added into cell lysate and samples were read out in luminator.

Nitric oxide measurement

NO was measured in conditioned medium indirectly using quantification of nitrites by total NO and nitrate/nitrite assay kit per protocol. NO level of both treated and nontreated cells were measured.

Von Willebrand factor measurement

Extracellular von Willebrand factor (vWF) was measured in HUVCs embedded in 96-well plates pre- and post-ATP depletion through Asserachrom vWF kit per protocol (Diagnostic Stago, France).

Expression of telomerase alternative splicing variants after ATP depletion

About 6 hours after induction of ATP depletion, cellular RNA was extracted using RNA extraction kit, per protocol. The following primer pairs were used:^[22]

hTERT980S/hTERT1303A, hTERT2164S/hTERT 2620A, hTERT1241S/hTERT1835A, hTERT1783S/hTERT2247A

PCR conditions were as follows:

94°C for 60 seconds

33 cycles of (96°C for 20 seconds, 68°C for 50 seconds, 72°C for 50 seconds)

PCR products were electrophorezed on polyacrylamide gel.

As a positive control, we used a tissue with certain telomerase activity (colon cancer tissue). Beta-actin gene expression was used as a house-keeping gene to verify the

accuracy of RNA extraction. Beta-actin primer pairs were as follows:

Forward primer 1 GCACTCTTCCAGCCTTCCTTC 21

Template 862.882

Reverse primer 1 CAGCACTGTGTTGGCGTACA 20

Template 981.962

Statistical analysis was performed using SPSS version 17.00 by K² test.

Results

ATP depletion

ATP was reduced in treated cells by 43.9%. Luminescence change in ATP-depleted cells was 5082.5000 ± 2147.48329 (mean \pm SD). In these cells, ATP level was nonsignificantly reduced, which was due to the applied method to avoid cell apoptosis. In control cells, ATP level showed nonsignificant change. In cells of control group, ATP was $22711.5000 \pm 7845.34974$ (mean \pm SD).

ATP depletion and NO release

We observed a slight decrease (16.32%) in NO release between induced and uninduced endothelial cells (7.23 ± 1.13 vs. 8.64 ± 0.85 $\mu\text{m/L}$, $P = 0.09$, $n = 3$). Indeed, our data demonstrated a marginal decrease (15.73%) in secreted vWF between treated and untreated cells (0.0876 ± 0.0087 vs. 0.1056 ± 0.023885 $\mu\text{m/L}$, $P = 0.395$, $n = 3$) [Table 1].^[23]

Telomerase gene expression

In both groups, no expression of telomerase gene expression was found. Since no telomerase gene expression was found in human umbilical vein endothelial cells (HUVECs), we investigated telomerase gene expression in colon cancer tissue. In this tissue sample, the presence of telomerase gene expression (380 bp) and full length telomerase gene (475 bp) were seen [Figures 2 and 3]. Other variants were not found in this sample. Expression of beta-actin as a house keeping gene confirmed the accuracy of RNA expression method in our survey [Figure 4].

Discussion

Our data implies induced ATP depletion and consequently induced decreased energy level in treated cells. In this setting, NO and vWF factors were measured in which

full discussion in values are explained by Behjati et al.^[23] In the setting of both ATP depletion and normal energy balance, we observed no telomerase gene expression in HUVECs; thus we used a tissue with certain telomerase

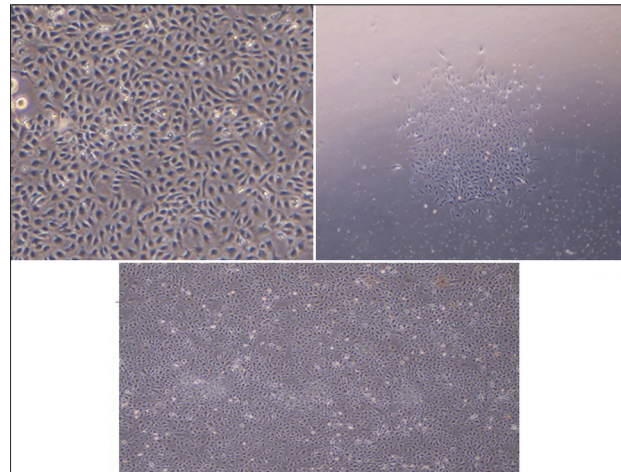


Figure 2: Cobble-stone morphology of HUVECs

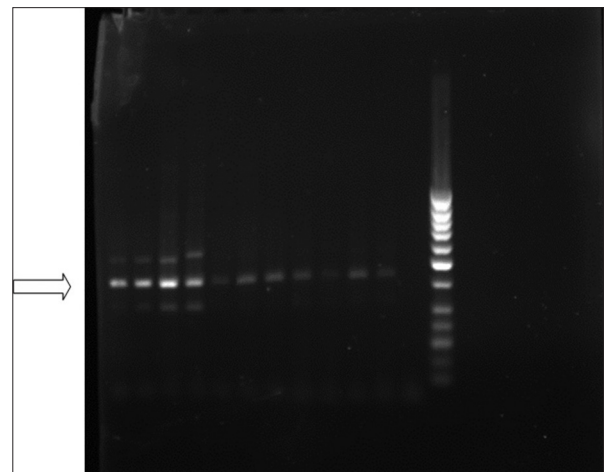


Figure 3: The photo indicates the presence of telomerase (323 bp) using hTERT980S and hTERT1303A Primer pairs (50 bp DNA ladder)

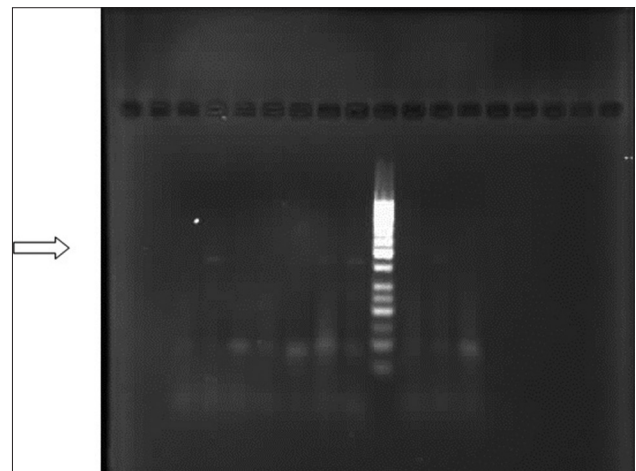


Figure 4: The photo indicates the presence of telomerase (475 bp) using hTERT2164S and hTERT2620A primer pairs (50 bp DNA ladder)

Table 1: Effect of ATP depletion on NO level and vWF secretion of human umbilical vein endothelial cells

Parameter	Mean \pm SD	
	Before	After
No	8.64 \pm 0.85	7.23 \pm 1.13
vWF	0.087 \pm 0.008	0.105 \pm 0.023

ATP: Adenosine tri-phosphate, vWF: von Willebrand factor

gene expression. Full length telomerase gene was found in this tissue. Other variants were negative in this tissue. Expression of beta-actin gene, confirms the accuracy of RNA extraction. These data demonstrate that telomerase gene expression is very low or zero in isolated HUVECs, which is undetectable by RT-PCR. These cells are mortal. In previous studies, immortalized HUVECs using transfected TERT or other vectors were used.^[24,25] In this way, endothelial cell lines were preferred. But since we aimed to evaluate the effect of impaired energy balance on telomerase activity, we were not allowed to use immortal cells. It may be possible that these cells possess very low amounts of telomerase activity, undetectable by RT-PCR. These cells may also have no telomerase activity.

In order to evaluate telomerase gene expression in HUVECs, various methods have been used. Quantitative real time RT-PCR, PCR-based TRAP analysis, linear range of TRAP assay are most often used methods.^[26-30] Some investigators have also used RT-PCR for assessment of telomerase gene expression in HUVECs in instances like assessment of telomerase activity by RT-PCR after induction of TERT over-expression.^[31,32] Indeed, RT-PCR was used to assess telomerase gene expression in HUVECs after administration of agents, which increase telomerase gene expression as 17beta-estradiol. Since both morphology and flow cytometry analysis, confirm the through isolation procedure of endothelial cells, it seems interesting that why telomerase gene expression was evaluated by semi-quantitative RT-PCR by some researchers but with similar applied method, it was undetectable in our survey. So, is semi-quantitative RT-PCR a suitable method for evaluation of telomerase gene expression in HUVECs?

Conclusions

The absence of telomerase gene expression in HUVECs might be due to the mortality of these cells or the low level of telomerase gene expression in these cells under normal circumstances.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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