BIOGERONTOLOGY

Epithalon Peptide Induces Telomerase Activity and Telomere Elongation in Human Somatic Cells

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Addition of Epithalon peptide in telomerase-negative human fetal fibroblast culture induced expression of the catalytical subunit, enzymatic activity of telomerase, and telomere elongation, which can be due to reactivation of telomerase gene in somatic cells and indicates the possibility of prolonging life span of a cell population and of the whole organism.

Key Words: *peptide; Epithalon; telomerase; telomeres; fibroblasts*

Epithalon peptide (Ala-Glu-Asp-Gly) was constructed and synthesized on the basis of amino acid composition of Epithalamine, a complex peptide preparation isolated from animal brain epiphysis [10]. Epithalon restores disordered neuroendocrine regulation in old monkeys [11], activates ribosomal genes [1]. Addition of the tetrapeptide cultured lymphocytes isolated from elderly and senile humans promoted decondensation of pericentrometric heterochromatin and activation of genes repressed because of age-associated condensation of chromosomal euchromatin regions [3]. Epithalon prolongs the mean life span of mice and rats and decreases the incidence of chromosome aberrations in rapidly aging SAM mice [2,4]. It is noteworthy that Epithalon prolongs the mean and maximum life span without development of malignant tumors in these animals [4-6]. The telomerase theory of aging attributes the age-associated decrease of the tissue proliferative potential to critical shortening of telomeres in the course of cell division [15]. Telomerase is a ribonucleic enzyme constructing the telomere repeats TTAGGG, which are lost because of asymmetry in the synthesis of the leading and lagging

DNA strands [13] and is encoded by two genes (for RNA component and protein component of the enzyme) [9]. In humans the expression of telomerase protein component and the corresponding enzyme activity are observed in the majority of malignant, sex, early embryonal, and, presumably, stem cells. Human somatic cells have no telomerase activity [12]. We investigated the effect of Epithalon on the expression of telomerase catalytical subunit, telomerase activity, and telomere elongation in human somatic cells.

MATERIALS AND METHODS

Telomerase-positive HeLa culture [9] and primary culture of human fetal lung fibroblasts 602/17 (24 weeks) were obtained from Laboratory of Cell Cultures of Institute of Influenza, Russian Academy of Medical Sciences. The cells were cultured in DMEM with 10% fetal calf serum, 2 mM L-glutamine, and 100 µg/ml gentamicin sulfate. Starting from passage 27, fetal fibroblasts were treated with Epithalon in a concentration of 0.05 µg/ml for 4 days and analyzed [3].

In order to detect telomerase protein catalytical subunit, fetal fibroblasts were put onto sterile slides (Menzek-Glaser). Immunohistochemical staining was carried out using murine monoclonal antibodies to human telomerase catalytical subunit NCL-hTERT

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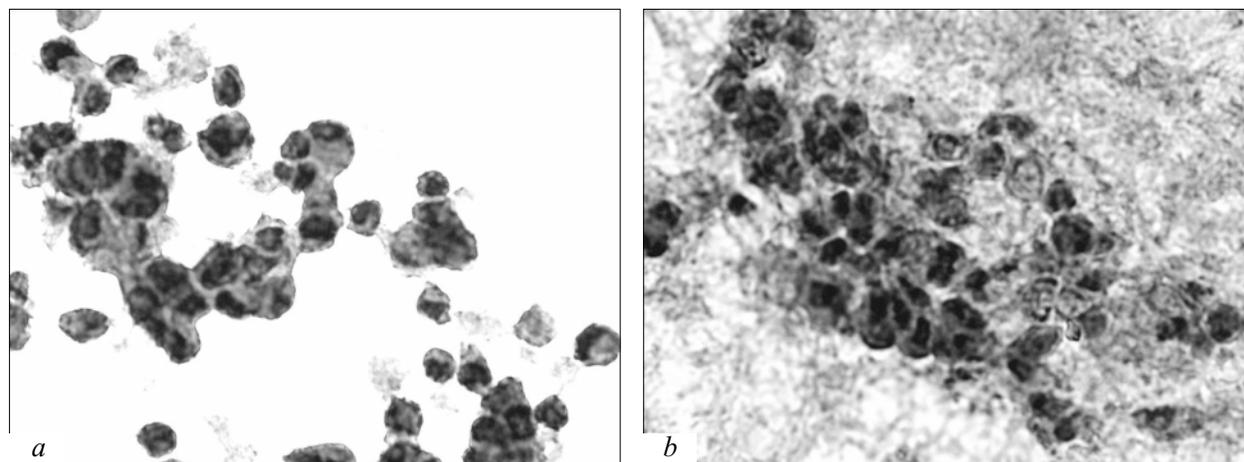
Fig. 1. HeLa cells (*a*) and fetal fibroblasts treated with Epithalon (*b*). Immunoperoxidase staining, ×400.

(Novocastra Laboratories Ltd.). Universal peroxidase ABC kit (Dako) served as second antibodies. The preparations were stained in accordance with manufacturer's instructions. Microscopy was carried out under a Nikon Eclipse E400 device with AST1 digital camera.

Telomerase activity was evaluated according to the protocol of telomere repeat amplification (TRAP) [9]. The cells (10^6) were washed with cold phosphate-buffered saline (PBS) and lyzed for 30 min in 100 µl cold CHAPS buffer (10% glycerol, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 0.1 mM PMSP, and 5 mM 2-mercaptoethanol). The extracts were centrifuged for 30 min at 12,000 rpm and 4°C. The supernatants were transferred into tubes and stored at -70°C. For *in vitro* analysis of telomerase activity, 2 µl supernatant was added to 46 µl 1 Taq buffer containing 2 µM TS primer (5'-AAT CCG TCG AGC AGA GTT-3'), 50 µM dNTPs, and incubated at 30°C for 30 min. Then 2 µM CX primer (5'-CCC TTA CCC TTA CCC TTA CCC TTA-3'), 5 U Taq-DNA polymerase (Promega) were added, and 35 PCR cycles with the following parameters were carried out: 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C on a DNA Engine PTC-200 thermocycler with warming cover (MJ Research). PCR products were electrophoretically separated in 10% nondenaturing polyacrylamide gel, stained with SYBR Green (Molecular Probes), and visualized in UV light.

The mean telomere length in individual cells was measured by fluorescent *in situ* hybridization with flow cytofluorometry (flow-FISH) [14]. To this end, the cells were twice washed in PBS containing 0.2% BSA and resuspended in hybridization solution containing 70% formamide, 1.0% BSA, 20 mM Tris-HCl (pH 7.0) (Sigma), and 0.3 µg/ml telomere-specific C_3TA_2 peptide-nucleic acid (PNA) FITC-labeled probe (PerSeptive Biosystems). The samples were subjected

to thermal denaturation at 80°C for 10 min with subsequent hybridization at 20°C for 2 h in the dark. Then cell suspension was centrifuged, supernatants were removed, and the cells were washed twice in a buffer containing 70% formamide, 10 mM Tris-HCl (pH 7.0), 0.1 BSA, 0.1% Twin-20 (Sigma), and once in PBS containing 0.1% BSA and 0.1% Twin-20. After washout the cells were resuspended in PBS containing 0.1% BSA, 10 µg/ml RNase A (Sigma), 0.06 µg/ml propidium iodide (Sigma), and incubated at 20°C in the dark for 2 h. Control hybridization without PNA was carried out in order to detect basal fluorescence and for estimating the fluorescence specific for telomeres in arbitrary units. The cells (10^4) were analyzed by flow cytofluorometry on a FACSCAN device (Beckton-Dickinson).
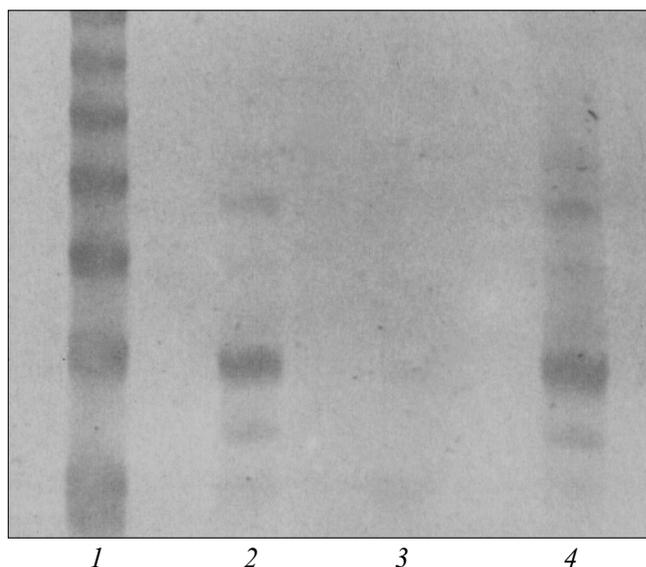

Fig. 2. Electrophoretic separation and detection of PCR-amplified elongated TS primers in PAAG. *1*) 10 b. p., DNA marker; *2*) HeLa cells; *3*) fibroblasts; *4*) fibroblasts treated with Epithalon.
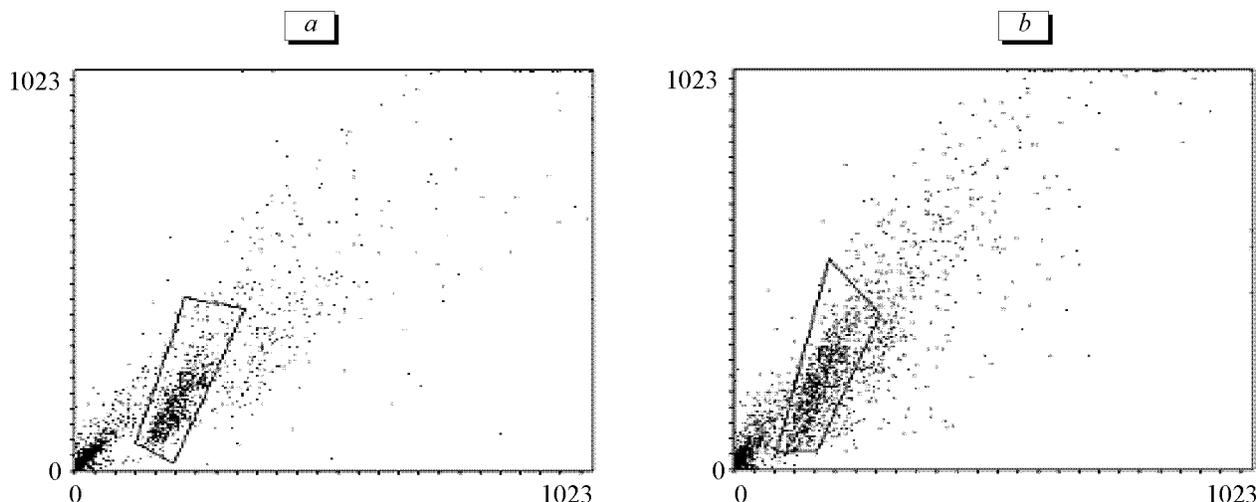
Fig. 3. Results of evaluation of telomere length by fluorescent *in situ* hybridization with flow cytofluorometry (flow-FISH). Fetal fibroblasts in control (*a*), Epithalon-treated fetal fibroblasts (*b*). Vertically: telomere staining with PNA-FITC probe (linear scale). Horizontally: cell DNA staining with propidium iodide (linear scale). Cells in the G1 phase of cell cycle are in the frame.

The results were statistically processed using Cell-Quest and XnView1.17 software. The significance of differences between the variants was evaluated using Student's *t* test.

RESULTS

Immunohistochemical study showed intense staining of telomerase-positive HeLa cells (Fig. 1, *a*) used as the positive control, and fetal fibroblasts treated with Epithalon (Fig. 1, *b*). No staining was seen in intact (control) fetal fibroblasts.

PCR also detected no telomerase activity in intact fetal fibroblasts, while HeLa cells and Epithalon-treated fibroblasts contained it (Fig. 2).

Fluorescent *in situ* hybridization with flow cytofluorometry demonstrated increased mean and maximum lengths of telomeres in G1 fetal fibroblasts treated with Epithalon in comparison with control cells (Fig. 3). The mean length of telomeres in G1 control cells was 180 arb. units. Epithalon increased the mean telomere length to 240 arb. units ($p < 0.05$).

These results indicate that in human somatic cells Epithalon can induce the expression of telomerase enzyme component, telomerase activity, and telomere elongation (by on average 33.3%). Hence, we for the first time demonstrated induction of telomerase activity with a peptide. These data largely explain the geroprotective effects of Epithalon in various experimental models [10]. It is known that critically short telomeres cannot prevent chromosome fusion, which can lead to activation of protooncogenes and malig-nant transformation [7], while activation of telomerase and telomere elongation under the effect of the peptide explain the mechanism of antitumor effect of Epithalon in old animals.

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