IDENTIFICATION OF GENES WHOSE mRNAs ARE SUBJECTED TO ALTERNATIVE SPLICING BY ENDONUCLEASE ENDOG ACTION IN HUMAN AND MURINE CD4+ T LYMPHOCYTES

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The aim of this work was to identify genes whose mRNAs were subjected to alternative splicing by apoptotic endonuclease EndoG in CD4+ T lymphocytes from healthy humans, mice, and rats. In order to induce EndoG, lymphocytes were transfected with an EndoG-containing plasmid, or a control pGFP plasmid, or were incubated with cisplatin. Efficiency of transfection, number of cells with DNA damages and the level of EndoG expression have been monitored. Total cell mRNA has been sequenced and the changes in proportion of splice variants of genes were analyzed. The changes in the proportion of 28 mRNA splice variants have been identified in human and murine lymphocytes in both transfected with EndoG gene or incubated with cisplatin. Thus, EndoG can be considered as a potent modulator of alternative splicing of mRNA of identified genes.

Key words: lymphocytes; EndoG; alternative splicing; sequencing

DOI: 10.18097/BMCRM00128

INTRODUCTION

Alternative splicing (AS) of mRNA is one of the ways of regulation of protein functions (including enzymatic activity) [1]. To date many of proteins which regulate this process have been identified; however precise mechanism of AS remains to be investigated. Previously, we showed that apoptotic endonuclease EndoG was capable of inducing AS of mRNAs of telomerase catalytic subunit hTERT (human Telomerase Reverse Transcriptase) [2,3] and deoxyribonuclease 1 (DNase I) [4] in human lymphocytes. In both cases induction of AS led to inhibition of the enzymatic activity. Later, the ability of EndoG to induce AS have been shown using mice and rat lymphocytes [5]. Thus, EndoG can be considered as a potent modulator of AS. Its mechanism of action is still poorly understood. It is of interest to study the ability of EndoG to modulate AS of mRNA of other genes. In this paper we have identified human and murine genes whose mRNAs are alternatively spliced by EndoG.

MATERIALS AND METHODS

Venous blood samples of four healthy donors were collected into tubes with K3EDTA as an anticoagulant («Greiner Bio-One», Austria). Blood samples from C57BL/6 mice and Wistar rats («Pushchino Breeding Center for Laboratory Animals», Russia) were obtained by cardiac puncture after euthanasia with CO2. CD4+ T cell purification was performed by magnetic selection using the human CD4+ Isolation Kit, mouse («Miltenyi Biotec»), Germany) according to the manufacturer’s protocol. CD4+ cells were seeded at a concentration of 5 × 105 cells/mL of the RPMI-1640 culture medium («Life Technologies», USA), and 0.02 mM 2-mercaptoethanol («Sigma-Aldrich»). Cells were cultivated in a CO2 incubator at 37°C, 5% CO2 and 90% humidity. Transfection of human, murine and rat CD4+ T cells was performed using Lipofectamine 2000 («Invitrogen», USA) according to the previously described protocol [2]. Transfection efficiency was estimated by flow cytometry by counting GFP-positive cells labeled with CD4-VioBlue («Miltenyi Biotec») using flowcyt MACS Quant Analyzer 10 («Miltenyi Biotec»). To induce DNA damage and EndoG expression, CD4+ T cells were cultivated with 60 μM cisplatin (cis-diamine dichloroplatinum (II), «Sigma-Aldrich») for 48 h [3, 7]. Determination of number of cells with damaged DNA was performed by the TUNEL assay (Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling) [8]therefore, is considered to be the gold standard for identification apoptotic cells. Several variants of the methodology that is based on fluorochrome-labeling of 3′-OH termini of DNA strand breaks in situ with the use of exogenous terminal deoxynucleotidyl transferase (TdT using the FlowTACSTM Apoptosis Detection Kit («R&D Systems») for flow cytometry.

Isolation of total RNA and EndoG expression analysis was performed according to the previously described protocol [2]. Total RNA was isolated from transfected, incubated with cisplatin or control cells followed by formation of cDNA libraries performed using Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat («Illumina Inc.», USA) [9,10]or how cells differ between a healthy state and a diseased state. With the advent and continuous refinement of
next-generation DNA sequencing technology. RNA-sequencing (RNA-seq). Each cDNA sample was diluted to a concentration of 6 pM and sequenced with HiScanSQ (Illumina Inc.) in the Total RNA-Seq application as described in [11]. Illumina RNA sequencing was used to profile two human normal control and two rheumatoid arthritis synovial fibroblasts (RASFs. The sequencing results were analyzed for the UCSC Homo sapiens reference genome hg19, Mus musculus reference genome mm10 and Rattus norvegicus reference genome rn10 using TopHat v1.3.0 [12] fast and mathematically principled analysis software. TopHat and Cufflinks are free, open-source software tools for gene discovery and comprehensive expression analysis of high-throughput mRNA sequencing (RNA-seq).

Statistical analysis of the results was performed using Student’s criterion by means of Statistica 9.0 (StatSoft Inc., USA) software. Results are presented as mean value ± standard error of mean. Numbers were considered statistically significant when \( p \leq 0.05 \).

RESULTS AND DISCUSSION

For identification of genes, whose mRNAs are subjected to alternative splicing regulated by EndoG, we have induced EndoG expression by transfection of human, murine and rat CD4+ T lymphocytes with plasmid constructs based on the pGFP-N1 vector. PGFP empty plasmid, which encodes only green fluorescent protein (GFP) was used for control transfection. Induction of EndoG was also performed by incubation of cells with the DNA-damaging agent cisplatin. Transfection efficiency was close to 100% in 48 h after transfection in human, murine and rat lymphocytes (Fig. 1 A–D). The number of cells, having DNA damages, which were determined by the TUNEL assay for flow cytometry, was 88.2–90.5% (fig. 1 E-F). 48 h after incubation with cisplatin (F). EndoG mRNA levels in cells determined by real time RT-PCR. mRNAs levels are normalized relative to the expression of the reference 18S gene. (n = 4).

Figure 1. Efficiency of transfection and EndoG induction in human, mice and rat lymphocytes. Results of flow cytometry for CD4+ T lymphocytes from human (A), mice (B) and rats (C) lymphocytes 48 h after transfection. Transfection efficiency determined by flow cytometry (D). Number of CD4+ T lymphocytes with damaged DNA, measured by TUNEL for flow cytometry (E). Induction of TUNEL-positive cells 48 h after incubation with cisplatin (F). EndoG mRNA levels in cells determined by real time RT-PCR. mRNAs levels are normalized relative to the expression of the reference 18S gene. (n = 4).

DNA damages, which were determined by the TUNEL assay for flow cytometry, was 88.2–90.5% (fig. 1 E-F). 48 h after incubation with cisplatin, EndoG mRNA was evaluated by real-time RT-PCR. Induction of DNA damage by cisplatin resulted in a significant increase in EndoG expression in lymphocytes of all species studied (Fig. 1G).

Figure 2. Identified genes whose mRNA splice variants levels changed after EndoG overexpression. Comparison of identified mRNA in CD4+ T lymphocytes after transfection with EndoG gene (A) or incubation with cisplatin (B).
<table>
<thead>
<tr>
<th>mRNA</th>
<th>AS Type</th>
<th>Biological role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARIH1 (Ariadne RBR E3 ubiquitin protein ligase 1)</td>
<td>Skipping of exons 1 and 2</td>
<td>Ubiquitination of target proteins. Plays a role in protein translation in response to DNA damage.</td>
<td>[14]</td>
</tr>
<tr>
<td>ARNTL (Aryl hydrocarbon receptor nuclear translocator-like)</td>
<td>Skipping of exon 3</td>
<td>Activates the transcription and translation of PER1, PER2, CRY1 and CRY2 proteins.</td>
<td>[15]</td>
</tr>
<tr>
<td>BCLX (B-cell Lymphoma extra)</td>
<td>Skipping of 189 nucleotides from 3'-end of exon 2</td>
<td>Regulate cell death by blocking the voltage-dependent anion channel (VDAC). Caspase activation inhibition.</td>
<td>[16]</td>
</tr>
<tr>
<td>BDKRB2 (Bradykinin receptor B2)</td>
<td>Inclusion of exon 2</td>
<td>Initiation of inflammatory processes: vasodilation, edema, smooth muscle spasm.</td>
<td>[17]</td>
</tr>
<tr>
<td>CASP-2 (Caspase 2)</td>
<td>Inclusion of exon 9</td>
<td>Mediation of apoptosis by proteolytic cleavage of specific protein substrates.</td>
<td>[18]</td>
</tr>
<tr>
<td>CLDN7 (Claudin 7)</td>
<td>Skipping of exon 3</td>
<td>Components of the membrane system of intercellular interactions of dense connective tissues.</td>
<td>[19]</td>
</tr>
<tr>
<td>CLOCK (CLOCK circadian regulator)</td>
<td>Skipping of exons 1-9</td>
<td>Regulation of circadian rhythms.</td>
<td>[20]</td>
</tr>
<tr>
<td>CRHR2 (Corticotropin releasing hormone receptor 2)</td>
<td>Skipping of exon 3</td>
<td>Coordination of endocrine, autonomic, immune and behavioral responses to stress.</td>
<td>[21]</td>
</tr>
<tr>
<td>DNASE I (Deoxyribonuclease 1)</td>
<td>Skipping of exon 4</td>
<td>Hydrolysis of DNA by phosphodiester bonds in the last stages of apoptosis.</td>
<td>[22]</td>
</tr>
<tr>
<td>DRG2 (Developmentally regulated GTP binding protein 2)</td>
<td>Skipping of exons 1 and 2</td>
<td>Regulation of cell proliferation and differentiation.</td>
<td>[23]</td>
</tr>
<tr>
<td>FOXP3 (Forkhead box P3)</td>
<td>Skipping of exon 4</td>
<td>Regulation of proliferation, differentiation and suppressor activity of regulatory T cells.</td>
<td>[24]</td>
</tr>
<tr>
<td>FLT1 (FMS-related tyrosine kinase 1)</td>
<td>Skipping of exons 15-30</td>
<td>Regulation of endothelial cells angiogenesis and migration.</td>
<td>[25]</td>
</tr>
<tr>
<td>GFAP (Glial fibrillary acidic protein)</td>
<td>Skipping of exons 8-9</td>
<td>Provides instructions for making a protein called glial fibrillary acidic protein.</td>
<td>[26]</td>
</tr>
<tr>
<td>GRIN 1 (Glutamate ionotropic receptor NMDA type subunit 1)</td>
<td>Skipping of exon 4</td>
<td>Play a key role in the plasticity of synapses, which is believed to underlie memory and learning.</td>
<td>[27]</td>
</tr>
<tr>
<td>GTPBP8 (GTP-binding protein 8)</td>
<td>Skipping of exon 2</td>
<td>GTP binding and ferrous iron transmembrane transporter activity.</td>
<td>[28]</td>
</tr>
<tr>
<td>IL18 (Interleukin 18)</td>
<td>Skipping of exon 3</td>
<td>Augments natural killer cell activity in spleen cells, and stimulates interferon gamma production in T-helper type 1 cells.</td>
<td>[29]</td>
</tr>
<tr>
<td>KHDRBS1 (KH RNA binding domain containing, signal transduction associated 1)</td>
<td>Skipping of exon 3</td>
<td>Regulation of alternative splicing, gene expression and cell cycle.</td>
<td>[30]</td>
</tr>
<tr>
<td>MGAT5 (Mannosyl (alpha-1,6-)glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase)</td>
<td>Skipping of exons 12-18</td>
<td>The addition of beta-1,6-N-acetylglucosamine to the alpha-linked mannose on the newly synthesized glycoproteins.</td>
<td>[31]</td>
</tr>
<tr>
<td>NOVA 1 (NOVA alternative splicing regulator 1)</td>
<td>Skipping of exon 4</td>
<td>Regulation of splicing and processing of mRNA in neurons.</td>
<td>[32]</td>
</tr>
<tr>
<td>NR1D2 (Nuclear receptor subfamily 1 group D member 2c)</td>
<td>Skipping of exons 2-6</td>
<td>Transcriptional repressor. Regulation of circadian rhythms, carbohydrate and lipid metabolism.</td>
<td>[33]</td>
</tr>
<tr>
<td>PECAM1</td>
<td>Skipping of exon 3</td>
<td>Regulation of integrin activation, transendothelial migration of leukocytes and angiogenesis.</td>
<td>[34]</td>
</tr>
</tbody>
</table>
Table 1. Identified genes whose mRNA AS changes upon overexpression of EndoG.

<table>
<thead>
<tr>
<th>Gene</th>
<th>AS Change</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNFI28</td>
<td>Skipping of exon 1</td>
<td>Inhibitor of cytokine gene transcription</td>
<td>[35]</td>
</tr>
<tr>
<td>SAMHD1</td>
<td>Skipping of exon 14</td>
<td>Regulation of the innate and adaptive immune response along the tumor necrosis factor-alpha signaling pathway.</td>
<td>[36]</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Skipping of exon 2</td>
<td>Deacetylation of proteins. Increasing cell sensitivity to insulin.</td>
<td>[37]</td>
</tr>
<tr>
<td>SREBF1</td>
<td>Skipping of exon 5</td>
<td>Regulation of gene expression of glucose metabolism proteins and the synthesis of fatty acids, cholesterol and lipids.</td>
<td>[38]</td>
</tr>
<tr>
<td>TERT</td>
<td>Skipping of exon 7-8</td>
<td>Regulation of telomerase activity.</td>
<td>[39]</td>
</tr>
<tr>
<td>TRIM23</td>
<td>Skipping of exon 2</td>
<td>Autophagy activation during viral infection.</td>
<td>[40]</td>
</tr>
</tbody>
</table>

Total cell RNA was sequenced. 30 million of 75-bp paired-end reads were sequenced. Analysis of mRNA levels of splice variants of different genes showed that in human, mouse, and rat cells transfected with pEndoG-GFP, and in cells incubated with cisplatin, the number of mRNA splice variants of 28 genes changed (Fig. 2, Table 1).

Defined shift in the proportion of splice-variants of mRNA was not associated with the changes of total mRNAs of these genes. Transfection of cells with control plasmid pGFP did not lead to the changes in the proportion of mRNA splice-variants of defined genes. Changes in the proportion of mRNA splice-variants was confirmed by real-time RT-PCR. Among defined mRNAs in all human, mice and rat lymphocytes, the levels of 9 of them (ARH11, BCLX, CASP2, CLDN7, DNASE1, GRIN1, IL18, SIRT1 and TERT) changed after transfection with EndoG gene (Fig. 2 A) and 6 (BCLX, CASP2, CLDN7, CLOCK, DNASE1, TERT) after incubation with cisplatin (Fig. 2 B). These results indicate the involvement of EndoG into the function of multiple proteins, which regulate diverse cellular processes.

Thus, we have shown that EndoG participates in modulation of alternative splicing of mRNAs of defined genes in human, mice and rats.

COMPLIANCE WITH ETHICAL STANDARDS

The study was approved by the Ethics Committee of the Institute of Biomedical Chemistry. Written consent was obtained from all donors participating in the research.

FUNDING

The work was performed in the framework of the Program for Basic Research of State Academies of Sciences for 2013–2020.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

ИДЕНТИФИКАЦИЯ ГЕНОВ, мРНК КОТОРЫХ ПОДВЕРГАЮТСЯ АЛЬТЕРНАТИВНОМУ СПЛАЙСИНГУ В РЕЗУЛЬТАТЕ ДЕЙСТВИЯ ЭНДОНОКУЛЛЕАЗЫ ENDOG В CD4+ Т-ЛИМФОЦИТАХ ЧЕЛОВЕКА, МЫШИ И КРЫСЫ

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Целью работы явилась идентификация генов, мРНК которых подвергается альтернативному сплайсингу в результате действия апоптотической эндонуклеазы EndoG в нормальных CD4+ Т-лимфоцитах человека, мыши и крысы. С целью индукции EndoG, лимфоциты трансфицировали плазмидой, содержащей ген EndoG, контрольной плазмидой pGFP или инкубировали с цисплатином. Оценивали эффективность трансфекции, количество клеток с повреждением ДНК и уровень мРНК EndoG. Тотальную мРНК клеток секвенировали и оценивали изменение уровней сплайс-вариантов различных генов. Обнаружили изменение пропорции сплайс-вариантов мРНК 28 генов в лимфоцитах человека, мыши и крысы, как в клетках, трансфицированных геном EndoG, так и в клетках, инкубированных с цисплатином. Следовательно, EndoG принимает участие в модуляции альтернативного сплайсинга мРНК идентифицированных генов.

Ключевые слова: лимфоциты; EndoG; альтернативный сплайсинг; секвенирование

ФИНАНСИРОВАНИЕ

Работа выполнена при финансовой поддержке Программы фундаментальных научных исследований государственных академий наук на 2013–2020 годы.

Поступила: 02.06.2020, после доработки: 17.06.2010, принята к печати: 18.06.2020