Alternative Splicing of Interleukin-6 mRNA in Mice

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Expression of mRNA for interleukin-6, interleukin-6Δ3, and interleukin-6Δ5 was detected in placental tissue (second and third trimesters of pregnancy) and spleen of mice immunized with sheep erythrocytes in high dose. We hypothesize that translation of mRNA yields proteins capable of binding to individual subunits of the interleukin-6 receptor and possessing effector functions.

Key Words: *interleukin-6; alternative splicing*

Interleukin-6 (IL-6) produced by various lymphoid and nonlymphoid cells belongs to the family of cytokines, which includes granulocyte colony-stimulating factor, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, and IL-11. Protein gp130 playing a role in signal transduction in cells serves as a component of cytokine receptors. IL-6 is involved in the regulation of immunopoiesis, hemopoiesis, inflammation, and oncogenesis. The effects of IL-6 depend on the type of cells and receptors [11]. Isoforms of cytokines and specific receptors provide diversity of the cytokine network and determine cell cooperation depending on the type of tissue and stage of ontogeny. Their expression undergoes changes under normal and pathological conditions [1]. The mechanism of alternative splicing is typical of the IL-6 gene (similarly to genes for other cytokines). Resulting transcripts encode proteins with a variety of properties. The are 5 variants of IL-6 mRNA expressed in humans: IL-6, IL-6alt, IL-6Δ2, IL-6Δ2,4, and IL-6Δ4. Recombinant proteins IL-6alt and IL-6Δ4 were obtained [2,8]. There are contradictory data on biological activity of these compounds. Further studies are required to evaluate their role in the cytokine network. Some similarity exists between the immune system in mice and humans. It is associated with the same genomic organization of immune components and similar diseases of mice and humans. Fundamental immunological studies are performed on mice. Here we studied alternative splicing of IL-6 mRNA in mice.

MATERIALS AND METHODS

Experiments were performed on female (CBA×C57Bl/6)F1 mice aging 3-6 months and obtained from the Laboratory of Experimental Biological Modeling (Tomsk Research Center). The animals were kept under standard conditions. Intact and pregnant mice were intraperitoneally immunized with sheep erythrocytes in doses of 4×10^9, 2×10^8, and 4×10^5 in 0.5 ml RPMI-1640 medium. The animals were killed by cervical dislocation. Embryos were killed by ether inhalation. The liver from intact mice, spleen from immunized animals, and placenta (first, second, and third trimesters of pregnancy) were removed on cold. Mouse tissues were frozen in liquid nitrogen and stored at -70°C.

RNA was isolated from mouse tissues [4]. The quality of RNA was evaluated by electrophoresis in 1.5% agarose gel. Its content was estimated spectrophotometrically at 260 nm. RNA was stored at -70°C.

cDNA was obtained from 1 μg total RNA using reverse transcription reaction with 5 μM $d(pT)_{18}$ primer and 100 U MoMLV RNA-dependent DNA polymerase (Institute of Biological Chemistry, Siberian Division of

Laboratory for Regulation of Immunopoiesis, Institute of Clinical Immunology, Siberian Division of the Russian Academy of Medical Sciences; *Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, Novosibirsk. ***Address for correspondence:*** ici@onlinensk.su. Sennikov S. V.

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the Russian Academy of Sciences) in 20 μl buffer containing 20 mM Tris-HCl (pH 8.3), 2 mM $MnCl_2$, 5 mM dithiothreitol, 10 mM KCl, and 400 μM dNTP.

The polymerase chain reaction (PCR) was conducted in 20 μl reaction mixture with 1 U Tag polymerase (Institute of Biological Chemistry, Siberian Division of the Russian Academy of Sciences). Amplification mixture contained 2 μl cDNA (~50 ng, matrix) and 200 μM dTNP in the standard buffer consisting of 67 mM Tris-HCl (pH 8.9), 16 mM ammonium sulfate, 1.5 mM $MgCl_2$, 0.05% Tween 20, and 20 pM amplification primers. First-round PCR involved primers U3 and R1. Two-stage PCR with nested primers U1 and R3 was performed to increase sensitivity and specificity of the method. PCR was conducted at 95, 60, and 72°C for 3 min, 7 sec, and 15 sec, respectively. First-round and second-round PCR was performed in 41 and 25 cycles, respectively. The final cycle of elongation was performed at 72°C for 3 min. Deoxyribooligonucleotide primers were synthesized at the Institute of Biological Chemistry and had the following nucleotide sequences: 5'-CGCT ATGAAGTTCCTCTCTGC-3' (U1), 5'-ATTAAAAA TAATTAAAATAGTGTCCCAAC-3' (R1), 5'-GAGC CCACCAAGAACGATAGTC-3' (U3), and 5'-CTAG GTTTGCCGAGTAGATCTC-3' (R3). Products of the amplification reaction (10 μl) were separated by electrophoresis in 6% polyacrylamide gel. The gel was stained with ethidium bromide (0.5 mg/ml). DNA was visualized in UV light. Images were recorded using a Water AD-901 CD camera (Watec Co., LTD).

Amplification products were eluted from the gel for sequencing. Sequencing was performed with a BigDye set (Amersham) according to manufacturer's recommendations. Sequencing products were electrophoretically separated on an automatic ABI310 DNA sequencer. Nucleotide sequences were analyzed using VectorNTI software (Informatics).

RESULTS

Two-round PCR and electrophoretic separation allowed visualizing the major band of DNA (640 bp) corresponding to full-length mRNA for mIL-6 and several minor fragments of smaller size (Fig. 1). We

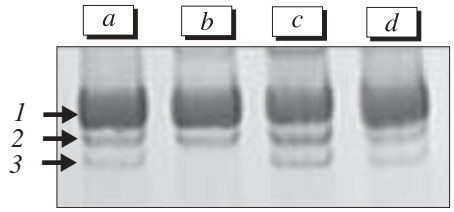

Fig. 1. Electrophoretogram of amplification products with primers R3 and U1: mouse spleen cDNA 24 h after immunization with sheep erythrocytes in a dose of 4×10^9 (*a*); liver cDNA of intact mouse (*b*); mouse spleen cDNA in the second trimester of pregnancy (*c*); mouse placental cDNA in the third trimester of pregnancy (*d*). Fragment of 640 bp (IL-6, *1*), fragment of 582 bp (IL-6Δ3, *2*), and fragment of 526 bp (IL-6Δ5, *3*).

hypothesized that these minor fragments are splice variants of IL-6 mRNA. DNA fragments were sequenced to test this hypothesis. Fragments *2* and *3* included deletions of exons 5 (58 bp) and 3, respectively (114 bp, Fig. 2). These isoforms were designated as mIL-6Δ5 and mIL-6Δ3, respectively.

IL-6 consists of 4 densely packed peptide α-helixes A, B, C, and D that are connected by 3 loops. Longer loops A-B and C-D connect parallel chains [10,12].

Translation of mIL-6Δ5 mRNA with a reading frame shift and formation of a new stop codon in the 3'-region can result in the appearance of a polypeptide consisting of 165 amino acids (*vs*. 211 amino acids in the full-length structure) and includes deletion of the C-terminal domain with the D-helix. Deletion of exon 3 in mIL-6Δ5 mRNA did not change the reading frame and translation of this mRNA yields protein consisting of 173 amino acids without AB-loop.

Domains responsible for the interaction of IL-6 with the α-subunit of its receptor are localized in C-terminal regions of the AB-loop and D-helix [10,12]. They received the name site I.

Residues of A- and C-helixes (Tyr31 and Gly35; and Ser118 and Val121, respectively) constitute the gp130-binding site II. Biological activity of mutant protein hIL-6 containing Y31 and G35 significantly decreased, which was associated with reduced binding to gp130. However, this protein retained high affinity for hIL-6-Rα [15]. The second gp130-binding site III is formed from amino acid residues of the N-terminal region in the AB-loop, C-terminal region in the C-loop, and N-terminal region in the D-helix [6,7]. Vari-

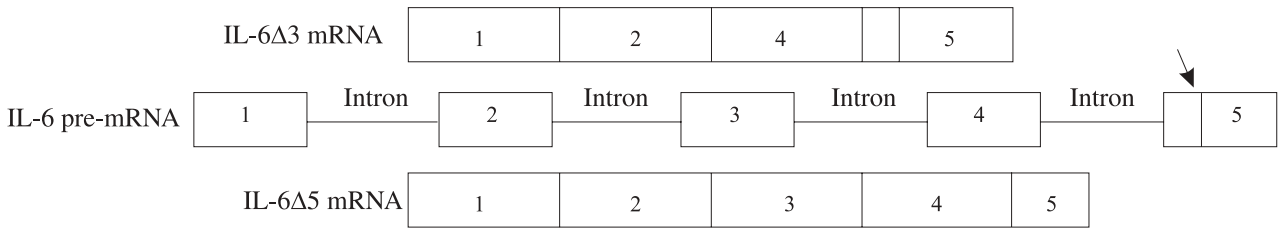

Fig. 2. Scheme for alternative splicing of mouse IL-6 pre-mRNA. Arrow: alternative splicing site in exon 5.

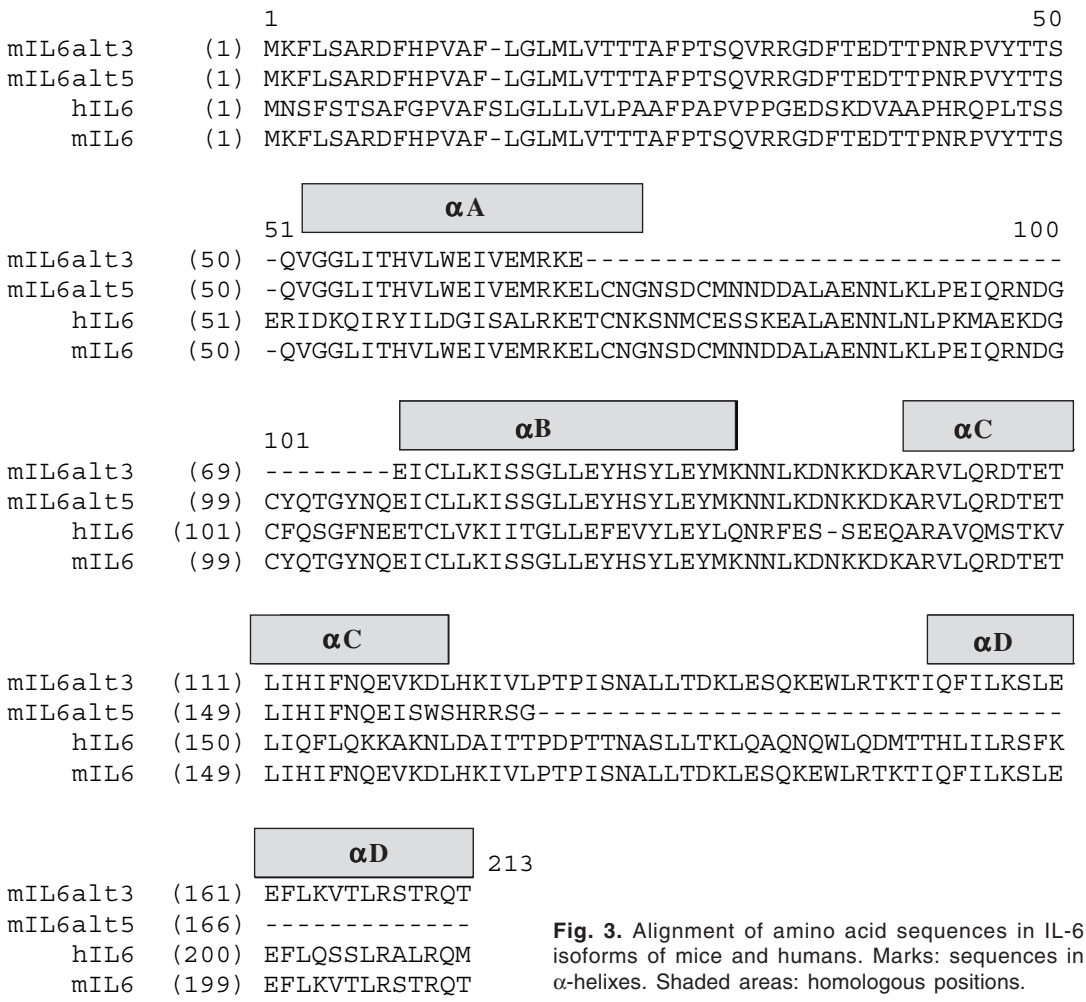

Fig. 3. Alignment of amino acid sequences in IL-6 isoforms of mice and humans. Marks: sequences in α-helixes. Shaded areas: homologous positions.

ants of hIL-6 mutant by this site exhibited antagonistic activity [3,5,13].

It is unlikely that the isoform of mIL-6Δ5 will bind to the receptor α-subunit (Fig. 3).

Probably, D-helix-lacking protein can retain its tertiary structure. These peculiarities contribute to preservation of site II that plays a role in binding to protein gp130. This protein inhibits the assembly of a hexa-subunit receptor or performs effector function, which differs from that of a full-length structure. Other results were obtained in studying the isoform of mIL-6Δ3 (Fig. 3). Deletion of the AB-loop can impair the tertiary protein structure. It is unclear which binding sites in the isoform of IL-6 retain functional activity. Probably, this protein binds to the receptor α-subunit and gp130 via site III and possesses effector function.

mRNA for mIL-6Δ3 and mIL-6Δ5 was detected in mouse spleen 24 h after immunization with sheep erythrocytes (4×10^9). These mRNA were found in the placenta in the second and third trimesters of pregnancy. mIL-6Δ5 mRNA was present in the liver of intact mice.

Various species express both similar and different isoforms of cytokines [1]. Alternative splicing can serve as a mechanism for the formation of cytokines with new regulatory properties during evolution. When the phylogenetically appeared isoform has a specific activity, it is stored in the evolutionary order and plays a new function in certain tissues or at certain stages of ontogeny. The isoform of mIL-6Δ5 mRNA found in mice is absent in humans, since the sequence of human IL-6 gene includes nucleotide exchanges eliminating alternative splicing site. It is hypothesized that the isoform similar to mouse mIL-6Δ3 mRNA can be detected in humans.

REFERENCES

1. S. V. Sennikov, A. N. Silkov, and V. A. Kozlov, *Med. Immunol.*, **3**, No. 3, 389-400 (2001).
2. M. Bihl, K. Heinimann, J. Rudiger, *et al.*, *Am. J. Respir. Cell Mol. Biol.*, **27**, 48-56 (2002).
3. J. P. Brakenhoff, F. D. de Hon, V. Fontaine, *et al.*, *J. Biol. Chem.*, **269**, No. 1, 86-93 (1994).
4. P. Chomczynski and N. Sacchi, *Anal. Biochem.*, **162**, No. 1, 156-159 (1987).
5. A. Demartis, F. Bernassola, R. Savino, *et al.*, *Cancer Res.*, **56**, No. 18, 4213-4218 (1996).

6. M. Ehlers, F. D. de Hon, H. K. Bos, *et al*., *J. Biol. Chem*., **270**, No. 14, 8158-8163 (1995).
7. M. Ehlers, J. Grötzinger, F. D. de Hon, *et al*., *J. Immunol*., **153**, No. 4, 1744-1753 (1994).
8. D. Kestler, S. Agarwal, J. Cobb, *et al*., *Blood*, **86**, No. 12, 4559-4567 (1995).
9. D. Kestler, K. Goldstein, S. Agarwal, *et al*., *Am. J. Hematol*., **61**, 169-177 (1999).
10. N. Q. McDonald, N. Panayotatos, and W. A. Hendrickson, *EMBO J*., **14**, 2689-2699 (1995).
11. T. Naka, N. Nishimoto, and T. Kishimoto, *Arthritis Res*., **4**, Suppl. 3, S233-S242 (2002).
12. N. Panayotatos, E. Radziejewska, A. Acheson, *et al*., *J. Biol. Chem*., **270**, No. 23, 14,007-14,014 (1995).
13. C. Renne, K. J. Kallen, J. Mullberg, *et al*., *J. Biol. Chem*., **273**, No. 42, 27,213-27,219 (1998).
14. M. Sato, D. Sawamura, Sh. Ina, *et al*., *Arch. Dermatol. Res*., **291**, 400-404 (1999).
15. R. Savino, A. Lahm, A. L. Salvati, *et al*., *EMBO J*., **13**, No. 6, 1357-1367 (1994).