Alternative Splicing of Murine Interleukin-4 mRNA

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We found an alternative form of mRNA with spliced second exon (IL-4δ2 mRNA) in mouse bone marrow and splenic cells. At rest, the amount of IL-4 mRNA markedly surpassed that of IL-4δ2 mRNA. Stimulation increased the content of both mRNA forms, but the alternative variant is accumulated more intensively and rapidly. We did not detect predominance of IL-4δ2 mRNA over full-lenght mRNA variant in the studied mouse tissues.

Key Words: *interleukin-4; alternative splicing*

Interleukin-4 (IL-4) is a key type Tx2 cytokines. In humans *IL-4* gene is expressed in two mRNA forms: full-lenght form containing all 4 exons and alternatively spliced mRNA form without exon 2: IL-4δ2 [3,9, 13]. IL-4δ2 mRNA was detected in higher primates and rabbits [8,11], as well as one more mRNA isoform IL-4δ3 with exon 3 deletion. Recombinant human protein IL-4δ2 (rhIL-4δ2) can bind to IL-4 receptor and inhibit the effect of recombinant human IL-4 (rhIL-4) on immunocompetent cells [4,5]. On the other hand, rhIL-4δ2 acts as IL-4 agonist in human lung and skin fibroblasts and stimulates α2 collagen(I) mRNA transcription [6]. Hence, we can speak about tissue-specific effects of IL-4 isoforms.

Tissue-specific distribution of IL-4 mRNA isoforms was discovered. Normally IL-4δ2 mRNA is present in human peripheral blood mononuclear cells (MNC) in minor amounts [3], though in some donors it can be dominant in comparison with the full-lenght form [3,5]. IL-4δ2 mRNA predominated over the full-lenght form in the thymus and bronchoalveolar lavage cells [5], in M95/8 and HJ60 cells [10]. The ratio of two IL-4 mRNA forms is changed in some diseases [9,12-14], which is believed to be an important factor in the pathogenesis of these diseases. Based on the data on tissue-specific distribution and activity of IL-4 isoforms and suggesting that the regulation of this gene splicing is similar in different species of mammals, we hypothesized the presence of an alternative IL-4 variant in mice — a convenient object for studies of this variant expression.

We studied IL-4 mRNA isoforms in mice, evaluated their levels, and investigated their tissue-specific expression in ontogeny.

MATERIALS AND METHODS

Intact (DBA/2J×C57BL/6J)F1 animals were used in the study: embryos, newborns, and male mice aged 3-6 months, bred in Tomsk Research Center and Laboratory of Experimental Animals (Models) of Institute of Clinical Immunology. Organs from animals were homogenized with a syringe, resuspended in RPMI-1640, cells were counted and their viability was evaluated. Bone marrow MNC were isolated in Ficoll-urografin 1.082 density gradient (Sigma). The isolated cells were cultured or placed into a lyzing solution (1×10^6 cells) [7] and stored at -20°C until use. Liver, thymus, lung, lymph node, Peyer's patches, intestinal wall, muscle tissues, placenta, and embryonal tissues (embryonal liver on days 11 and 15 and brain after 14-day gestation) were taken on cold for investigation of tissue-specific expression. Tissue collected from each mouse was frozen in liquid nitrogen and stored at -70°C.

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The cells (5×10^6) were cultured *in vitro* in Petri dishes in RPMI-1640 with 2 mM L-glutamine, 5% inactivated fetal calf serum, 50 μM β_2-mercaptoethanol, and gentamicin (80 μg/ml) for 48 h in a CO_2 incubator at 37°C. Mitogens were added into culture medium once at the start of culturing: concanavalin A (con A, Sigma) in a dose of 10 μg/1 million cells and *E. coli* 055:B5 LPS (Sigma) — 30 μg/1 million cells.

RNA was isolated from mouse tissues as described previously [7]. The quality of summary RNA was evaluated by electrophoresis in 1.5% agarose gel, its quantity was measured by spectrophotometry at 260 nm. RNA was stored at -70°C. cDNA was isolated from 1 μg summary RNA in reverse transcription (RT) reaction with 5 μM $d(pT)_{18}$ primer and MoMLV RNA-dependent DNA polymerase (100 U, Institute of Chemical Biology and Fundamental Medicine) in 20 μl buffer containing 20 mM Tris-HCl (pH 8.3), 2 mM $MnCl_2$, 5 mM dithiotreitol, 100 mM KCl, and 400 μM dNTP.

PCR was carried out in 20 μl reaction mixture with 1 U Taq polymerase (Institute of Chemical Biology and Fundamental Medicine). Amplification mixture contained 2 μl (approximately 50 ng) cDNA for matrix, 200 μM dNTP solution in standard buffer: 67 mM Tris-HCl (pH 8.9), 16 mM ammonium sulfate, 1.5 mM $MgCl_2$, 0.05% Twin-20, and 20 pM each primer for amplification. PCR conditions were as follows: 3 min denaturing at 95°C, 40 cycles of 7 sec at 95°C, 7 sec at 62°C, and 12 sec at 72°C, followed by the final elongation cycle (3 min at 72°C).

Deoxyribooligonucleotide primers were synthesized at the Institute of Chemical Biology and Fundamental Medicine and had the following nucleotide sequences: IL-4m-d CAGGAGAAGGGAACACCAC, IL-4m-n TGGATGTGCCAAACGTCC, IL-4m-r G(G,C)TCTTTAG(G,C)CTTTCCA(A,G)GAAG.

Competitive PCR was carried out as described previously [2]. Competitive standard DNA for measuring IL-4 and IL-4δ2 cDNA were obtained by amplification of phage T7 genome DNA with primers specific for the studied cDNA using low annealing temperature for the first cycles.

Amplification products (10 μl) were separated by electrophoresis in 6% PAAG or 1.5% agarose gel. The gel was stained with ethidium bromide (0.5 mg/ml) and DNA was visualized in UV light. The image was fixed using Watec AD-901 CD camera (Watec Co. Ltd.).

DNA content was calculated by band density using ScionImage software. A curve was plotted, presenting the linear relationship between the logarithm of the ratio of the level of competitive DNA to the studied cDNA to the logarithm of the initial concentrations of competitive DNA, in which it was added to the PCR mixture. The equivalence point was determined (zero ratio of the logarithm of competitive DNA to cDNA) and the initial level of the studied cDNA corresponded to the initial level of competitive DNA. The mean value and the standard error in the mean were calculated for each cDNA by the results of 3 repeated measurements.

Amplification products were eluted from the gel as described previously [1]. Sequencing was carried out using BigDye kit (Amersham) according to the manufacturer's recommendations. Electrophoretic separation of sequencing products was carried out on an automated ABI310 DNA sequenator. The resultant nucleotide sequences were analyzed using Vector NTI software (Informatics).

RESULTS

Deoxyribooligonucleotide primers were constructed for selective detection of full-lenght IL-4 and IL-4δ2 mRNA. The primer for full-lenght IL-4 variant contained a sequence complementary to the region of the end of exon 1 and beginning of exon 2, that for IL-4δ2 included a sequence of the end of exon 1 and beginning of exon 3. The reverse primer, common for both forms, was situated in exon 4 region (Fig. 1).

In order to detect mouse IL-4δ2 mRNA, summary cDNA were obtained from mouse splenocytes and MNC and cultured *in vitro* with Con A, LPS, and without mitogens. Electrophoretic separation of amplification products of the above cDNA with primers IL-4m-d/IL4m-r showed a fragment of expected size (254 n. p.) in Con A-stimulated splenic and bone marrow cells (Fig. 2). The nucleotide structure of this DNA fragment was determined by direct Senger sequencing; it corresponded to mouse IL4δ2 mRNA and was called mIL-4δ2. This product was not detected in cDNA samples from cell cultures without mitogens or those stimulated with LPS. On the other hand, PCR

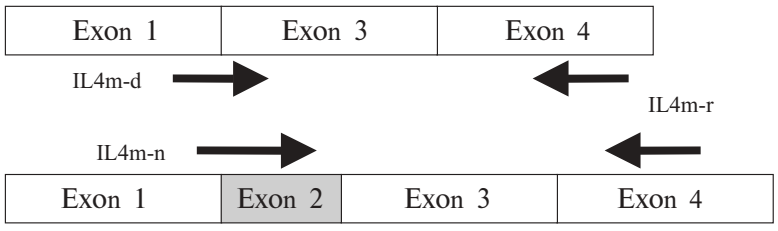

Fig. 1. Scheme of oligonucleotide primers.

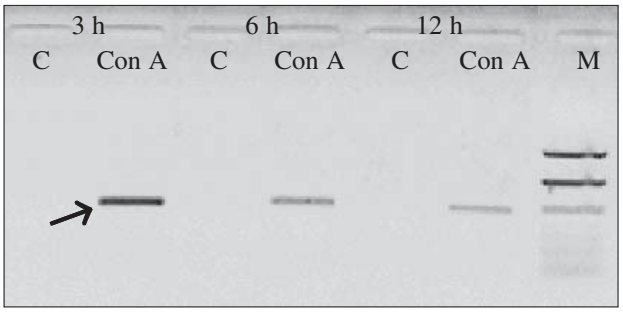

Fig. 2. Electrophoregram of amplification products with IL4m-d and IL4m-r for mouse splenocyte cDNA. C: without mitogen stimulation; Con A: stimulation with concanavalin A; M: BluscriptIIKS/HaeII. Arrow: 254 n. p. DNA fragment corresponding to IL-4δ2 cDNA.

products identical to IL-4 mRNA (264 n. p.) were present in all samples. We hypothesized that the alternative form is also expressed in cell resting, but in quantities below the sensitivity of RT-PCR method. Later the presence of minor amounts of IL-4δ2 mRNA in intact bone marrow and splenic cells was confirmed by inserted PCR, which was more sensitive (data not presented).

The study of the kinetics of IL-4mRNA and IL-4δ2mRNA expression in mouse splenocyte culture showed that Con A stimulation resulted in induction of both IL-4 and IL-4δ2 mRNA. The maximum level of IL-4δ2 mRNA was observed 3 h after addition of Con A, but then it decreased. Minor amounts of IL-4δ2 mRNA were detected in cells until 48 h in culture. The peak of IL-4 mRNA induction was observed 6 h after the start of stimulation, after which mRNA level gradually decreased (Fig. 3). Competitive quantitative PCR showed that the content of full-lenght mRNA at the peak of induction (6 h) 14 times surpassed the level of IL-4δ2 mRNA.

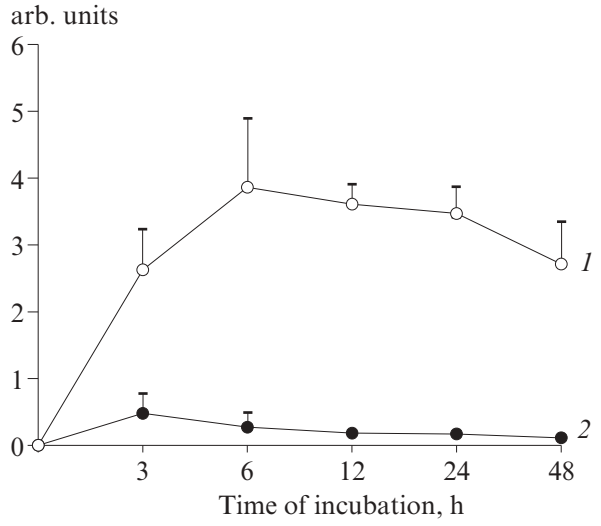

Fig. 3. Kinetics of IL-4 mRNA (*1*) and IL-4d2 (*2*) expression in mouse splenocyte cultures.

In order to study the tissue-specific distribution of mouse IL-4 mRNA isoforms, we isolated RNA and synthesized cDNA from the liver, thymus, lung, lymph nodes, Peyer's patches, intestinal wall, muscle tissues, placenta, and embryonal tissues (on days 11 and 15 of gestation from the liver and on 14 day from the brain). We found no IL-4δ2 mRNA in native tissues by single-stage PCR, while inserted PCR detected IL-4δ2 in minor quantities in all the studied samples (data not presented).

Our findings indicate that in mammals (starting from mice) IL-4 gene is expressed in 2 forms of mRNA (full-lenght and alternatively spliced by exon 2). In contrast to humans, alternative mRNA form (IL-4δ2) in mice is not tissue-specific and is expressed in lymphoid and hemopoietic tissue as a minor form in comparison with full-lenght variant. The same is true for the expression of two IL-4 mRNA forms in ontogeny.

It is noteworthy that stimulation of mouse splenocytes by Con A mitogen led to an increase in the content of IL-4 and IL-4δ2 mRNA, similarly as in human peripheral blood MNC [10], but even in this case the level of IL-4δ2 mRNA was considerably lower than that of the full-lenght IL-4 mRNA.

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