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Expression and secretion of human recombinant LIF by genetically modified mammalian cells

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Aim. *The aim of this work was to express the human LIF gene in mammalian cells and to study the secretion of recombinant LIF into culture medium.* ***Methods***. *Recombinant LIF was detected by Western blot analysis and immunoprecipitation in culture medium of CHO-K1, L-M (TK$^-$) (ins$^+$), 293T cells, transfected with recombinant plasmids containing human LIF gene.* ***Results***. *The recombi- nant plasmids. containing human gene LIF, were constructed. The cells of three (CHO-K1, L-M (TK$^-$) (ins$^+$), 293T) mammalian lines were transfected with these plasmids. It was shown that the transfected mammalian cells secreted recombinant human LIF which was characterized by variable degree of glycosylation including completely glycosylated form (approximately 68 kD).* ***Conclusions***. *The conditioned medium of developed cell lines can be used as a source of human recombinant LIF for different purposes, including purification of human recombinant LIF and as an additional supplement for cell culturing.*

Keywords: recombinant LIF, expression, secretion, transfection, cell lines.

Introduction. Leukemia inhibitory factor (LIF) is a polyfunctional cytokine of IL-6 family. It affects different tissues and types of cells, including hematopoietic, neural and muscle cells, hepatocytes, adipocytes, osteoblasts and osteoclasts. LIF affects proliferation of germ cells and maintenance of pluripotent embryonic stem cells. It plays a key role in the implantation of a blastocyst, participates in the hypothalamus-pituitary-adrenal axis activation, and development of pituitary, kidneys, energetic homeostasis, etc. [1].

The impact of LIF on the main processes in different cells is a reason to use this protein as a growth factor during cultivation of stem cells of various origin and their directed differentiation into cells of various types.

LIF is known as an absolutely necessary component of a medium, required to support pluripotency of mouse embryonic stem cells. These cells are widely used for investigations of pluripotency mechanisms and biology of embryonic stem cells [2, 3]. LIF supports the multiplication of human neural stem cells, providing their long-term self-renewal (up to 110 doublings) [4] and keeping their multipotent properties for a year [5]. It is also added into media for the cultivation of mesenchymal stem cells (MSC), that improves their osteogenetic differentiation [6, 7], as well as for MSC differentiation into cardiomyocytes [8, 9]. The non-glycosylated, *Escherichia coli* expressed, human recombinant LIF has a biological

activity, which is manifested by its ability to induce differentiation of murine myeloid leukemic M1 cells [10]. It is used in investigations *in vitro* and *in vivo*, though the desired results are not always achievable *in vivo* [11]. One of the main reasons of such negative result is the lack of glycosylation of recombinant proteins. The glycosylation increases protein stability and its lifetime in bloodstream, influences resistance to proteases, antigenicity and specific activity which is important for therapeutic proteins [12]. This fact is the reason of searching for special ways to increase the level of glycosylation of recombinant proteins in the process of their expression in mammalian cells [13, 14]. For cell cultivation *in vitro* the purified recombinant growth factors, cytokines, and other biologically active molecules are added to the medium or conditioned media are used [15, 16]. Inactivation of growth factors requires regular replacement of the medium. Therefore, other approaches to provide efficient microenvironment for the cultivated cells are elaborated too, in particular, using immobilized growth factors [17] and transgenic cell lines as feeder cells [18–20]. The development of transgenic cell lines of different origin allows solving a number of tasks, related to the study of a transgene effect on the recipient cell. Besides, expression of recombinant proteins in mammalian cells is a main source of producing glycosylated proteins. Thus, in 2007 70% of world production of therapeutic recombinant proteins were obtained in Chinese hamster cells [21]. Because of the significance of LIF for investigations *in vivo* and *in vitro*, the aim of this work was to express human recombinant LIF in the cells of transgenic mammalian cell lines and to study its secretion into the culture medium.

Materials and Methods. Plasmids *pUC28*, *pBluScrII (KS$^+$)*, *pEGFP-C1*; strains of *E. coli* XL1, DH10B; cell lines CHO-K1 (Chinese hamster, ovary), L-M(TK$^-$) (ins$^+$) (mouse, subcutaneous connective tissue), transfected with recombinant plasmid, containing human insulin gene, and 293T, which is a variant of line HEK293 (human embryonic kidney), expressing large T-antigen SV40 and resistant to G418, were used in the work.

The isolation of plasmid DNA, treatment with restriction endonucleases, ligation, obtaining competent *E. coli* cells, analysis of recombinant plasmids were performed by standard methods [22].

The following primers were used to obtain cDNA LIF: 5'-TCTGAGGTTTCCTCCAAGG-3' and 5'-TGCTCAGCTTCATCACAGC-3';
5'-ATGAAGGTCTTGGCGGCAGG-3' and 5'-ACCTCCTGCTAGAAGGCCTG-3'.

cDNA of *LIF* gene was obtained by reverse transcription reaction according to the manufacturer instructions, using cDNA synthesis kit (*Fermentas*, Lithuania). PCR was performed in standard buffer conditions for Taq and Pfu polymerases using the optimal program in accordance with specific tasks.

Mammalian cells were transfected with recombinant plasmids using polyethyleneimine (PEI) [23]. Cells were kept with the complexes DNA-PEI for 1 hour. Selection of CHO-K1 cells, transfected with *pC1-L*, was conducted using G418 (200 мg/ml). After transfection with *pC1-IL* the cells were selected with hygromycin (200 мg/ml). L-M(TK$^-$)(ins$^+$) and 293T cells were transfected only with *pC1-IL*, as they were resistant to G418. In starting experiments an optimal concentration of antibiotics required to kill host cells was determined.

The expression and secretion of LIF into culture medium were studied by Western-blot analysis and immunoprecipitation. The cells were grown on DMEM medium, containing 20% fetal calf serum, washed with DMEM (serum-free) three times, and then grown in the same medium during 1.5-2 days. Next, the culture medium was collected, proteins were precipitated by 10% solution of TCA in the presence of 0.015% sodium deoxycholate. After protein separation by SDS-PAGE [24] Western-blot analysis was performed in accordance with the online protocol of *Millipore* company (USA) (millipore.com/immunodetection /id3/westernblotting), using polyclonal anti-human LIF antibodies (developed in goat) and anti-goat IgG peroxidase conjugate (*Sigma*, USA).

Immunoprecipitation was performed according to the protocol of *Abcam* company (USA) (abcam.com/ps/ pdf/protocols/immunoprecipitation). 1 ml of the conditioned DMEM medium, obtained after cultivation of $2.5 \cdot 10^5$ cells, was mixed with 3 мg of primary antibodies and incubated for 4 hours while stirring at 4?C, then 70 мl of protein G-sepharose were

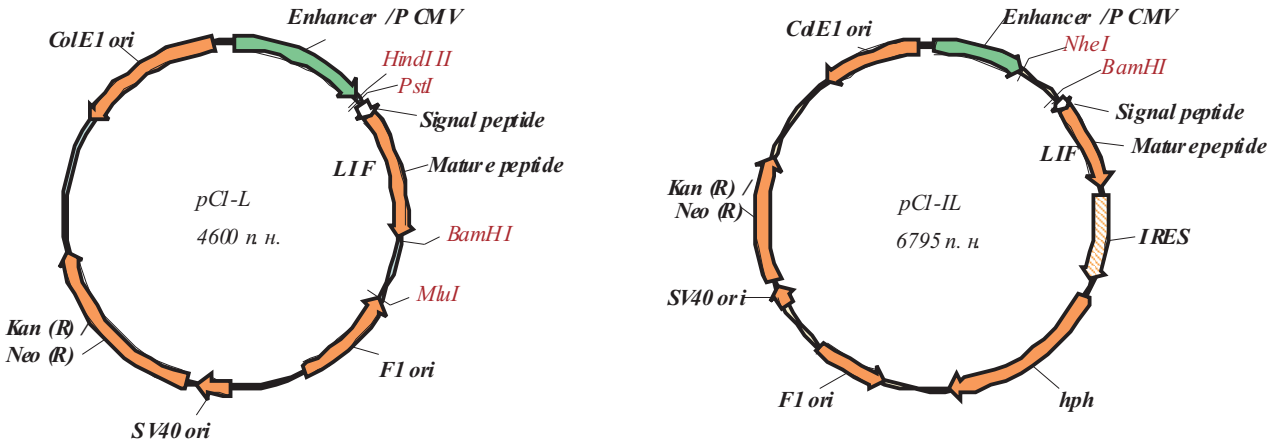

Fig.1 Physical maps of recombinant plasmids

added and incubated again (4?C, 4 hours). Samples were centrifuged, supernatant was removed and protein G-sepharose was washed three times with the following buffer: 50 mM tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS. 25 мl of loading buffer were added to protein G-sepharose and boiled (5 min). Western-blot analysis was performed after electrophoresis in SDS-PAGE..

Results and Discussion. At first the *LIF* coding sequence with signal sequence [Human *LIF* gene, coding LIF with signal peptide, was cloned via RT-PCR methodology using mRNA, extracted from human placenta, and corresponding primers. LIF cDNA, amplified in PCR, was cloned in *pBluScrII* (KS^+), and its nucleotide sequence was confirmed by sequencing. Two plasmids – *pC1-L* and *pC1-IL* (Fig.1) – were constructed based on the expression vector for mammalian cells *pEGFP-C1* (*Clontech*, USA). *LIF* gene was introduced to *pC1-L* under the control of cytomegalovirus promoter, while a fragment of DNA, containing IRES (internal ribosome entry site) of encephalomyocarditis virus and hygromycin resistance gene were introduced into *pC1-IL*.

The expression of *LIF* gene, inserted in these plasmids, in eukaryotic cells should result in secretion of the glycosylated protein. LIF has six sites of glycosylation [25]. The molecular weight (m.w.) of the protein is in the range from 32 kDa to 73 kDa depending on the glycosylation degree. Western-blot analysis of the media, conditioned by the cells of three cell lines, transfected with recombinant plasmids, showed that they secreted LIF which was likely glycosylated to a variable degree. The proteins with m.w. about 30 kDa, 55 kDa and 68–72 kDA were identified (Fig.2–4).

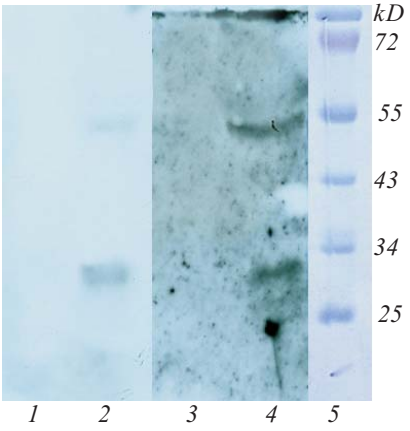

Fig.2 Western-blot analysis of conditioned media (1st passage cells): *1* – CHO-K1 (control, transfection with the vector, serum- free DMEM , 24 hours); *2* – CHO-K1 (transfection with *pC1-L*, serum-free DMEM, 24 hours); *3* – CHO-K1 (control, transfection with the vector, serum-free DMEM, 48 hours); *4* – CHO-K1 (transfection with *pC1-L*, serum-free DMEM , 48 hours); *5* – molecular weight marker, kDa

The protein with the highest molecular weight form likely corresponds to completely glycosylated protein. Bovine serum albumin, presented in the serum, interfered with the visualization of completely glycosylated LIF. Due to this fact the LIF secretion was studied via cultivation of washed cells in serum-free DMEM. Under these conditions the cells lost their

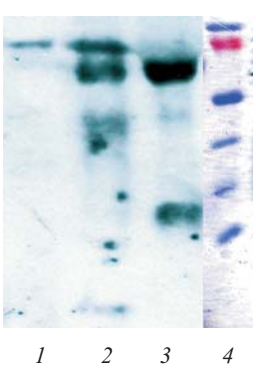

Fig.3 Western-blot analysis of conditioned media (cells of 2nd and 3rd passages, cultivated in serum-free DMEM for 48 hours); *1* – CHO-K1 (control, transfection with the vector); *2* – CHO-K1 (transfection with *pC1-L*); *3* – CHO-K1 (transfection with *pC1-IL*); *4* – molecular weight marker, kDa

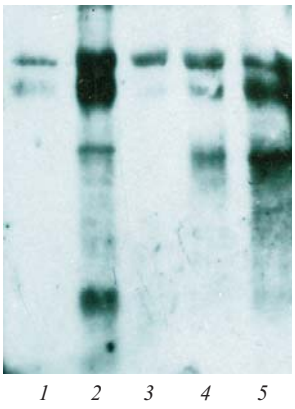

Fig. 4 Western-blot analysis of conditioned media (cell of 2nd — 4th passages, cultivated in serum-free DMEM for 48 hours); *1* – L-M(TK⁻)(ins⁺) (control, transfection with the vector); *2* – L-M(TK⁻)(ins⁺) (transfection with *pC1-IL*); *3* – CHO-K1 (control, transfection with the vector); *4* – CHO-K1 (transfection with *pC1-IL*); *5* – CHO-K1 (transfection with *pC1-L*); 6 – molecular weight marker, kDa

viability in 2–3 days and the level of expression of cellular proteins including recombinant protein decreased correspondingly. The viability of L-M (TK⁻)(ins⁺) line cells, producing human insulin, was higher in comparison with CHO-K1 and 293T. Besides, the controls may have positive signals specified by endogenous LIF because the degree of homology of human LIF with murine one is 79%. According to the analysis of experimental data from different laboratories, the molecular mass of human LIF isolated from various cells is diverse. For instance, the recombinant LIF with m.w. about 45 kDa was expressed in the Chinese hamster cells [26]. LIF with m.w. 73 kDa was isolated from the conditioned medium of the bladder carcinoma cell line 5637 [27]. Other authors isolated LIF with m.w. 43 kDa from the same source [28], LIF with m.w. 38 kDa was obtained from the conditioned medium of T-lymphocytes [29].

For Western-blot analysis LIF, secreted into conditioned medium and concentrated with immunoprecipitation using protein G-sepharose, was used. The results obtained with genetically modified lines CHO-K1, L-M (TK⁻)(ins⁺) and 293T, are presented in Fig.5. The Western-blot analysis revealed that mobility of some forms of LIF (in particular with m.w. about 30kDa and 55 kDa) coincided with that of light and heavy chains of immunoglobulins, added to the sample during immunoprecipitation. It resulted in masking some forms of LIF by immunoglobulins. The level of signals derived from the light and heavy chains of immunoglobulins in the immunoprecipitation of conditioned medium by the cells, transfected with vector plasmids (without *LIF* gene), was significantly lower. There was an additional intensive band, corresponding to the protein with m.w. 65–68 kDa, which was likely a completely glycosylated form of LIF, and a less intensive band, corresponding to the protein with m.w. about 48 kDa. The LIF expression in cells CHO-K1 (Fig.3, 4) transfected with *pC1-L* using G418 selection was more efficient than LIF expression in cells, transfected with *pC1-IL* using hygromycin selection. The efficiency of LIF expression in L-M (TK⁻)(ins⁺) cells, transfected with *pC1-IL*, was

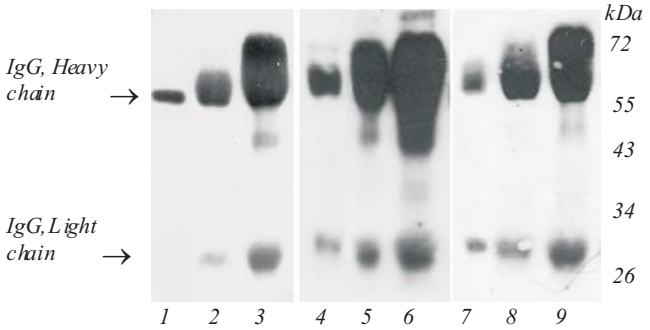

Fig.5 Determination of LIF in conditioned media (immunoprecipitation) (cells of 2nd – 4th passages, cultivated in serum-free DMEM for 48 hours, except for L-M(TK⁻)(ins⁺)): *1* – control of immunoprecipitation (immunoprecipitation without medium); *2* – 293T (control, transfection with the vector); *3* – 293T (transfection with *pC1-IL*); *4* – CHO-K1 (control, transfection with the vector); *5* – CHO-K1 (transfection with *pC1-IL*); *6* – CHO-K1 (transfection with *pC1-L*); *7* – L-M (TK⁻)(ins⁺) (control, transfection with the vector); *8* – L-M(TK⁻)(ins⁺) (transfection with *pC1-IL*, serum-free DMEM, 15 hours); *9* – L-M(TK⁻)(ins⁺) (transfection with *pC1-IL*, serum- free DMEM, 48 hours); *10* – molecular weight marker, kDa

sufficiently high like that in CHO-K1 cells, transfected with *pC1-L*) (Fig.4). It may be explained by the fact that the inhibition of metabolism in insulin-producing cells in serum-free media is weaker than in CHO-K1 and 293T cells.

Conclusions. The recombinant plasmids *pC1-L* and *pC1-IL*, constructed by us, provide expression and secretion of human recombinant LIF in the medium of three mammalian line cells (two murine and one human) transfected with these plasmids. The recombinant human LIF produced with mammalian transgenic cells is glycosylated to different degree, likely including completely glycosylated form. The medium conditioned by genetically modified cells may be used as a source of LIF for growth of different cells requiring this growth factor as well as for its isolation.

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Экспрессия и секреция рекомбинантного белка LIF человека генетически модифицированными клетками млекопитающих

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Резюме

Цель *работы состояла в получении экспрессии гена LIF человека в генетически модифицированных клетках млекопитающих и изучении секреции рекомбинантного белка этими клетками в культуральную среду.* ***Методы****. Для выявления рекомбинантного LIF в кондиционированной среде, полученной в результате культивирования клеток, трансфецированных рекомбинантными плазмидами, содержащими ген LIF, использовали Вестерн-блот-анализ и иммунопреципитацию.* ***Результаты****. Сконструированные рекомбинантные плазмиды обеспечивают експрессию и секрецию рекомби- нантного LIF человека клетками трех линий (CHO-K1, L-M(TK^-)(ins^+) и 293T), трансфецированными этими плазмидами. Степень гликозилирования продуцируемого такими клетками рекомбинантного LIF варьирует, при этом наблюдается секреция полностью гликозилированного LIF (с молекулярной массой около 68 кДа).* ***Выводы****. Кондиционированную среду, полученную вследствие культивирования трансфецированных клеток, можно использовать как источник LIF человека для культивирования клеток, нуждающихся в этом ростовом факторе, и для его выделения в чистом виде.*

Ключевые слова: рекомбинантный LIF, экспрессия, секреция, трансфекция,клеточные линии

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Експресія і секреція рекомбінантного білка LIF людини генетично модифікованими клітинами ссавців

Резюме

Мета. *Мета роботи полягала в одержанні експресії гена LIF людини в генетично модифікованих клітинах ссавців і вивченні секреції рекомбінантного білка цими клітинами в культуральне середовище.* ***Методи.*** *Для визначення рекомбінантного LIF в кондиціонованому середовищі, одержаному в результаті культивування клітин, трансфекованих рекомбінантними плазмідами, що містять ген LIF, використовували Вестерн-блот аналіз та імунопреципітацію.* ***Результати****. Сконструйовані рекомбінантні плазміди забезпечують експресію і секрецію рекомбінантного LIF людини клітинами трьох ліній (CHO-K1, L-M(TK^-)(ins^+) і 293T), трансфекованих цими плазмідами. Ступінь глікозилювання рекомбінантного LIF, продукованого такими клітинами, варіює, при цьому спостерігається секреція повністю глікозильованого LIF (з молекулярною масою близько 68 кДа).* ***Висновки****. Кондиціоноване середовище, одержане внаслідок культивування трансфекованих клітин, можна використовувати як джерело LIF людини для культивування клітин, яким потрібен цей ростовий фактор, а також для його виділення в очищеному стані.*

Ключові слова: рекомбінантний LIF, експресія, секреція, трансфекція, клітинні лінії.

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