CROATICA CHEMICA ACTA
CCACAA **81** (1) 177–182 (2008)
ISSN-0011-1643
CCA-3228
Original Scientific Paper

Effects of LIF on Neuromuscular Junction Formation in Co-cultures of Rat Spinal Cord Explant and Human Muscle

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RECEIVED APRIL 2, 2007; REVISED SEPTEMBER 24, 2007; ACCEPTED OCTOBER 10, 2007

Leukemia inhibitory factor (LIF) is a multifunctional cytokine important in nerve and muscle cells development and maintenance. The effects of LIF on the formation of neuromuscular junction (NMJ) have not been studied directly, that is why we employed the *in vitro* model of human muscle cell cultures innervated by rat embryonic motor neurons. In this model, several aspects of neuromuscular junction formation can be studied. The aim of the study was to test if LIF promotes the formation of functional NMJ. The effects of LIF on neuronal outgrowth, acetylcholine receptor accumulation and the formation of functional contacts were tested. Co-cultures treated with higher concentrations of LIF have increased neuronal outgrowth, higher number of acetylcholine receptor clusters and finally at later stages also a higher degree of functional innervation. LIF positively promotes the formation of neuromuscular junction in specific concentration range. Effects appeared to be stronger at later stages of NMJ formation in our co-cultures. The model of *in vitro* innervated human muscle cell can be a useful experimental system for testing toxic and therapeutic effects of substances on the formation and development of neuromuscular systems at cellular level.

Keywords
neuromuscular junction
leukemia inhibitory factor
motor neuron
human muscle cell

INTRODUCTION

Neurotrophic factors play a profound role in the development, maintenance and regeneration of the nervous system. Further more they are involved in the formation and maturation of neuromuscular junction, where appropriate trophic interactions between motor neurons and muscle are very important for successful formation of functional neuromuscular system.[1–3] Neurocytokine family of neurotrophic factors includes ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF).[4] LIF is synthesized in great variety of cell types and as well as in motor neurons and skeletal muscle.[5,6] Intracellular actions of LIF are mediated via transmembrane receptor complex (LIFR) activation.[7] Expression and synthesis of LIF and its receptor in motor neurons, skeletal muscle and other tissue is precisely regulated depending on the developmental and physiological situation.[8] LIF is ubiquitous multifunctional cytokine [9,6] and unique in its independent neurotrophic and myotrophic actions.[10] Neurotrophic effects of LIF promote survival of embryonic motor neurons *in vitro* and it is essential factor in the process of motor neuron regeneration.[11,12] Several studies also support an active role of LIF in skeletal muscle development and regeneration. LIF promotes myoblast proliferation and growth.[13] In regenerating skeletal muscle LIF promotes different aspects of this process as shown in the study by Kurek *et al*.,[14,15] further it increases the size of regenerating muscle fibers[16] and it might be essential in functional recovery.[17]

The role and effects of LIF and other neurotrophic factors on the formation of neuromuscular junction (NMJ)

are still poorly understood partly due to a lack of appropriate experimental models. The experimental model of *in vitro* innervated human muscle enables studying more closely the different aspects of NMJ formation. In this experimental model human muscle are innervated with embryonic rat spinal cord motor neurons which results in the formation of functional and long lived NMJ and highly mature and contracting myotubes.[18–20] In the presented study, the *in vitro* model of innervated human muscle was used in order to find out if LIF promotes the formation of NMJ.

EXPERIMENTAL

Preparation of Human Muscle Cell and Embryonic Rat Spinal Cord Co-culture

All studies reported here were approved by the Ethical Commission at the Ministry of Health of the Republic of Slovenia (permit No: 63/01/99). Co-cultures were prepared as described before.[18–20] Human myoblast cultures were derivered from muscle pieces routinely discarded at the orthopaedic operations on patients that did not suffer from any muscular disease. Briefly, human muscle tissue was cleaned of adhering connective tissue, cut to 0.5–1 mm pieces and trypsinized to release muscle satellite cells. Cells were grown to clonal density in 100 mm Petri dishes in Minimum Essential Medium (MEM; Invitrogen, UK) with 10 % Fetal Bovine Serum (FBS; Invitrogen, UK) at saturated humidity in a mixture of 5 % CO_2 and air at 37 °C. Myoblast colonies identified by morphology and devoid of fibroblast contamination were trypsinized and further expanded in 75 cm^2 flasks containing MEM with 15 % FBS. Confluent myoblast cultures were harvested by trypsinization prior to myoblast fusion and were plated on glass coverslips coated with 1:2 mixture of gelatine (1.5 g dissolved in 100 ml of water; Sigma) and human plasma, in 35 mm six-well plates. Myoblasts were grown in F-14 medium (Invitrogen, UK) supplemented with 10 % FBS, 50 ng/ml Fibroblast Growth Factor (Sigma), 10 ng/ml Epidermal Growth Factor (Sigma) and 10 μg/ml Insulin (Sigma). Timed pregnant Wistar female rats were sacrificed by CO_2 narcosis at 14 days of gestation, as specified by the Veterinary Administration of the Ministry for Agriculture, Forestry and Food of Slovenia (permit No: 323-02-74/00) and in accordance with European Communities Council Directive (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering. The embryos were removed by caesarean section and placed in Earle's Balanced Salt Solution (Invitrogen, UK). Spinal cords were aseptically dissected from E14 embryos cut in 1-mm thick cross-sections. For each section, the anatomical connections with dorsal root ganglia and the integrity of meninges were preserved. Four to five explants were placed on to a monolayer of fused myoblasts grown on glass coverslips. After 1 week, co-cultures were grown in F-14 medium containing 5 % of FBS and devoid of FGF and EGF to promote further differentiation of muscle cells into myotubes. In order to study the effects of LIF we tested five different concentrations of LIF (1, 5, 10, 20 and 50 ng/ml) (R&D Systems, MN, USA), same concentrations were already tested in long term spinal cord cultures.[21]

Quantitative Analysis of Functional Innervation

The effects of LIF on neuronal outgrowth were determinated by counting the neurite roots growing out of spinal cord explant at various time intervals during the experiment and as described before.[20] The effect of LIF on acetylcholine receptor (AChR) accumulation was determinated by counting the number of AChR clusters. AChR clusters were vitally labelled by rhodamine-conjugated α-bungarotoxin (Molecular Probes, OR, USA) for 1 hour at the concentration of 1 μg/ml diluted in culture medium with 1 % bovine serum albumine and without serum. Co-cultures were then washed with F-14 medium without serum and α-bungarotoxin three times and fixed in 4 % paraformaldehyde. For easier identification of myotubes we stained nuclei with Hoechst 33342 (Molecular Probes, OR, USA) for 5 minutes at the concentration of 1 μg/ml. Coverslips were mounted on glass slides with Vectashield. The number of AChR clusters per microscopic field was counted as already performed in other studies.[22] AChR clusters were viewed under fluorescent microscope (Axioscope Zeiss, Germany) with 40x objective. Between 20 to 40 different randomly chosen microscopic fields (final magnification of microscopic field was 400x) were examined in the innervation areas[20] of the contraction-positive explants for each set of control and LIF treated co-cultures. The efficiency of functional neuromuscular junction formation was quantitated by determining i) the number fraction (expressed in percents) of contraction-pos itive explants, and ii) the number of contracting units within the innervation areas of individual contraction-positive explants. The explant was considered contraction-positive if it exhibited at least one contraction unit in its area of innervation. The fraction of contraction-positive explants was determined in the population of explants placed on human myotubes at the first day of co-culture. The number of contraction-positive explants was counted at certain time points during the experiment and from this data an average fraction of contraction-positive explants for each set of co-cultures was determined. Contracting unit was defined as a distinct group of muscle fibers contracting simultaneously at a frequency which was different from the frequencies of contractions of other contracting units. By counting these units we were not directly counting the number of NMJs since the number of fibers belonging to each contracting unit is difficult to asses. Results are given as mean ± SD. Statistical analysis was performed using Student *t*-test, statistical significance was accepted at $P<0.05$.

RESULTS

In most of the co-cultures, neurons started to grow out of spinal cord explants in the first days after establishment of the co-cultures. Neuronal outgrowth was quantitated by counting the number of neuronal roots per spinal cord

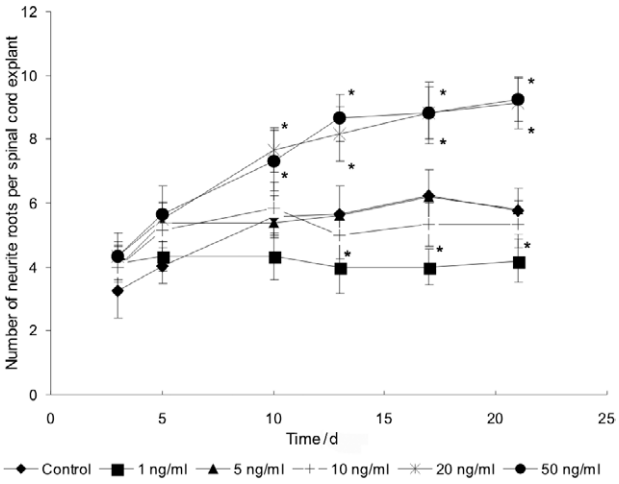

Figure 1. Neuronal outgrowth in control and LIF treated co-cultures at different time points after establishment of co-cultures. Asterisks denote significant differences from the control ($P<0.05$; *t*-test).

explant at various time points. This number was continuously increasing from day 3 (3.25 ± 0.84) to day 17 after establishment of co-cultures with 6.25 ± 0.81 neuronal roots per spinal cord in control co-cultures. Later on, the number of neuronal roots per spinal cord became stabilized at the same level or even slightly but not significantly decreased (Figure 1). Similar results were observed in co-cultures treated with lower concentrations of LIF (5 and 10 ng/ml). In co-cultures treated with 1 ng/ml of LIF decreased neuronal outgrowth at all time points after day 10 was observed. In comparison with control co-cultures at day 21 neuronal outgrowth reached the value 4.21 ± 0.69, which is 33 % less than in controls. Neuronal outgrowth was significantly higher from day 5 in co-cultures treated with higher concentrations of LIF (20 and 50 ng/ml). At day 21 there was 9.24 ± 0.68 neuronal roots per spinal cord, which is 1.77 times higher than in control co-cultures at this time point.

Further we investigated the effects of LIF on AChR clustering by counting the AChR clusters in microscopic fields in close vicinity of spinal cord explants in 15 days old co-cultures. For each set of co-cultures treated with different concentrations of LIF twenty to thirty different microscopic fields were examined. The average number of AChR clusters per microscope field in control co-cultures was 7.8 ± 0.8, similar results were obtained in co-cultures treated with lower concentrations of LIF (6.9 ± 1.2 in 1 ng/ml, 7.4 ± 1 in 5 ng/ml and 8.5 ± 0.8 in 10 ng/ml), Figure 2. The number of AChR clusters was significantly higher in co-cultures treated with 20 ng/ml of LIF (11.3 ± 1.0) which is 45 % higher and in co-cultures treated with 50 ng/ml of LIF (10.9 ± 0.9) 39 % higher than in control co-cultures.

We did not observe any contractions prior to day 7 after the establishment of co-cultures. After that, progressively more explants became contraction-positive. The average fraction of contraction-positive explant through time course of experiment in control co-culture was 40 % (Figure 3). No statistically significant difference in this respect could be observed between control co-cultures and co-cultures treated with 1 ng/ml of LIF, where the average fraction of contraction-positive explants was 40 % and co-cultures treated with 5 ng/ml of LIF was 43 %. Statistically significant higher average fraction of contrac-

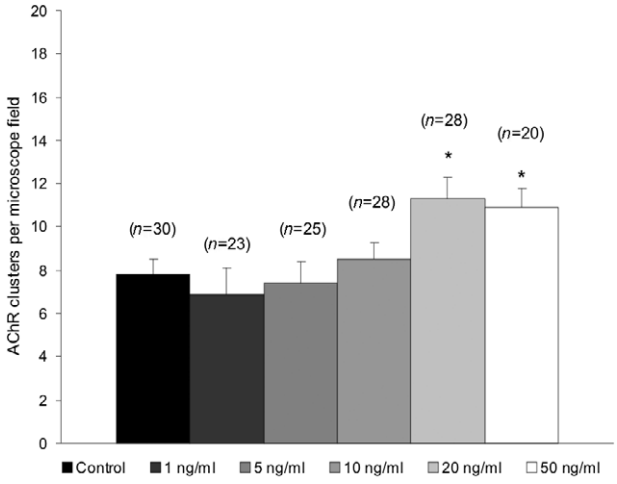

Figure 2. AChR clustering in control and LIF treated 15 days old co-cultures. Histograms present an average number of AChR clusters per microscope field (400x) examined in the area of innervation. Number of fields examined in type of co-cultures is indicated above bars. Asterisks denote significant differences from the control ($P<0.05$; *t*-test).

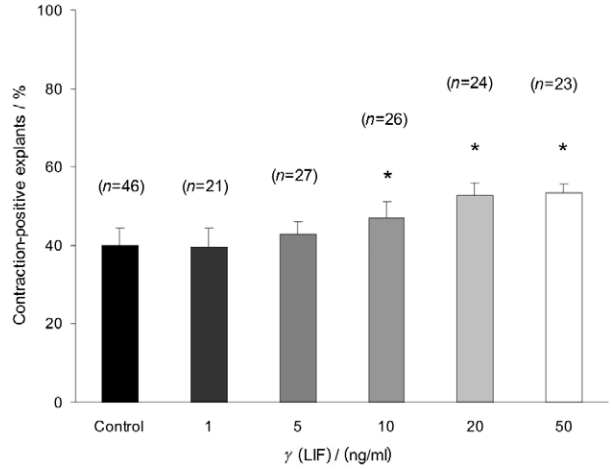

Figure 3. Number fraction of contraction-positive explants expressed in percents during the time course of experiment in control and LIF treated co-cultures. Number of spinal cord explants followed in each set of co-cultures is indicated above bars. Explant was considered contraction-positive if at least one contraction unit had been detected in the area of innervation. Contraction unit was defined as distinct group of muscle tubes contracting simultaneously at frequency, which was unique for this contracting unit and different from the contraction frequencies of other units. Asterisks denote significant differences from the control ($P<0.05$; *t*-test).

tion-positive explants in co-cultures treated with 10 ng/ml (47 %), 20 ng/ml (52 %) and 50 ng/ml of LIF (53 %) in comparison with control and co-cultures treated with lower concentration of LIF was observed.

Efficiency of innervation was further determinated by counting the number of contraction units per contraction-positive explant. In all co-cultures an increasing number of contraction units per spinal cord explant could be observed from day 7, when the first contraction was observed. The average number of contracting units in all co-cultures at the beginning of contraction was 2.05 ± 0.46 till day 17 after establishment of co-cultures (Figure 4). Later on, no increase could be observed, however the number of contracting units stayed at the same level with 3.86 ± 0.23 in control co-cultures, 3.33 ± 0.58 in co-cultures treated with 10 ng/ml and 3.65 ± 0.47 contracting units in co-cultures treated with 10 ng/ml of LIF. In co-cultures treated with 1 ng/ml of LIF the number of contraction units never reached the number observed in control co-cultures and was even statistically significantly lower at later time points. We observed a significantly higher number of contracting units firstly at day 14 after establishment in co-cultures treated with 20 ng/ml of LIF than in control co-cultures. This number stayed at the higher level after that time point. Significantly, a higher number than in the control co-culture was also observed in co-cultures treated with 50 ng/ml of LIF, but firstly at day 17 after establishment.

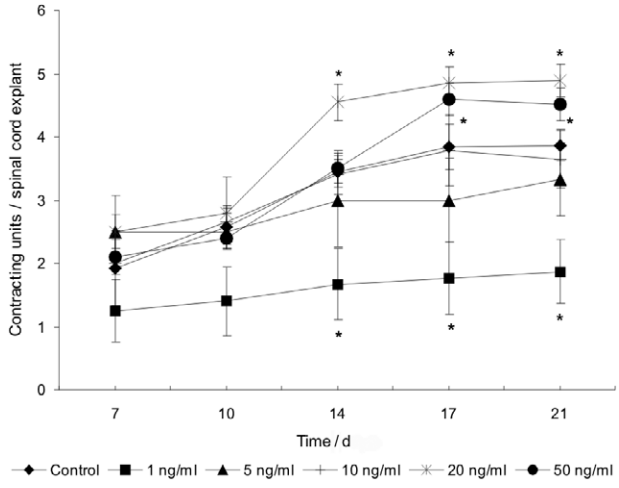

Figure 4. The number of contracting units per contraction-positive spinal cord explant at different time in co-cultures. Means ± S.D. was determined for individual contraction positive explants of control and LIF treated co-cultures. Asterisks denote significant differences from the control ($P<0.05$; *t*-test).

DISCUSSION

In the study presented here the effects of LIF on the formation of NMJ in *in vitro* model of innervated human muscle was studied. The experimental model employed is so far the only model where early events in synaptogenesis of NMJ could be followed and functional innervation could be easily quantitated.[20] The effects of LIF on the formation of NMJ have not yet been systematically studied. We found, that certain concentrations of LIF promote processes in NMJ formation like neuronal outgrowth, AChR accumulation and functional innervation. These processes are important for successful NMJ formation. We decided to follow these parameters since they reflected on both neuronal and muscle development. Our results are consistent with previous studies of neurotrophic and myotrophic effects of LIF.

We found, an increased neuronal outgrowth in co-cultures treated with higher concentration of LIF but not in co-cultures treated with lower LIF concentrations. This result is consistent with study of Martinou *et al*.[23] where LIF supported motor neurons survival and increased cholinacetyltransferase activity in cultures of isolated rat embryonic motor neurons. LIF is the neurotrophic factor known to have a wide range of effects that promote neuronal outgrowth also in other nerve cell types.[24] Positive effects at higher concentrations could be explained by cell complexity found in our experimental model.[19] Complex interaction among neuronal and glial cell in our experimental model may influence LIF effects or it could be even mediated through Schwann cell[25,26] or via activation of mature astrocytes like it is in the case of CNTF.[27,28] White *et al*.[29] reported that beneficial effects of LIF at least on skeletal muscle development are mediated by modulation of the extracellular matrix. This kind of interaction requires higher concentration of LIF.[30] An increased neuronal outgrowth is also in accordance with findings that LIF rescues motor neurons from axotomy.[31,32] Motor neurons enter into the process of regeneration after nerve lesion, regeneration is successful only if proper trophic stimuli are supplied. Typical reaction to nerve lesion is an up-regulation of trophic factor receptors mRNAs like LIFR.[9,11] The regeneration process could be compared to the first stages of innervation, where motor neurons survival is dependent on proper target trophic survival stimulation. Survival of motor neurons is beside exogenously derivered LIF dependent also from endogenously synthesized LIF, which increases after nerve injury and where newly synthesized LIF is actively transported in retro- and anterograde way.[10,12,14]

No direct studies of LIF on NMJ, to the best of our knowledge, have yet to be performed. AChR clusters counted in our experiments are equivalent to NMJs during human muscle innervation. AChR cluster counting was performed at the stage when most of NMJ are already formed. However, at this stage we could not distinguish between functional and non functional NMJ. Higher concentrations of LIF caused an increase in number of AChR clusters. It was showed by Kwon *et al*.[33]

that LIF delays programmed withdrawal of newly formed synapses from muscle, these findings could partially explain increased number of AChR clusters in LIF treated co-cultures.

The higher average fraction of contraction-positive explants and increased number of contraction units in co-cultures treated with higher LIF concentrations is in agreement with several studies of LIF myotrophic effects.[13,34,35] Higher neuronal outgrowth means a greater possibility for nerve-muscle contacts to be formed, which in turn, results in a higher number of AChR clusters. Although AChR clustering is a function of muscle cells it is on the other hand strongly influenced by nerve derived factors.[2] This ability is exclusive function of cholinergic neurons. It was shown that LIF in immature neurons actively governs a switch from an adrenergic to a cholinergic phenotype.[36] Increased cholinergic activity however may influence the functional innervation of myotubes. In skeletal muscle development LIF importantly influence myoblast proliferation.[13,37] Firstly, successful myoblast proliferation and subsequent myotubes formation and survival are prerequisites for efficient innervation. Secondly, LIF promotes and is essential for muscle regeneration after denervation[38] or injury.[16,35,39] In this way LIF promotes not only muscle cell survival but also indirectly stimulates reinnervation or even new innervation of muscle. Lower degrees of successful innervation in co-cultures treated with a lower concentration of LIF could be explained either by the fact that neurotrophic factors trigger positive effects only in specific narrow concentration range[40] or by negative interactions of LIF with other endogenously secreted trophic factors. This peculiar mechanism has to be taken in account for possible therapeutic use of neurotrophic factors.

In summary, our results show that in our experimental model only specific concentrations of LIF positively promotes several processes important for NMJ formation and successful innervation which finally results in the appearance of contracting myotubes. This study could be an important contribution to the possible use of this *in vitro* model of innervated human muscle in studying other trophic substances on the formation and maintenance of NMJ and to possible use of LIF as therapeutic agent.

Acknowledgments. – This study was supported by a Fogarty International Research Collaboration Award from the Fogarty International Center and Slovenian Research Agency. The technical assistance by Mrs. Zvonka Frelih is gratefully acknowledged.

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SAŽETAK

Učinak LIF-a na stvaranje neuromuskularne spojnice u ko-kulturama eksplantata leđne moždine štakora i mišića čovjeka

Tomaž Marš

Leukemijski inhibicijski čimbenik (engl. *Leukemia Inhibitory Factor,* LIF) multifunkcionalni je citokin, važan za razvoj i održavanje živčanih i mišićnih stanica. Učinci LIF-a na stvaranje neuromuskularne spojnice još nisu sustavno istraživani. U ovom radu korišten je *in vitro* model kulture ljudskih mišićnih stanica inerviranih embrionalnim motoričkim neuronima štakora, što je omogućilo istraživanje različitih aspekata neuromuskularne spojnice. Cilj ovog istraživanja bio je ispitati potiče li LIF razvoj funkcionalne neuromuskularne veze. Ispitivani su učinci LIF-a na rast neurona, akumulaciju acetilkolinskih receptora te nastanak funkcionalnih neuromuskularnih spojnica. Ko-kulture podražene većim koncentracijama LIF-a pokazale su povećan rast neurona, povećan broj nakupina acetilkolinskih receptora, te u kasnijem periodu, nastanak većeg broja funkcionalnih sinapsi. LIF u točno određenom rasponu koncentracija promovira nastanak neuromuskularnih spojnica. U našim ko-kulturama učinci su bili izraženiji u kasnijim stadijima formiranja neuromuskularnih veza. Opisani model *in vitro* nastanka inerviranih ljudskih mišićnih stanica može biti učinkovit eksperimentalni sustav za testiranje toksičnih i terapeutskih učinaka različitih tvari tijekom nastanka i razvoja neuromišićnih veza na staničnoj razini.