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ORIGINAL PAPER

Effects of Interleukin-6, Leukemia Inhibitory Factor, and Ciliary Neurotrophic Factor on the Proliferation and Differentiation of Adult Human Myoblasts

Xuan Wang · Haitao Wu · Zhenxing Zhang · Shuhong Liu · Jian Yang · Xiaoping Chen · Ming Fan · Xiaomin Wang

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Abstract Our previous studies have demonstrated that ciliary neurotrophic factor, a member of the interleukin-6-type cytokine superfamily, could inhibit the differentiation of myoblasts into mature myotubes at a certain concentration. In this study, another two members, interleukin-6 and leukemia inhibitory factor, together with ciliary neurotrophic factor were tested their roles in the proliferation and differentiation of myoblasts derived from the adult human skeletal muscles, in order to confirm that these cytokines might be a new type of regulatory factors on the myoblasts. The results showed that the effects of interleukin-6, leukemia inhibitory factor, and ciliary neurotrophic factor on the proliferation and differentiation of adult human myoblasts were different. Leukemia inhibitory factor in the dose of 10 ng/ml could accelerate the cell proliferation. Leukemia inhibitory factor in the dose of 10 or 50 ng/ml and ciliary neurotrophic factor in the dose of 10 or 50 ng/ml could inhibit the myoblast differentiation. The inhibition mechanism might be that leukemia inhibitory factor and ciliary neurotrophic factor inhibited the expressions of transcription factor MyoD/myf5, which could regulate the myoblast differentiation. This study will provide the experimental and theoretic foundations for the basic and clinical researches about human myoblasts.

Keywords Myoblasts · Interleukin-6 · Leukemia inhibitory factor · Ciliary neurotrophic factor

X. Wang · J. Yang · X. Wang (✉)
Department of Physiology, Capital Medical University, Key Laboratory for Neurodegenerative Disorders of the Ministry of Education, 10# You An Men, Fengtai District, Beijing 100069, P.R. China
e-mail: xmwang@ccmu.edu.cn

H. Wu · Z. Zhang · S. Liu · X. Chen · M. Fan (✉)
Department of Brain Protection & Plasticity Research, Beijing Institute of Basic Medical Sciences, 27# Taiping Road, Haidian District, Beijing 100850, P.R. China
e-mail: fanming@nic.bmi.ac.cn

Introduction

The superfamily of interleukin-6-type cytokines regulates cell survival, proliferation, and differentiation, and plays an important role in anti-inflammation, hematopoietic, and neuronal differentiation. This subfamily includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), cardiotrophin-1 (CT-1) and so on (Heinrich et al. 2003; Murakami et al. 2004). Among them, IL-6, LIF, and CNTF are extensively studied with their roles in the hematopoietic and neural development (Nakajima et al. 1996; Gregg and Weiss 2005; Emery et al. 2006). It has been shown that CNTF receptors are expressed in the skeletal muscle tissue (Sleeman et al. 2000) and LIF is a trophic factor for cadiocytes and cardiomyoblasts (Austin et al. 2000). For embryonic stem cells, LIF inhibits their differentiation and maintains them in the primary status (Davey and Zandstra 2006).

The myoblasts provide the sufficient source for muscle formation, which is potentially useful for treatment of the muscular atrophy and non-muscle diseases, such as neurodegenerative disorders (Gussoni et al. 1992; Mignon et al. 2005). Furthermore, these cells can be obtained from the patients per se, providing the advantage for clinical use. It had been reported that LIF promoted the proliferation of myoblasts (Spangenburg and Booth 2002) and blocked the myogenic differentiation of murine myoblasts (Jo et al. 2005). CNTF had been reported that it inhibited the human myoblasts to differentiate into mature myotubes (Chen et al. 2005). However, whether IL-6 and LIF also affect proliferation and differentiation of adult human myoblasts remains unclear. Since, IL-6 and LIF share many common functions with CNTF, it is thus possible that these two cytokines may also affect human myoblasts. In the present study, we have studied the effects of IL-6, LIF, and CNTF on the myoblasts derived from the adult human skeletal muscles and found that these cytokines could affect the proliferation and differentiation of myoblasts. The molecular mechanisms by which the three cytokines regulate the proliferation and differentiation of myoblasts were also discussed.

Methods

Source of Muscle

Mixed primary cultures were obtained from the temporal muscle of an adult human autopsy tissue suffered with cerebral tumor. The procedure of myoblast isolation and purification was performed as previously described (Rando and Blau 1994). Briefly, the muscle was placed in a few drops of cold D-Hank's buffer to keep it moist, then was dissociated both enzymatically and mechanically by mincing the muscle into a coarse slurry with a razor blade in 2 ml of a solution of 2.4 U/ml dispase and 1% collagenase II, supplemented with $CaCl_2$ to a final concentration of 2.5 mM. The slurry was then maintained at 37°C for 45 min with occasional mixing. To remove the enzymatic solution, the slurry was centrifuged and resuspended in a selective medium containing 80% Ham's F-10 nutrient mixture, 20% fetal bovine serum (FBS), 2.5 ng/ml basic fibroblast growth factor (bFGF), penicillin G (200 U/ml), and streptomycin (200 μg/ml). Then, the suspension was plated on the 1% poly-L-lysin-coated dishes growing in a humidified incubator containing 5% CO_2 at 37°C. This medium is selective for the growth of myoblasts while inhibiting fibroblast growth. After 2 h, supersuspension was collected and replaced into new lysine-coated dishes. Then supersuspension was removed into new dishes again after 24 h, and the procedure of the above continued for 3 days. After contiguous adhesion experiments, myoblasts would be highly purified, and the fibroblasts contaminated could shrink to the least extent.

Myoblast Cloning

After above primary purification cultivation, a selective medium containing growth medium (GM) for primary myoblasts was used. It consisted of 40% Ham's F-10 nutrient mixture, 40% DMEM, and 20% FBS, supplemented with 2.5 ng/ml bFGF, penicillin G (200 U/ml), and streptomycin (200 μg/ml). This medium was selective for the growth of myoblasts while inhibiting fibroblast growth. In order to purify the primary cultured myoblasts further, myoblasts were serially diluted in GM and plated onto 96-well plates, and allowed to adhere overnight, after which the well containing the individual cell was marked. After 1-week proliferation, progenies derived from single one cell were passaged and seeded into one well of 24-well plate for 1 week, then of 6-well plate. After grown to subconfluence, these monoclonal cells were passaged and part of them were stained with anti-desmin antibody (see Immunocytochemistry assay). Only the desmin + clones were selected and seeded into the 25-cm^2 tissue culture flasks in GM for further proliferation and passages.

IL-6/LIF/CNTF on the Proliferation and Differentiation of Myoblasts

Cloned myoblasts were seeded in the 21 wells of 24-well plates. The cell density was adjusted to 10^4/ml. For the proliferation experiment, GM was added into the wells as the control group. Cells in other groups were treated with different doses of IL-6, LIF, or CNTF, respectively. The concentration of cytokines was 0.1, 1, 10, and 50 ng/ml. Cell number in three wells were counted ($n = 3$) by trypan blue staining every 24 h for 7 days, and then cell proliferation curve was drafted. The different effects of IL-6, LIF, and CNTF on the day 5 in vitro (DIV) were analyzed and compared with each other. Cell cycle was analyzed by flow cytometry for further testing the cell proliferation. Briefly, cloned myoblasts were treated with cytokines, respectively for 48 h. Then they were trypsinized, spun, washed in cold phosphate-buffered salt (PBS) solution, and fixed in ethanol for 2 h. The cells were resuspended in 0.1% Triton X-100 in PBS with 0.2 mg/ml RNase A and 20 ng/ml propidium iodide solution. Cell percentage of G0/G1 phase, S phase, and G2-M phase among the whole cell cycle were recorded and analyzed. For differentiation experiment, GM was replaced with differentiation media (DM) containing DMEM with 2% horse serum, penicillin G (200 U/ml), and streptomycin (200 μg/ml). Different doses of IL-6, LIF, or CNTF were added into the culture media, respectively as above-mentioned. The extent of myoblast myogenic differentiation was measured by the number of multinucleated myotube forming cells containing more than three nuclei were regarded as multinucleated myotubes. The expressions of mature myogenic differentiation marker proteins, myogenin and myosin were examined by immunofluorescent staining, and the expression levels of myogenic regulatory factors, MyoD and myf5 were also tested by Western blot analysis.

Immunocytochemistry Assay

Cells on coverslips were fixed by 4% paraformaldehyde, blocked with 10% normal goat serum and incubated with rabbit anti-desmin (1:500, Chemicon), mouse anti-myogenin (1:500, Santa Cruze), and mouse anti-myosin (1:500, Santa Cruze) primary antibodies and then with TRITC or FITC-conjugated secondary antibodies. Images were examined under confocal lazer scanning microscope (CLSM, Leica, Heideberg, Germany).

Western Blot Analysis

Cells were washed twice with 0.1 M PBS and lysed with 200 μl lysis buffer containing 0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, 0.5% deoxycholic acid, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, and 1% NP-40. Equal amounts of protein were loaded under reducing conditions onto a 10% SDS gel. After electrophoresis, the protein was blotted onto a polyvinylidene difluoride membrane. The membrane was blocked by skimmed milk and incubated with mouse anti-MyoD (1:1000, Santa Cruze) and rabbit anti-myf5 primary antibodies (1:1000, Santa Cruze). Signals were visualized by incubating with horse radish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagent (NENTM Life Science Products), then photographed using Kodak 1D image analysis software. Mouse anti-β-actin (1:1000, Santa Cruze) was used as inner parameter.

Statistical Analysis

All data were expressed as the mean ± SEM. One-way analysis of variance (ANOVA SPSS software) was used to compare the differences among groups, followed by a Bonferroni (Dunn) comparison using least squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

Results

Isolation and Characterization of Myoblasts

We first isolated the human myoblasts from adult skeletal muscle tissues and cultured the cells and found that the cells could survive for 6 months in vitro. As shown in Fig. 1a, the cell phenotypes were not uniform although the sequential adhesion method had eliminated fibroblast cells mixed with the myoblasts. In the monoclonal experiment, individual myoblasts were seeded in the 96-well plates by limited dilution. After cultured in GM for 4 weeks, six cell clones were obtained and named as human myoblast (HM) A8, HMB6, HMB8, HMB10, HMC7, and HME9. These clones were then immunostained with the antibody against desmin, a marker known for myogenic lineage-committed myoblasts. Only the desmin-positive clones were propagated. Among these clones, the HMA8, HMB8, HMB10, and HMC7 were desmin-positive. Since the different clones responded similarly to cytokines, and the HMC7, a clone with the most uniform phenotype (Fig. 1b–f), was used in our studies.

IL-6/LIF/CNTF Promoted Proliferation of the Myoblasts

HMC7 cells were treated with different doses of IL-6, LIF, and CNTF and the growth of the cells was examined. As shown in Fig. 2, treatment of the cells with IL-6 (10 ng/ml), LIF (10 and 50 ng/ml), and CNTF (0.1 and 1 ng/ml) for 5 days promoted proliferation of the myoblasts, especially with LIF at 10 ng/ml. Furthermore, flow cytometry was used to test the effects of three factors on the cell cycle at the 48 h incubation. In the different dose-treated group of IL-6 or CNTF, cell percentage of S and G2-M phase was not influenced apparently (data not shown). Among the different doses of LIF, 10 ng/ml LIF increased the cell percentage of S phase (27.25 ± 4.55%) and G2-M phase (11.8 ± 0.87%) significantly ($P < 0.01$, $n = 3$) contrary to the control group (S: 9.45 ± 0.99%; G2-M: 7.28 ± 0.99%).

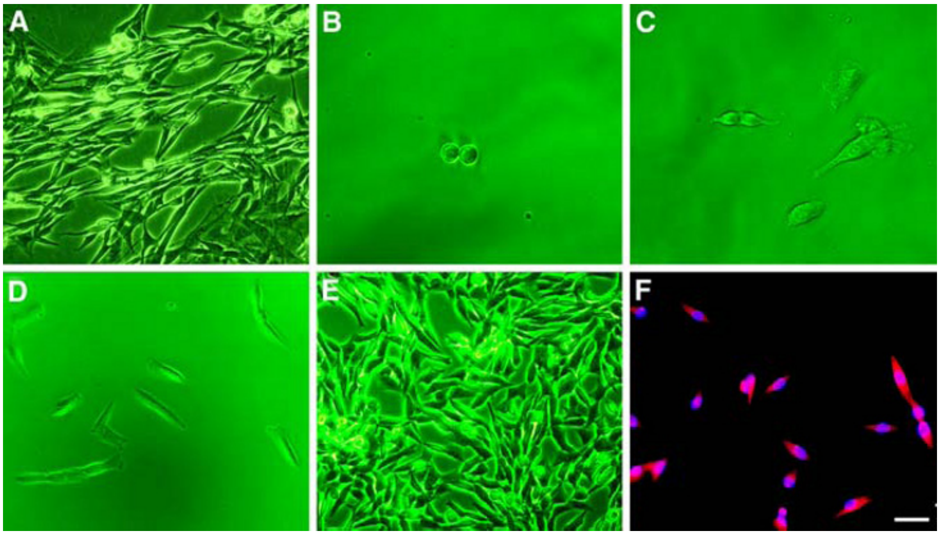
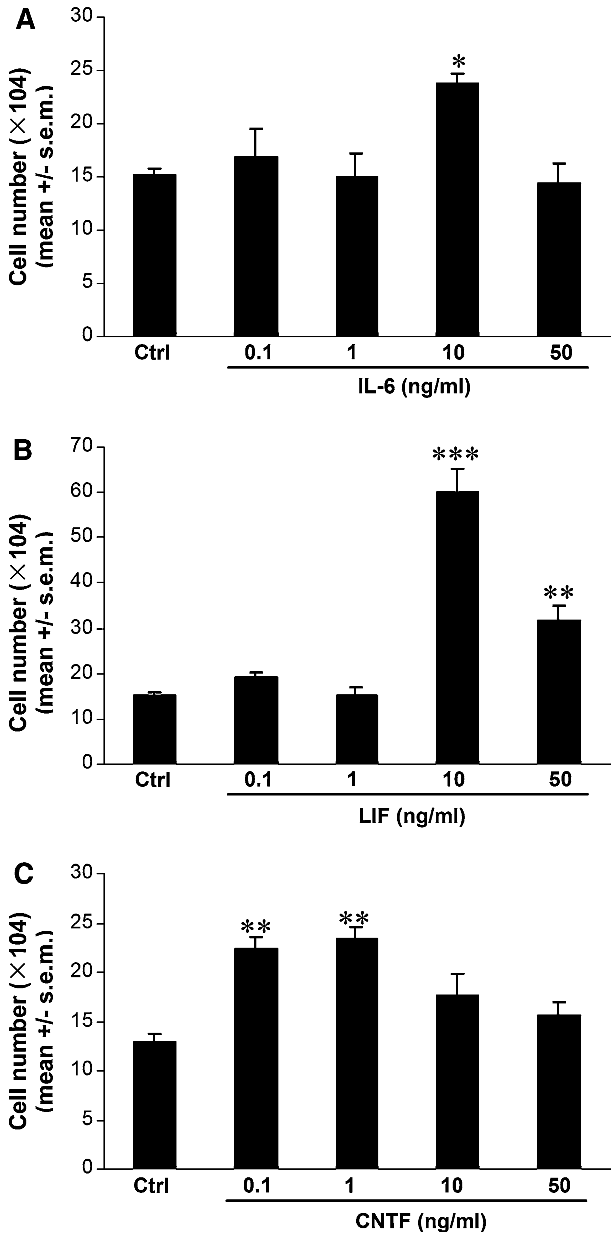


Fig. 1 Proliferation, clonalization, and identification of human myoblasts. Myoblasts derived from human muscle tissues proliferated in the DMEM/F10 (1:1) growth media with 2.5 ng/ml bFGF mitogen (**a**), possessing diverse cell shapes. Single cell began to divide after 2 days in vitro (DIV) cultivation in the 96-well culture plate (**b**). The number increased apparently after 3 DIV (**c**), and proliferated in a large-scale after 1 week (**d**). At the 2nd week of cultivation, human myoblasts derived from single one cells filled in the whole well (**e**). These cloned cells were uniformity compared with cells in Fig. 1a. Pure degree examination of cloned human myoblasts by immunostaining desmin with contrastaining the cell nuclear with DAPI. All the DAPI positive cells expressed the desmin (**f**). Bar = 10 μm

IL-6/LIF/CNTF on the Differentiation of Myoblasts

We then examined whether these three cytokines could affect the differentiation of the myoblasts. The mononucleated myoblasts in vitro can be fused into multinucleated myotubes, a process known as myogenic differentiation of myoblasts. Furthermore, the expression of myogenin and myosin proteins can be regarded as differentiation makers for myoblasts (Hasty et al. 1993; Odelberg et al. 2000; Parker et al. 2003). We added the different concentrations (0.1, 1, 10, 50 ng/ml) of IL-6/LIF/CNTF to subconfluent myoblasts cultured in DM for 14 days, and then analyzed their ability to undergo myogenic differentiation. The differentiation extent of myoblasts was expressed as fusion index indicating a percentage of the number of nuclei in the fused cells among the total number of nuclei. Cells containing more than three nuclei are regarded as fused cells. In the control group, the fusion rate was 50.7 ± 4.97%. After addition of different concentration of three factors (0.1, 1, 10, 50 ng/ml), the fusion index was 57.3 ± 6.01%, 61.3 ± 6.68%, 42.7 ± 1.08%, 46.0 ± 5.79% in the IL-6 treated group (Fig. 3a), 43.7 ± 4.71%, 37.3 ± 8.44%, 15.3 ± 3.19%, 35.7 ± 5.35% in the LIF treated group (Fig. 3b), and 46.3 ± 3.19%, 44.7 ± 2.94%, 32.3 ± 3.90%, 18.5 ± 4.02% in the CNTF treated group (Fig. 3c), respectively. Contrary to the control, 10 ng/ml and 50 ng/ml LIF or CNTF inhibited the myogenic fusion into multinucleated myotubes apparently, especially 10 ng/ml LIF treatment. In the control group, myoblasts in the DM differentiated for DIV 14 and expressed myogenic differentiation marker proteins myosin (Fig. 4a–c) and myogenin (Fig. 5a–c). Addition of 10 ng/ml IL-6 did not impact on the expression of above two proteins (Figs. 4d–f, 5d–f). In the group of 10 ng/ml LIF or 50 ng/ml CNTF treatment, the myoblasts fused into smaller myotubes with less expression of myosin (Fig. 4g–l), and the

Fig. 2 Proliferation of human myoblasts induced by different cytokines with different doses at the 5 DIV. Human myoblasts proliferated more rapidly when treated with 10 ng/ml IL-6 (**a**), 10 ng/ml LIF (**b**), 50 ng/ml LIF (**b**), 0.1 ng/ml CNTF and 1 ng/ml CNTF (**c**), respectively compared with the control. Ctrl: control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 3$



percentage of myogenin-positive cells was 26.31 ± 5.93% or 35 ± 3.84%, respectively (Fig. 5g–l), more lower than that of control group (50.93 ± 1.38%, $P < 0.05$). Western blot analysis also indicated that the 10 ng/ml LIF or 50 ng/ml CNTF treatment resulted in significant decrease in the expression levels of myogenic regulatory factors MyoD and myf5 compared with the control cells (Fig. 6).

Discussion

Activated muscle satellite cells were considered as myoblasts when cultured in vitro and these precursor cells are very useful for degenerative diseases. The chief problem during studies of

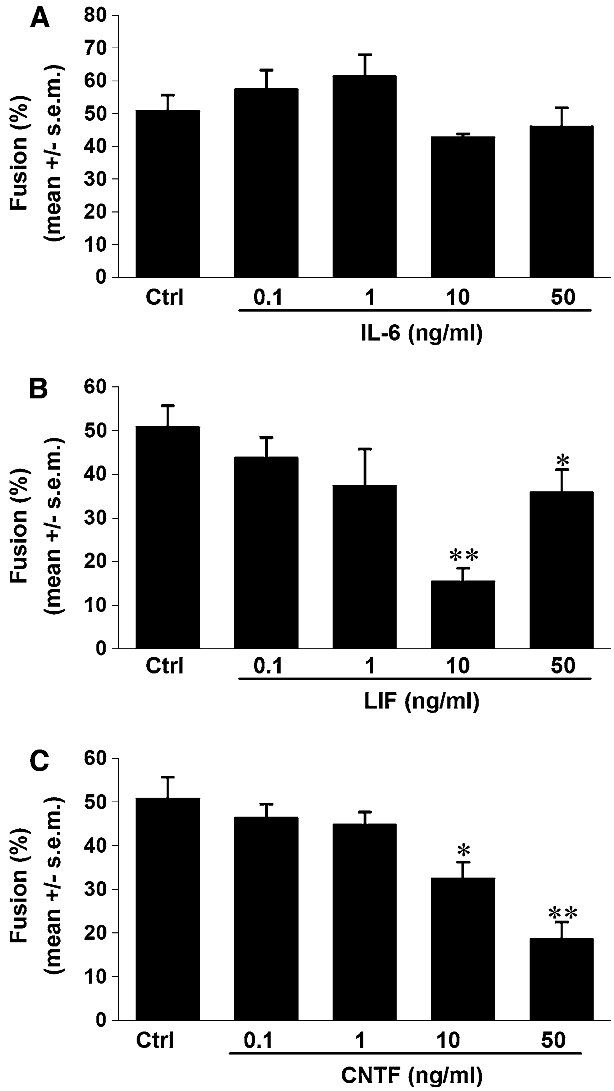


Fig. 3 The effects of different doses of IL-6, LIF, and CNTF on the membrane fusion ability of human myoblasts. IL-6 in any concentration could not influence on the fusion rate (**a**). Contrary to the control respectively, 10 ng/ml LIF, 50 ng/ml LIF (**b**), 10 ng/ml CNTF and 50 ng/ ml CNTF (**c**) inhibited the fusion, thereby inhibited the differentiation of human myoblasts into myotubes. Other concentrates of LIF and CNTF could not play any role in the myotube differentiation. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$

adult human myoblasts is that how to obtain homogeneous cell population without fibroblasts mixture (Rando and Blau 1994; Yablonka-Reuveni et al. 1987; Fukada et al. 2004; Qu-Petersen et al. 2002). In the present study, the desmin + monoclone of individual myoblasts was used for experiments, and these cloned cells were homogeneous, proliferated for a large scale and for a long time with preserving their myogenic lineage-committed phenotypes. In order to confirm the function of IL-6, LIF, and CNTF on the proliferation and differentiation of myoblasts, different concentration of three factors were added into the culture media. The results showed that among these cytokines, certain concentration of LIF could promote the cell proliferation, and certain concentration of LIF and CNTF could inhibit the cell differentiation into matured myotubes by inhibiting the expression of myogenic regulatory factors.

Contrary with those derived from rodent muscle tissues, cell proliferation ability of adult human myoblasts in our study was lower, which indicated that different genus derived myoblasts might have different cell growth checkpoint mechanism. Many experiments have confirmed that LIF, as an activator of human telomerase reverse transcriptase gene

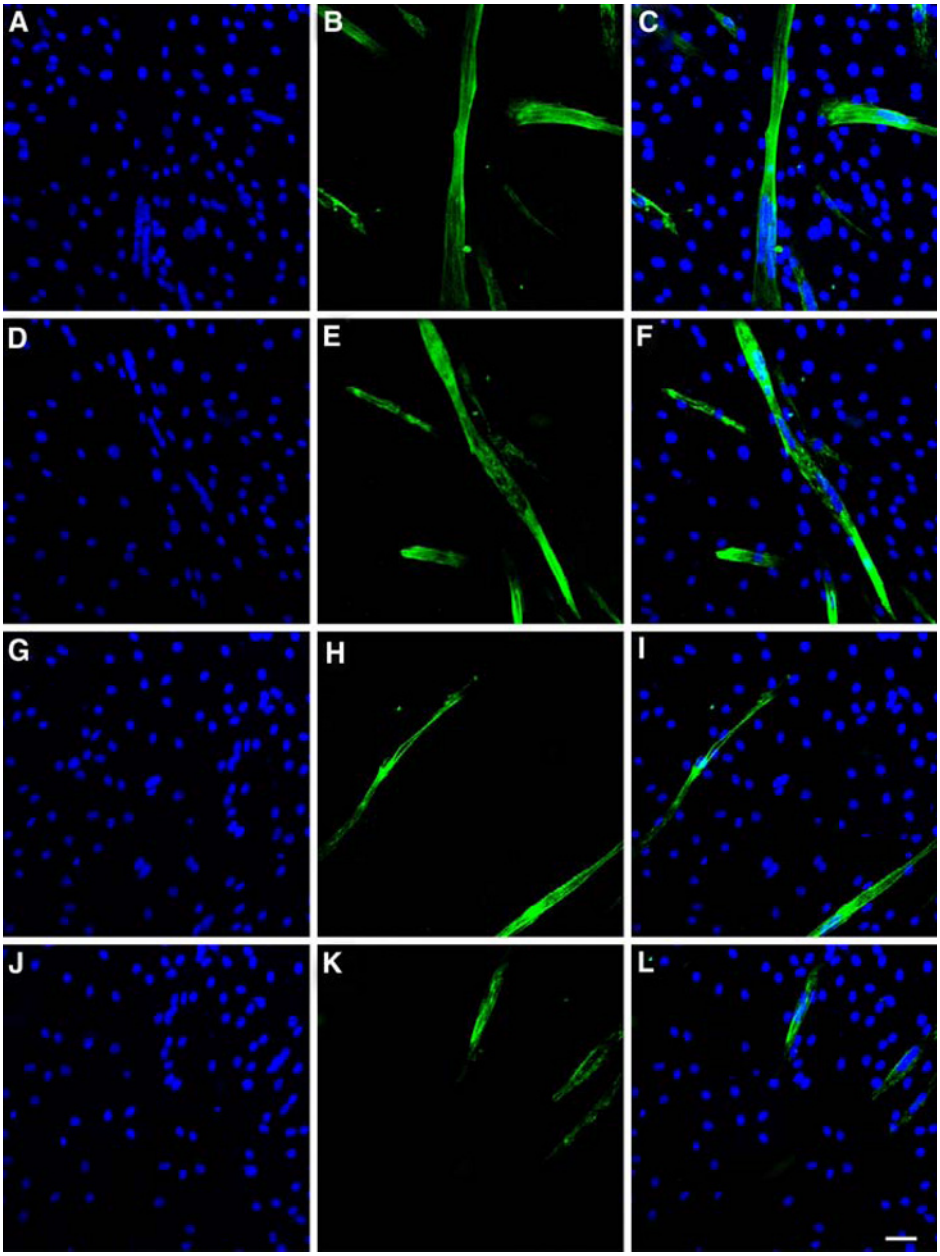


Fig. 4 IL-6 (10 ng/ml), LIF (10 ng/ml), or CNTF (50 ng/ml) on the myosin expression of differentiated human myoblasts. Contrary to the control (**a–c**), IL-6 did not influence on the myosin expression (**d–f**), while LIF (**g–i**) or CNTF (**j–l**) inhibited the myosin expression apparently. **a**, **d**, **g**, **j**: DAPI to stain the nuclear (blue), **b**, **e**, **h**, **k**: myosin (green), **c**, **f**, **i**, **l**: merge image. Bar = 10 μm

(Ostenfeld et al. 2000; Caldwell 2001), played a broad role in promoting the proliferation of kinds of stem cells such as embryonic stem cells or neural stem cells (Metcalf 2003; Uchida et al. 2000). Our results suggested that addition of LIF into the cultivation system of adult human myoblasts; it could resolve the problem of long-term proliferation and passage of cells.

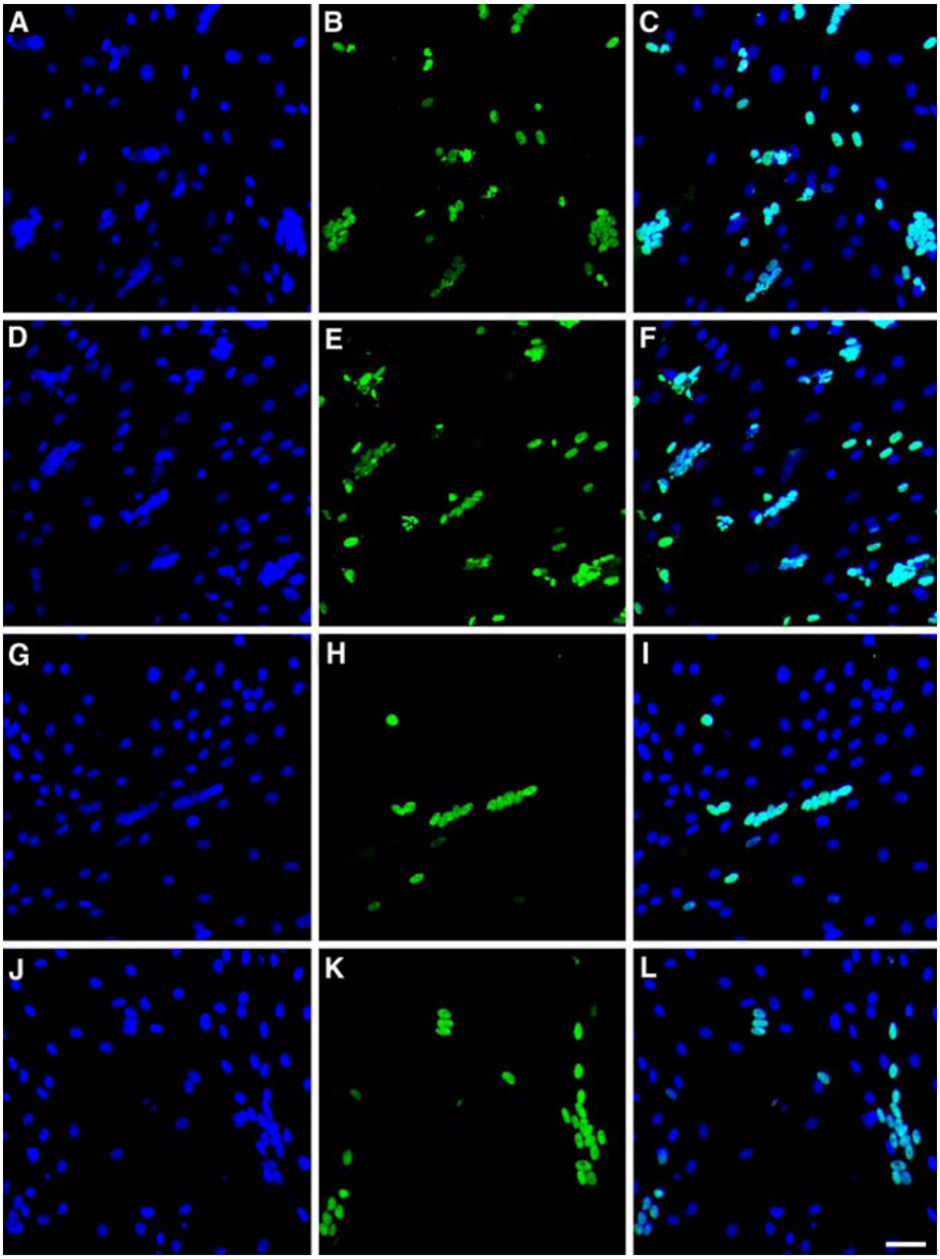


Fig. 5 IL-6 (10 ng/ml), LIF (10 ng/ml), or CNTF (50 ng/ml) on the myogenin expression of differentiated human myoblasts. Contrary to the control (**a**–**c**), IL-6 did not influence on the myogenin expression (**d**–**f**), while LIF (**g**–**i**) or CNTF (**j**–**l**) inhibited the myogenin expression apparently. **a**, **d**, **g**, **j**: DAPI to stain the nuclear (blue), **b**, **e**, **h**, **k**: myogenin (green), **c**, **f**, **i**, **l**: merge image (bright blue). Bar = 10 μm

Activation and differentiation of myoblasts are regulated by basic helix-loop-helix (bHLH) transcription factors—MyoD, myf5, myogenin, and MRF4, which determine the myogenic destiny of myoblasts (Seale and Rudnicki 2000; Sabourin et al. 1999; Arnold and Winter 1998). In the present experiment, when culture media containing the high level (20%) serum

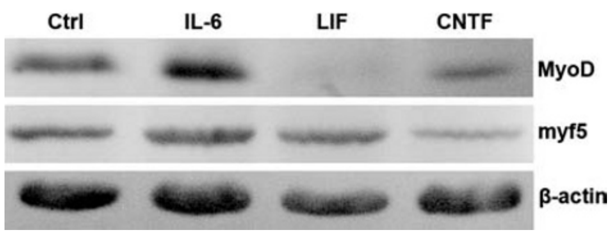


Fig. 6 MyoD and myf5 expression tested by Western blot analysis. IL-6 (10 ng/ml) did not inhibit the expression of MyoD and myf5, while LIF (10 ng/ml) inhibited the MyoD expression greatly and the myf5 expression lightly, and CNTF (50 ng/ml) inhibited the MyoD and myf5 apparently relative to the control

converting into low level (2%), membranes of myoblasts could fuse into multi-nuclei myotubes with expressing myogenic differentiation specific proteins myosin and myogenin. When the differentiating myoblasts were treated with different doses of IL-6, LIF, or CNTF, certain concentration of LIF or CNTF could inhibit the cell fusion, resulting in the less formation of multi-nuclei myotubes apparently. At the same time, the expression of myosin and myogenin sharply decreased, accompanied with the down-regulation of MyoD and myf5 expression. IL-6 did not affect on the differentiation of human adult myoblasts. These results indicated that during the differentiation regulation of human adult myoblasts, LIF and CNTF played the similar negative roles, whereas, IL-6 could not inhibit the differentiation and maturation of myoblasts. There have been other reports confirming that IL-6 may promote the differentiation and maturation procedure of myoblasts (Okazaki et al. 1996).

Presently, it has been very clear that IL-6, LIF, or CNTF play their biologic functions through combining with corresponding receptor compounds. The structures of receptor compounds of these three factors are very distinct. IL-6 receptor compound is a dimer consisting of IL-6 receptor and gp130, LIF receptor compound contains LIF receptor and gp130, and CNTF one contains CNTF receptor, LIF receptor, and gp130 (Heinrich et al. 1998). From the compositions of these receptor compounds, it is found that LIF receptor exists in the compounds of CNTF receptor, as well as LIF one (März et al. 2002), whereas default in the IL-6 receptor compounds. This suggested that LIF receptor might offer the most critical contribution to the differentiation inhibition of human adult myoblasts (Spangenburg and Booth 2002).

After combination with receptor compounds on the cell membrane, LIF, or CNTF activated inner-cellular signal transduction pathway, resulting in transcription and expression of target genes (Kami and Senba 2002). Our result had showed that LIF and CNTF could inhibit the expressions of MRFs—MyoD and myf5, therefore inhibiting the differentiation of human adult myoblasts into mature myotubes. Either LIF or CNTF possessed the ability of differentiation limitation on myoblasts, however, it seemed that the mechanism was slightly different. LIF inhibit the expression of MyoD mainly, and CNTF affected the myf5 expression. The reasons interpreting this disparity need to be investigated further. In addition to above interpretations on the differentiation inhibition efficacy of cytokines, the promoted proliferation by them followed by increasing cell number could not be ignored.

In conclusion, present studies demonstrated that IL-6, LIF, and CNTF, members of IL-6-type cytokine superfamily, were important regulation factors on the proliferation and differentiation of myoblasts derived from adult human skeletal muscles. Three factors promoted the cell growth, and certain doses of LIF or CNTF inhibit the myogenic differentiation. The different signal transduction procedures of three factors should be the next study focus in order to explore the molecular mechanisms of regulating the proliferation and differentiation of myoblasts.

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