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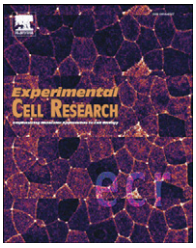

Research Article

Leukemia inhibitory factor-dependent increase in myoblast cell number is associated with phosphotidylinositol 3-kinase-mediated inhibition of apoptosis and not mitosis

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ABSTRACT

Leukemia inhibitory factor (LIF) is an important regulator of skeletal muscle regeneration and has been suggested to be mitogenic for myogenic cells because it has been shown to increase the quantity of myoblast cells grown in culture over extended periods of time. Using the established C2C12 murine myoblast cell line, we observed that LIF treatment did not significantly increase the rate at which myoblasts synthesise DNA under conditions which increased cell quantity by 73% above control, whilst the known mitogen fibroblast growth factor-2 significantly increased DNA synthesis under these conditions. Consequently, we examined the capacity of LIF to prevent apoptotic cell death. LIF treatment significantly reduced staurosporine-induced apoptotic DNA fragmentation by 37% compared to control and also reduced the proteolytic activation of caspase-3 by 40% compared to control. This effect of LIF was completely abolished by addition of the phosphatidylinositol 3-kinase inhibitor wortmannin, indicating that the phosphatidylinositol 3-kinase signalling pathway, previously shown to be linked to LIF-dependent increases in cell number, is necessary in mediating the anti-apoptotic effects of LIF. LIF treatment was also associated with increased levels of Bcl-xL and XIAP transcripts compared to control. Therefore, we suggest that the role of LIF in skeletal muscle regeneration and myogenesis is that of a survival factor rather than a mitogen.

Introduction

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine belonging to the interleukin-6 (IL-6) family of cytokines that share similar activities and receptors [1]. LIF is expressed in multiple tissues and involved in many biological processes, but its increased expression in dystrophic and injured skeletal muscle [2-4] indicates an important role in skeletal muscle regeneration. LIF is actively involved in regeneration of skeletal muscle, with LIF knockout mice showing a decrease in the area occupied by regenerating myofibres after crush injury compared to wild-type, which is restored by administration of exogenous LIF [5]. Administration of LIF to the site of crush injury in wild-type mice increased the area occupied by regenerating fibres with an associated increase in average myofibre diameter [2,5]. These original studies suggested that enhanced regeneration and increase in fibre size occurred, at least in part via stimulation of proliferation of the muscle forming myoblast cells, thus providing more cells to fuse to and increase the size of regenerating fibres.

Earliest descriptions of LIF as a possible mitogen for myoblasts suggested that LIF treatment increased the number of human and mouse derived primary myoblast cells present in culture in a

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dose-dependent manner after several days of culture, with the earliest increases noticeable after 6 days [6,7]. It was also shown that short periods of exposure to LIF (4 h) produced similar increases in cell number after 10 days compared to periods of exposure for all 10 days [7]. LIF binds to a heterodimer of the LIF receptor (LIFR) and gp130 receptor subunits [8], which leads to activation of multiple signalling pathways within the target cell including signal transducer and activator of transcription-3 (STAT3), phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) [9-11]. Although some of these signal transducers are shown to be activated shortly after LIF treatment (15–30 min), the increase in cell number is not observed until at least 48 h later [10]. This effect of LIF on cell number is consistently observed in myoblasts cultured under sub-optimal conditions where low serum concentrations are used (5% FBS) and frequently only after several days of culture [6,7,10,12]. Mitogens generate rapid responses [13]. That this effect occurs under conditions which are not optimal for cell viability and after extended periods of culture suggests that LIF may be increasing cell number not by increasing replication but by promoting survival of myoblasts.

There is evidence to suggest that LIF promotes survival of myoblasts and other cell types [14-16]. It has been demonstrated that LIF treatment maintained viability of primary myoblasts over long periods of culture under sub-optimal growth conditions [14]. Similarly, LIF enhanced the survival of rhabdomyosarcoma cell lines treated with etoposide [15]. LIF treatment was also capable of inhibiting doxorubicin induced apoptosis of cardiac myocytes via activation of PI3K [16], a pathway shown to be involved in LIF-dependent increases in myoblast cell number [10]. Administration of LIF in conjunction with myoblast transfer therapy showed an increase in dystrophin expression of *mdx* mice [17,18] and may be due to LIF promoting survival of the transferred myoblasts, which otherwise die within 24 h of injection in the host environment [19]. Therefore we hypothesise that LIF inhibits apoptosis of myoblasts and that this accounts for the enhanced viability and increased cell number previously observed.

Materials and methods

Cell culture and reagents

C2C12 myoblast cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Recombinant murine LIF was purchased from Millipore (Billerica, MA, USA). FGF-2 was obtained from Invitrogen (Carlsbad, California, USA). All reagents were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise stated.

MTT assay of cell number

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based assay was employed which is commonly used to measure cell viability or number. C2C12 myoblasts were seeded at a density of 700 cells per well in 96-well plates. Cells were incubated with concentrations of LIF (0.001–10 ng/mL) in sub-optimal growth media (5% (v/v) FBS in DMEM (Invitrogen, Carlsbad, California, USA) for up to 5 days. At each time point, the cultures were incubated with 20 μL of CellTiter96 AQUeous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) for 3 h at 37 °C. The absorbance was measured at 450 nm, which is representative of the relative number of cells in each well.

BrdU incorporation assay of DNA synthesis

C2C12 myoblasts were seeded at a density of 3000 cells per well in 96-well plates. Myoblasts were then treated with a range of LIF and FGF-2 concentrations in media containing 5% FBS or lacking serum for 24 h. For the final 2 h of treatment, cells were incubated with BrdU labelling reagent from Cell Proliferation ELISA, colour metric BrdU (Roche Applied Science, Basel, Switzerland) and the standard protocol followed. The substrate reaction was stopped with 1 M sulphuric acid and relative amount of BrdU incorporated was measured by the absorbance at 450 nm, which corresponds to the number of cells synthesising DNA over the 2-h period.

Active caspase-3 immunocytochemistry

C2C12 myoblasts seeded on 1% (w/v) gelatin coated glass cover slips were subjected to staurosporine and LIF treatments, as described in results, and subsequently fixed with 4% paraformaldehyde. Active caspase-3 was detected with Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb #9664 (Cell Signaling Technologies, Danvers, MA, USA) and AlexaFluor-488 donkey anti-rabbit IgG (Invitrogen, Carlsbad, California, USA) following the recommended standard protocol for immunofluorescence and visualised with standard fluorescein isothiocyanate (FITC) filters. Coverslips were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) mounted and visualised by fluorescence microscopy. Imaging and counting was conducted blind with a minimum of 400 nuclei counted per replicate.

DNA fragmentation assay

Myoblast cultures were seeded, treated as described above for caspase-3 assays and lysed in 96-well plates. DNA fragmentation in the lysates was assessed by sandwich ELISA for mono- and oligo-nucleosomal DNA fragments using Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Basel, Switzerland). The final absorbance was measured at 405 nm, which corresponds to the relative proportion of DNA fragments present.

Quantitative PCR assay

Total RNA was extracted from cultures grown in 6-well plates using SV Total RNA Isolation System (Promega, Madison, WI, USA). One microgram of RNA from each sample was used for reverse transcription with M-MLV Reverse Transcriptase, RNase H Minus (Promega, Madison, WI, USA). The cDNA synthesised was subjected to real-time quantitative PCR using SYBR Green qPCR Supermix UDG (Invitrogen, Carlsbad, California, USA) on a Stratagene Mx3000z real-time PCR unit. The derived cycle threshold (Ct) values were analysed using the relative expression software tool (REST) [20] and changes in regulation normalised to cyclophilin A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and statistical difference derived. Oligonucleotide primer