Insulin-like growth factor-I protects myoblasts from apoptosis but requires other factors to stimulate proliferation

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Abstract

Insulin-like growth factor-I (IGF-I) has been shown to stimulate myoblast proliferation for a limited time after which serum is required to reactivate IGF-I-stimulated myoblast proliferation. The aim of these studies was to determine whether IGF-I can stimulate myoblast proliferation and/or inhibit apoptosis alone or whether co-factors are necessary. This was achieved by investigating the proliferative response of L6 myoblasts to IGF-I and horse serum (HS) and by examining the status of cells in terms of cell number, substrate adherence, cell viability and DNA laddering following incubation with IGF-I and HS.

L6 myoblasts proliferate in response to IGF-I for 36 h; failure to respond to IGF-I after 36 h is not due to accumulation of waste products or lack of IGF-I. The addition of a low level (1% v/v) of HS restores the ability of myoblasts to proliferate in response to IGF-I and this supports the existence of a mitogenic competence factor. Furthermore, myoblasts failing to proliferate in response to

IGF-I after 36 h regain the capacity to respond to IGF-I for a further period of 36 h when exposed to fetal bovine serum.

Following the initial (36 h) phase of IGF-I-stimulated proliferation, removal of both IGF-I and HS led to a dramatic (60%) reduction in the number of cells fully attached to the culture vessel, with 60% of the completely detached cells dead. Agarose gel electrophoresis of extracts from these detached cells revealed higher levels of DNA laddering than extracts prepared from attached cells with IGF-I present. This suggests that IGF-I acts as a survival factor by protecting cells from apoptosis. In conclusion these experiments support the presence of a mitogenic competence factor in horse serum, which restores the ability of cells to proliferate in response to IGF-I. Unlike proliferation, protection against apoptosis is achieved by IGF-I or HS independently of each other.

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Introduction

Growth and development of muscle requires the proliferation, differentiation and fusion of myoblasts. Insulin-like growth factors (IGFs) are unique amongst growth factors in that they stimulate both proliferation and differentiation (Ewton & Florini 1981, Ballard *et al.* 1986) suggesting that other factors may determine the response of myoblasts to IGF.

McWade *et al.* (1995) reported in an abstract that L6A1 myoblasts proliferate in response to IGF-I but only for 36 h. They showed that cessation of proliferation was neither due to depletion of IGF-I nor to accumulation of waste products of cell metabolism secreted into the media. IGF-I-stimulated proliferation was restored by incubating cells with horse serum (HS) for 12 h. This activity, which they named mitogenic competence factor (MCF), also inhibited IGF-I-stimulated differentiation.

The IGFs are also known to function as survival factors in several cell types. D'Mello *et al.* (1993) have shown that IGF-I will protect cultured rat cerebella granule neurons from low potassium-induced apoptosis. Stewart and Rotwein (1996) were able to block cell death in IGF-II antisense clones of the C2 muscle cell line using des[1–3]IGF-I, IGF-I, IGF-II or insulin with a dose potency appropriate for activation of the IGF-I receptor. These latter workers were also able to demonstrate that IGF-I added to low-serum differentiation media led to a 25–50% increase in the number of adherent myoblasts.

The aim of these studies was to determine whether IGF-I can stimulate myoblast proliferation and/or inhibit apoptosis alone or whether co-factors are necessary. This was studied by investigating the proliferative response of L6 myoblasts to IGF-I and HS and by examining the status of cells in terms of cell number, substrate adherence and viability following incubation with IGF-I and HS in order

to determine the effect of IGF-I on proliferation and cell death of myoblasts.

Materials and Methods

Cell culture

L6 myoblast cultures (Yaffe 1968), maintained at subconfluence by regular passaging, were allowed to proliferate in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Ltd, Gaithersburg, MD, USA) buffered with NaHCO_3 (41.9 mmol/l) and gaseous CO_2 and containing phenol red (7.22 nmol/l) as a pH indicator. Foetal bovine serum (FBS, 10% v/v, Life Technologies Ltd, Auckland, New Zealand), penicillin (1×10^5 IU/l, Sigma, St Louis, MO, USA) and streptomycin (100 mg/l, Sigma) were routinely added to media. Cell culture flasks and microtitre plates were obtained from Nunc, Roskilde, Denmark. Recombinant human IGF-I was a generous gift from Ciba Geigy, Basel, Switzerland. Horse serum (HS) was obtained from Life Technologies Ltd, New Zealand. DMEM-FBS refers to buffered DMEM media containing FBS as described above, whereas DMEM refers to buffered media without FBS. Before treatment, cells were allowed to attach to the culture vessels overnight at 37 °C in DMEM-FBS media at a plating density of 15 243 cells per cm^2 (corresponding to 5000 per microtitre plate well). This cell density, considered to be substantially less than 10% of confluence, was selected because it was sufficiently low to allow subconfluent growth in positive control wells treated with DMEM-FBS over the course of the experiments. Following attachment and before the addition of treatment media, wells were thoroughly washed using 3 changes of pre-warmed DMEM media.

In experiments carried out following withdrawal of initial (36 h) IGF-I, the following procedure was adopted: cells were allowed to attach to flasks or microtitre plates overnight in DMEM-FBS. Media were washed from the cells using three changes of pre-warmed DMEM media and replaced with DMEM containing 100 ng/ml IGF-I. Cells were allowed to proliferate for 36 h at 37 °C, then media were washed from the cells using three changes of pre-warmed DMEM media. Test media were then added to the flasks or microtitre plates. In the case of the experiment designed to test the effect of adding back FBS following 36 h of IGF-I treatment, DMEM-FBS was added back for 16.25 h (i.e. for the same time that cells were exposed to DMEM-FBS during overnight attachment), media were then washed from the cells using three changes of pre-warmed DMEM media and DMEM with and without 100 ng/ml IGF-I was added back for up to 67 h at 37 °C.

Cell proliferation assay

Cell proliferation was determined by a modification of the method of Oliver *et al.* (1989). Briefly, growth media were

decanted, cells were washed in Dulbecco A phosphate buffered saline, (Oxoid Ltd, Basingstoke, Hants, UK; PBS) and then fixed for 30 min in 10% (v/v) formaldehyde in saline. The fixed cells were stained for 30 min with 10 g/l methylene blue in 0.01 M borate buffer (pH 8.5). Excess stain was removed by four sequential washes in borate buffer. Methylene blue was eluted from the cells by the addition of 100 μl 1:1 (v/v) ethanol/0.1 M HCl. The plates were gently shaken and optical density was determined at 655 nm using a microplate photometer (BioRad model 3550 microplate reader, BioRad, Hercules, CA, USA). A plot of absorbance versus cell number was found to be linear in the range of interest (4000 to 30 000 cells per cm^2 , Oliver *et al.* 1989).

Cell adherence

When cell suspensions were required for cell counting, cells were washed with PBS then stripped from the plastic surface using 0.25% trypsin (1:250 grade, Sigma Cell Culture Ltd, St Louis, MO, USA) in PBS. For this, cells were incubated at room temperature until they were observed by microscopic examination to begin to pull away from the surface of the flask; this took approximately 1 min. Trypsin was decanted from the flask and complete detachment of cells from the vessel surface allowed to proceed at 37 °C. This second incubation, also monitored microscopically, took 7–8 min. When a determination of the number of lightly attached cells was required, the second trypsin incubation was omitted, and the lightly attached cells decanted carefully from the surface of the flask. When a determination of the number of free floating cells was required no enzymatic digestion was carried out, media were carefully decanted and wells were washed with PBS with washings added to the decanted cells. Counting was performed using a haemocytometer.

Cell viability

Cell viability was determined using the trypan blue assay (Sigma Cell Culture Catalogue 1999).

Apoptosis assay

Sufficient cells for the determination of apoptosis were obtained by seeding 175 cm^2 flasks (Nunc) with L6 myoblasts at the same cell density as for microtitre plate wells in the other experiments, i.e. 15 243 cells per cm^2 in DMEM-FBS. This larger-scale cell production was required in order to generate sufficient cells in the 'IGF-treated combined lightly attached and floating populations' for the apoptosis assay. Following washing with pre-warmed DMEM to remove FBS, proliferation was allowed to proceed for 36 h in the presence of 100 ng/ml IGF-I in DMEM. Media were washed from the plates as before and cells were exposed to DMEM with or without

100 ng/ml IGF-I for a further 12 h at 37 °C. Apoptosis was determined in combined lightly attached and floating populations of cells and in fully adherent populations according to the method of Hermann *et al.* (1994). This method may underestimate the level of apoptosis, because cells which are in the G2/M phase of the cell cycle and about to divide will not be as adherent as those in other phases and may also lift off with the dying cells following short exposure to trypsin. Briefly, harvested cells (1×10^6) were washed with PBS and lysed using NP-40 detergent in Tris-HCl buffer, pH 7.5. RNA was digested using ribonuclease A (Boehringer Mannheim, Mannheim, Germany) and protein was digested using proteinase K (Boehringer Mannheim). The cell digest was loaded according to cell number only onto a 1% agarose gel, containing 200 ng/ml ethidium bromide and electrophoresed for 1 h using a constant voltage of 105 V. Bands of DNA were visualised using a Transilluminator (UVP Inc, Upland, CA, USA) and photographed using a Direct Screen Instant Camera (Polaroid, UK).

Data analysis

Microtitre plate well optical density values for each treatment were averaged and tested for differences using the Student's *t*-test. Methylene blue assay data is expressed as the mean of eight replicates obtained from the same microtitre plate within the one assay, and are representative of the results obtained from several assays.

Results

The effect of IGF-I and HS was examined in myoblasts to determine whether IGF-I can stimulate myoblast proliferation alone or whether co-factors are required.

Proliferation with IGF-I

The proliferative response of L6 myoblasts to IGF-I at 10, 100 and 1000 ng/ml was determined over 48 h following cell attachment to microtitre plates. Figure 1 shows that L6 myoblasts were dose responsive to IGF-I by 72 h ($P < 0.001$); however this trend was identified from 30 h. By 48 h, cell proliferation in response to IGF-I had stopped and cell numbers were very low compared with the positive control DMEM-FBS treatment, despite the fact that cell cultures remained subconfluent, the addition of further IGF-I did not restore proliferation and harvested conditioned media were equipotent with IGF-I in stimulating proliferation when added to naive cultures. DMEM alone was used as the negative control media in which no cell proliferation was observed.

Proliferation with horse serum

Horse serum promoted L6 myoblast proliferation in a dose-dependent fashion ($P < 0.001$, Fig. 2) and this was

Figure 1 L6 myoblast proliferation IGF-I dose-response curve. Results are means of eight replicate optical density (655 nm) measurements $\pm$ S.E.M. following staining of cells by methylene blue, and are representative of several assays. Symbols represent positive control media (DMEM-FBS) ($\bullet$), 1000 ng/ml IGF-I (∇), 100 ng/ml IGF-I ($\blacktriangledown$) and 10 ng/ml IGF-I ($\circ$). Mean negative control (DMEM alone) values have been subtracted from each value to obtain the 'Net' optical density value.

Figure 2 L6 myoblast proliferation horse serum dose-response curve. Results are means of eight replicate optical density (655 nm) measurements $\pm$ S.E.M. following staining of cells by methylene blue. Symbols represent positive control media (DMEM-FBS) ($\bullet$), and DMEM containing 10 ($\blacklozenge$), 5 ($\square$), 2 ($\blacksquare$), 1 (∇), 0.5 ($\blacktriangledown$) and 0.3% (v/v) ($\circ$) horse serum. Mean negative control (DMEM alone) values have been subtracted from each value to obtain the 'Net' optical density value.

maintained for 72 h, unlike IGF-I which no longer stimulated proliferation past 30 h. In future trials, 1% (v/v) HS was used because of its low effect on cell proliferation.

Proliferation following withdrawal of IGF-I for 36 h

Myoblasts remain essentially quiescent in the presence of either 100 ng/ml IGF-I or 1% HS alone (Fig. 3), but

Figure 3 The proliferative effect of IGF-I and horse serum either alone or in combination following withdrawal of initial (36 h) IGF-I from growth media. Results are means of eight replicate optical density (655 nm) measurements $\pm$ S.E.M. following staining of cells by methylene blue and are representative of several assays. Symbols represent proliferative response to DMEM media containing 100 ng/ml IGF-I (●), 1% (v/v) horse serum (○), 100 ng/ml IGF-I plus 1% (v/v) horse serum (▼) and DMEM alone (▽). L6 myoblasts were plated onto microtitre plates and allowed to attach overnight at 37 °C.

proliferate in the presence of both HS and IGF-I together ($P < 0.001$). When DMEM alone was added (without either HS or IGF-I) there was a dramatic reduction ($P < 0.001$) in cell numbers after 8 h, after which time cell numbers were stable. When cells were exposed again to DMEM-FBS for a time equivalent to the attachment time, and then had media removed, their capacity to respond to IGF-I was restored, but again only for 36 h ($P < 0.001$, Fig. 4).

Cell attachment, viability and apoptosis

To examine the reduction in cell numbers observed in Fig. 3 with DMEM culture media alone, viability, attachment status and apoptosis of myoblasts were measured.

Flasks of cells were raised specifically to study the change in attachment status and cell death induced by incubation with DMEM alone. After 8 h incubation with DMEM alone, approximately 46% of the cells were no longer firmly attached to the surface of the culture vessel (Table 1). Of the total cells present following this treatment, 13% had completely detached from the surface and were floating free in the media, 33% of the total cells were attached only very weakly and, as discussed in the Materials and Methods section, required minimal exposure to trypsin digestion in order to detach them from the surface. Of the 13% of cells that were floating free, 60% were dead as determined by the trypan blue assay.

Figure 4 The effect of a second exposure to fetal bovine serum on the capacity of L6 myoblasts to respond to IGF-I. Results are expressed as the means of eight replicate optical density (655 nm) measurements $\pm$ S.E.M. following staining of cells by methylene blue, and are representative of several assays. Following overnight attachment in positive control media (DMEM-FBS), L6 myoblasts were exposed for 36 h to DMEM-FBS (●) or to DMEM containing 100 ng/ml IGF-I (▼ and ○). Media were then washed from the wells (A), and DMEM-FBS added back to each treatment group for 16.25 h. Media were again washed from the wells (B) and DMEM-FBS (●) or DMEM with (▼) or without (○) 100 ng/ml IGF-I was added back for a further 67 h. Mean negative control (DMEM alone) values have been subtracted from each value to obtain the 'Net' optical density value.

Table 1 shows the cell numbers present following each treatment regime and is in agreement with the results presented in Fig. 3.

Apoptosis

Apoptosis was determined (see Fig. 5) in cell populations following 12 h treatment with DMEM alone or with 100 ng/ml IGF-I after withdrawal of the initial (36 h) IGF-I treatment. DNA laddering, as a measure of apoptosis, was much greater in combined floating and lightly attached populations of cells in the absence of IGF-I than in the other treatment groups.

Discussion

L6 myoblast proliferation in these studies was dose-responsive to IGF-I up to 48 h, confirming earlier work (Ballard *et al.* 1986, Ewton *et al.* 1987, Moore *et al.* 1993). Although myoblasts proliferated in response to IGF-I, they did so for only 30 to 36 h, regardless of concentration. This confirms the earlier findings in L6A1 myoblasts by McWade *et al.* (1995) who found that IGF-I stimulates myoblast proliferation for only 36 h.

L6 myoblast proliferation was dose-responsive to horse serum and this is supported by the findings of Allen *et al.*

Table 1 L6 myoblast cell numbers following different treatment regimes

	100 ng/ml IGF-I	1% (v/v) HS	100 ng/ml IGF-I + 1% (v/v) HS	DMEM alone
Population				
Firmly attached	152 133 (1555)	157 080 (0)	196 000 (0)	70 933 (1867)
Lightly attached	22 333 (1680)	23 000 (2000)	5667 (333)	43 040 (1144)
Floating	7667 (3333)	19 680 (4000)	1000 (0)	17 667 (10667)
Total	182 133 (5013)	199 760 (6000)	202 667 (333)	131 640 (13678)

Figures in parentheses represent the number of dead cells as determined using the trypan blue method. Cells were allowed to attach to the culture vessel overnight in the presence of DMEM-FBS. Media were washed from the cells, replaced with DMEM containing 100 ng/ml IGF-I, and incubation was allowed to proceed for 36 h after which the starting cell number was 177 333 per well. Media were washed from the cells and DMEM containing either 100 ng/ml IGF-I, 1% (v/v) HS, both IGF-I and HS, or neither was added and incubation allowed to proceed for 12 h. Cells were counted using a haemocytometer and 'Firmly attached', 'Lightly attached' and 'Floating' refer, respectively, to populations of cells that required long (8–9 min), short (1 min) or no exposure to trypsin to detach from the surface of the vessel.

(1982) for HS with chick embryo myogenic cells, White *et al.* (1988) for adult porcine serum with L6M1 myoblasts and Kotts *et al.* (1987) for swine serum with a clone of L6 myoblasts. We found that whereas IGF-I stimulated L6 myoblasts for 36 h, the cells remained responsive to HS for at least 48 h.

When both IGF-I and HS were added to cells that had stopped proliferating after an initial 36 h of IGF-I treat-

ment, proliferation was restored; however HS had little or no mitogenic activity in the absence of IGF-I in these cells. This is consistent with the findings of McWade *et al.* (1995), in which they describe an activity in HS as a mitogenic competence factor (MCF) which was able to restore IGF-I-stimulated proliferation of L6A1 myoblasts, but which had no mitogenic activity on its own. When neither IGF-I nor HS were added back to the L6 cells a dramatic decline in cell numbers was identified using the methylene blue assay as well as by direct cell counting and no mention was made of this finding in McWade and colleagues' 1995 abstract. Stewart and Rotwein (1996), however, using direct counting of stably transfected IGF-II antisense C2 cells, reported a similar effect when they changed from 10% FBS-containing media to differentiation media (containing 2% HS). They were able to arrest the rapid decline in the number of adherent, viable myoblasts using either IGF-I or cycloheximide. Clearly IGF-I in some way protects the cells from a decline in number. In our studies we were also able to arrest the decline using HS.

Our results clearly show that myoblasts require some factor or factors that are to be found in horse serum in order to proliferate in response to IGF-I. It appears from first sight that IGF-I alone is able to stimulate cell proliferation for the first 36 h. This would indicate that (i) actively growing cells do not require a competence factor to proliferate in response to IGF-I, (ii) that sufficient of this factor is taken up from FBS at the time of attachment to last for 36 h or (iii) that FBS at attachment moves cells into the cell cycle where they can respond to the proliferative signal of IGF-I. Following 36 h of serum starvation, the addition of HS may serve to move the cells back into the cell cycle where they can respond to IGF-I. This effect of serum is likely to be multi-factorial. MCF has been described as being distinct from transforming growth factor- β , platelet-derived growth factor-BB, transferrin, known IGF binding proteins, fibroblast growth factor, fetuin or a general effect of proteins (McWade *et al.* 1995, 1997).

Figure 5 1% Agarose gel showing DNA fragmentation as a determination of apoptosis. Lanes show DNA from cells treated as follows: lane 1, DMEM (combined lightly attached and floating populations); lane 2, DMEM (fully attached cells); lane 3, IGF-I (combined lightly attached and floating populations); lane 4, IGF-I (fully attached cells); lane 5, marker (1 kb plus ladder from Life Technologies Inc, Gaithersburg, MD, USA). The gel contained ethidium bromide to allow visualisation of DNA bands.

Agarose gel electrophoresis established that the combined population of floating and lightly attached cells underwent significant apoptosis compared with fully attached cells as determined by the characteristic laddering profile. When taken together with the fact that IGF-I protects cells from declining in number past 36 h, this suggests that IGF-I protects the cells from apoptosis. There appears to be two populations of cells, as initially there is a significant loss of cells up to 8 to 12 h after removal of IGF-I from the media; after that time the cells are quiescent but do not die, and proliferation can be restored if the cells are stimulated with FBS. There may be different cell types, or cells that are at different stages of the cell cycle within the L6 cultures. Apoptosis under conditions of substrate detachment is consistent with the findings of Stewart and Rotwein (1996) who showed that apoptosis in C2 myoblasts could be arrested with IGF-I, des[1-3]IGF-I, IGF-II or insulin, with a dose potency consistent with the activation of the type 1 IGF receptor. In addition to this work, other investigators have reported IGF-I as protecting other cultured cell lines from apoptosis (Rodriguez-Tarduchy *et al.* 1992, Muta & Krantz 1993, Sell *et al.* 1995).

While we did detect apoptosis in combined lightly attached and floating populations following IGF-I treatment, the total number of cells in this treatment group was very low.

This study has shown that IGF-I does require a co-factor to stimulate proliferation of myoblasts under specific circumstances. Whether a co-factor is always required for maximum stimulation of proliferation is not known; however a reduced external co-factor requirement in early proliferation is at least indicated from our results. IGF-I-mediated myoblast survival is not necessarily associated with proliferation. This is in agreement with Harrington *et al.* (1994) who have shown a separation of function for IGF-I in fibroblasts. Our results clearly show that IGF-I-stimulated proliferation and cell survival can be separated and that proliferation requires another factor or factors. In conclusion we have described two distinct actions for IGF-I. IGF-I stimulates proliferation of subconfluent L6 myoblasts in the presence of a mitogenic competence factor and it is also able to inhibit apoptosis on its own.

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