FEBS Letters 522 (2002) 156–160 FEBS 26223

Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation

Shi Yu Yang, Geoffrey Goldspink*

Molecular Tissue Repair Unit, Department of Surgery, Royal Free and University College Medical School, University College London, Rowland Hill Street, London NW3 2PF, UK

Received 14 March 2002; revised 7 May 2002; accepted 27 May 2002

First published online 10 June 2002

Edited by Veli-Pekka Lehto

Abstract The physiological function of a recently cloned splice variant of insulin-like growth factor-I (IGF-I; mechano growth factor (MGF)) was studied using an in vitro cell model. Unlike mature IGF-I, the distinct E domain of MGF inhibits terminal differentiation whilst increasing myoblast proliferation. Blocking the IGF-I receptor with a specific antibody indicated that the function of MGF E domain is mediated via a different receptor. The results provide a basis for localized tissue adaptation and helps explain why loss of muscle mass occurs in the elderly and in dystrophic muscle in which MGF production is markedly affected.

Key words: Insulin-like growth factor-I splice variant; Muscle differentiation; Proliferation; C2C12 myoblast

1. Introduction

Insulin-like growth factor-I (IGF-I) has been shown to be involved in several cellular processes including tissue differentiation [1], maintenance of tissue mass [2] and tissue repair [3]. However, the IGF-I gene is spliced differently in response to different signals. Different peptides resulting from this alternatively splicing have been reported to have different modes of action [4].

Although the mature systemic type of IGF-I is a simple 70 amino acid peptide, its gene is unexpectedly large spanning a region of over 90 kb of genomic DNA. Alternative splicing of IGF-I gene results in different transcripts encoding several IGF-I precursor proteins. Two of them produced by active muscle in rodents are positive regulators of muscle hypertrophy [5,6]. One of these, IGF-I Ea, has been given various names including muscle liver-type IGF-I [5] and muscle IGF [7]. The other isoform, which is the IGF-I Eb in rodents, corresponds to IGF-I Ec in human, and is only markedly up-regulated in exercised and damaged muscle. Therefore it has been named mechano growth factor (MGF) [4,6]. Although it has been known that IGF-I gene gives rise to different peptides, the differences in their physiological function have not been investigated.

Mammalian skeletal muscle differentiation is a useful model system for studying the molecular mechanisms that switch the cellular program from proliferation to differentiation. The murine C2C12 cell line is a pure, immortalized mouse skeletal muscle cell line, the cells of which are morphologically similar to primary myoblasts [8–10] and can be grown without contamination from other cell types. When grown to cellular confluence and deprived of certain growth factors, C2C12 myocytes enter the terminal differentiation pathway and begin to fuse to form myotubes and express muscle-specific proteins. This differentiation process is apparently regulated by several growth factors including IGF-I. However, IGF-I is unique in that it stimulates both proliferation and differentiation of skeletal muscle cells in culture [11].

The aim of this study is to use murine C2C12 skeletal muscle cell line to investigate potential physiological function of IGF-I Ea and MGF as well as their interactions by determining the mitotic index and creatine kinase (CK) expression as a marker of terminal differentiation [12,13].

2. Materials and methods

2.1. Cell culture and transfection

Proliferating C2C12 mouse myoblasts were maintained and passaged as subconfluent monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, plus 0.5% ampicillin and gentamicin (GM) at 37°C, with 5% CO_2. MGF and IGF-I Ea cDNAs [4] were inserted in frame with the N-terminal His tag and the enterokinase site downstream of the human cytomegalovirus promoter of the pcDNA 3.1/His vector (Invitrogen). Following digestion with *Nro*I (208 bp) and *Bst*II071 (3319) the backbone of the pcDNA3.1/His Vector containing the ampicillin resistance and the Co1EI site was removed. The remaining sequence containing the MGF or L.IGF-1 cassette was used in stable transfection of C2C12 mouse muscle myoblast cell line (European Collection of Cell Cultures) with Superfect Transfection Reagent (Qiagen). The stable expression of MGF or IGF-I Ea clones was grown in a selective medium containing neomycin (G418) for 4 weeks.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to test which C2C12 cell clones positively expressed MGF or IGF-I Ea, the total cellular RNA was prepared from cells transfected with MGF, IGF-I Ea and non-transfected C2C12 cells using a total RNA isolation kit (Promega). The sequences of the primers used were: forward primer is 5′-TAAGGTACCCGGACCGGAGACGCTCTGCGGTGCT-3′; and reverse primer is 5′-TCAGTCTAGAATGTTTACTTGTGTATTTCATTGG-3′. As both of the primers included part of the vector and part of the insert, only the mRNA of the introduced gene was amplified and not the endogenous MGF and IGF-I Ea cDNA in the transfected C2C12 cells. Using these primers a RT-PCR was performed.

2.3. MGF peptide synthesis

A predicted peptide resulting from the IGF-I Ec [14] was synthesized by Applied Biosystems (Eurogentec, Belgium) using standard solid-phase methodology and purified to $>95\%$ by HPLC. Validation of synthetic peptide was accomplished by total amino acid composi-

*Corresponding author. Fax: (44)-20-7830 2917.
E-mail address: g.goldspink@rfc.ucl.ac.uk (G. Goldspink).

PII: S0014-5793(02)02918-6

tion and HPLC. The sequence of the synthetic peptide was NH_2-Try-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-His-Lys-COOH.

2.4. Analysis of CK activity

Cells were passaged and maintained in 12-well plates in GM medium until 70% confluent (3 days). They were then induced to differentiate in Dulbecco's modified Eagle's medium supplemented with 2% horse serum, plus 0.5% ampicillin and gentamicin (DM). At the indicated times, cells in DM medium were washed three times with PBS, lysed by incubating with reporter lysis buffer (Promega) for 15 min at room temperature, and harvested by scraping. The concentration of total protein was determined by BCA protein assay (Sigma). CK activity was determined using the creatine phosphokinase assay kit (Sigma Diagnostics). Briefly, 0.1 ml of cell lysate was reacted with phosphocreatine and ADP–glutathione for 30 min at 37°C. The reaction was stopped by adding *p*-hydroxymercuribenzoate and the color reaction was induced by addition of α-naphthol and 0.05% diacetyl. Spectrophotometric reading was taken at 520 nM, and CK units were determined from a calibration curve generated by creatine standards. All data points represent the means of triplicate determinations from two repeated independent experiments.

2.5. Cell proliferation assay

In order to determinate the extent of cellular proliferation, the incorporation of 5-bromo-2′-deoxyuridine (BrdU) was quantified using BrdU labelling and detection kit (Roche) as previously described [15]. Briefly, 5000 cells were cultured with GM in 96-well microtiter plates at 37°C under 5% CO_2. At indicated times after plating, 10 μM BrdU was added into each well. The cells were continuously cultured under the same conditions for 2 h; during which time BrdU was incorporated into freshly synthesized DNA. Following fixation of cells, cellular DNA was partially digested by nuclease treatment. The incorporated BrdU was detected and quantified by the peroxidase-labelled antibody to BrdU. The peroxidase catalyzes the cleavage of the substrate which produces a color reaction. The absorbance at λ 405 nm is directly correlated to the level of BrdU incorporated into cellular DNA was measured using a microplate reader (Bio-Rad) and indicated the extent of myoblasts proliferation. All data points represent the means of eight measurements from four repeated experiments on each cell clone.

2.6. Investigation of proliferation mechanism of MGF action on C2C12 cells

Normal C2C12 cells were plated (each well containing 5000 cells) in 96-well microtiter plates and maintained in GM for 3 days. Cells were then placed in serum-free medium for 12 h before they were exposed to different concentrations of MGF peptide in serum-free Dulbecco's modified Eagle's medium containing 100 μg/ml of BSA for 24 h. For the purpose of comparison, a mature human IGF-I peptide (Pharmacia) was included in the experiments. In order to investigate pathway though which the MGF act on in C2C12 myoblast, a specific anti-IGF-I receptor (Ab-1) antibody (Oncogene) was included in medium at the same time when cells were exposed to different concentration of MGF or IGF-I peptide. The concentration of the antibody (1000 ng/ml) was used according to the manufacturer's recommendation. All the measurement was repeated four times using different plates and eight wells were used for each concentration of the growth factor.

2.7. Statistical analysis

ANOVA was used to analysis the significance of the differences in the means for CK activity, and the rate of incorporation of BrdU in transfected clones and treated cells. In the figures the mean values are expressed as means ± S.E.M.

3. Results

3.1. MGF inhibits terminal differentiation of C2C12 cells

Two of MGF-positive C2C12 clones and two IGF-I Ea clones were used to study the effects of MGF and systemic IGF-I Ea on myoblast proliferation and differentiation. Fig. 1 demonstrates that positive MGF and IGF-I Ea C2C12 clones expressed transfected MGF or IGF-I Ea mRNA, whilst no exogenous MGF mRNA was detected in control C2C12 cells. When cells were induced to undergo myogenesis in the differentiation medium (DM), it was found that positive terminal differentiation was prevented in MGF-positive cells. Even these cells kept in DM for 14 days, remained as myoblasts (Fig. 2A). In contrast, those of the IGF-I Ea-positive clone formed myotubes (Fig. 2B). The normal C2C12 cells showed less cellular proliferation as well as forming myotubes (Fig. 2C).

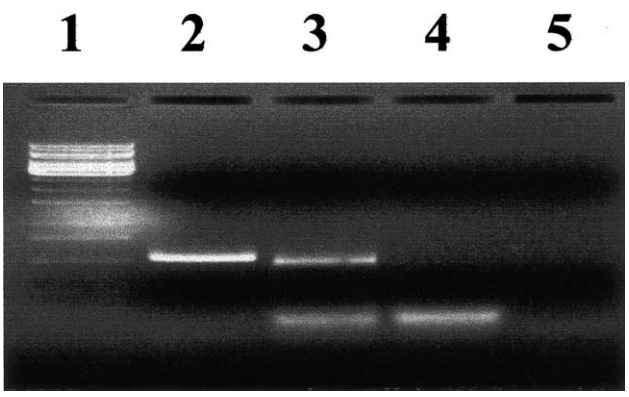

Fig. 1. Detection of the introduced MGF or IGF-I Ea mRNA in MGF- or IGF-I Ea-transfected cell clones. The results showed that in the MGF-positive cells (lane 2) transfected MGF mRNA and in IGF-I Ea-positive cells (lane 3) transfected IGF-I Ea mRNA was detected using the specific primers. These primers did not detect any endogenous MGF or IGF-I Ea mRNA in control C2C12 cells as shown in lane 4. Lane 1 is a 1 kb DNA ladder; lane 5 is a non-cDNA negative control.

In order to further test that the inhibition of cell differentiation was due to the effects of MGF, normal C2C12 cells were treated with either synthetic MGF peptide or the medium harvested from MGF-transfected cells. Fig. 2 shows that both synthetic (D) and MGF condition medium (E) inhibited cell differentiation. It is interesting to note that this inhibition was reversed when the synthetic MGF peptide (G) or MGF conditional medium (H) was withdrawn and the cells started fusing and forming myotubes in a similar way to untreated C2C12 cells (F).

Fig. 3 shows that the induction of CK activity was inhibited ($P < 0.001$) in MGF-positive cell clones and that similar results were obtained when normal C2C12 cells were treated with MGF peptide.

3.2. MGF increases C2C12 cell proliferation

Cellular proliferation of MGF-positive clones was analyzed using BrdU incorporation assay. The incorporated BrdU was measured at 12, 24, 36 and 48 h after plating. There was a significant increase in BrdU uptake in MGF cells at 24, 36 and 48 h after plating comparing to the non-transfected C2C12 cells (Fig. 4). IGF-I Ea also increased BrdU uptake into new synthetic DNA comparing to normal cell after 24 h of plating but it is not quite as effective as MGF in stimulating cell proliferation.

3.3. MGF increases cell proliferation but not via the IGF-I receptor

Fig. 5 shows that MGF stimulated normal C2C12 cells proliferation in a dose-dependent manner (A). Interestingly, this stimulation was not inhibited by blocking IGF-I receptors

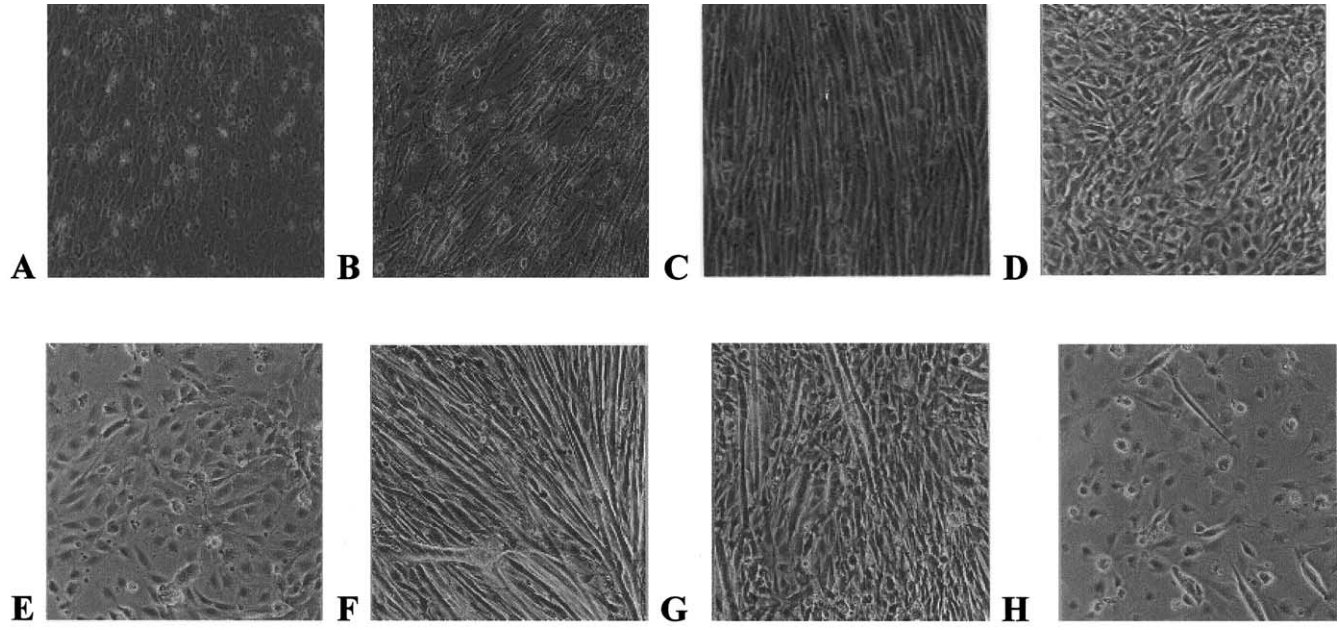

Fig. 2. Cell profile of MGF-positive (A), IGF-I Ea-positive (B) and normal control C2C12 (C) cells grown in differentiation media for 14 days. This demonstrates that MGF-positive cells stayed as mononuclear cells whilst almost all normal control C2C12 cells had by 14 days fused to form multinuclear myotubes. In the IGF-I Ea-positive clone, most cells had already fused and formed myotubes, but some were still in the myoblast state. To confirm the inhibition of myoblast differentiation by MGF, normal C2C12 cells were treated with either synthetic MGF peptide or the medium harvested from MGF-transfected cells (ratio 1:1). When MGF peptide (100 ng/ml) was added to the differentiation medium (D) or the medium from the MGF-transfected clone was added in a 1:1 ratio (E), the normal C2C12 cells lost their capability to form myotubes. When the MGF peptide (G) or MGF-transfected conditional medium (H) were withdrawn from the above differentiation medium after 24 h, the cells again began to fuse and formed myotubes in a similar way to untreated C2C12 cells (F).

(B) using an anti-IGF-I receptor (Ab-1) antibody (Oncogene) which has been used previously in similar studies. On the other hand, IGF-I also increased normal cells proliferation (Fig. 6A). This stimulation was blocked by the IGF-I receptor antibody (Fig. 6B). The results strongly suggest that MGF inducing C2C12 myoblast proliferation was not via the type 1 IGF-I receptor and involves a different signalling pathway.

4. Discussion

Several growth factors are initially synthesized as pro-peptide precursors that are post-translationally processed to pro-

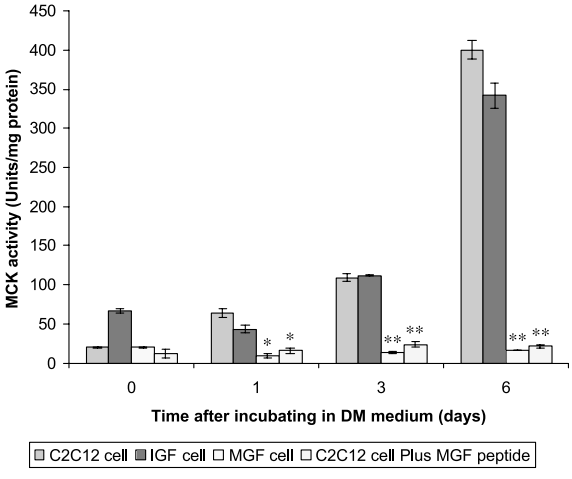

Fig. 3. Muscle CK (MCK) activity in normal C2C12 cells, IGF-I Ea- and MGF-positive cells and normal C2C12 cells plus the MGF peptide (100 ng/ml) for up to 6 days incubation in differentiation medium (DM). All data points represent the means of enzymatic activity determinations carried out in triplicate from two duplicate experiments. Values shown are the mean ± S.E.M. for $n = 6$. The lack of MCK as a marker of terminal differentiation was very significant in MGF-transfected cells and those treated with MGF peptide (ANOVA $P < 0.001$ when comparing MGF clone and MGF treated cells with control IGF-I Ea clone and control C2C12 cells).

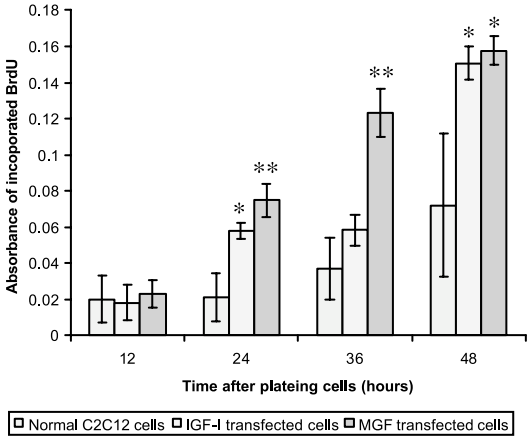

Fig. 4. The comparison of the number of cells in a proliferative state (determined by incorporation of BrdU shown as absorption at 405nm) within MGF-transfected, IGF-I Ea-transfected and control C2C12 cells at 12, 24, 36 and 48 h after plating. The results showed that after 24 up to 48 h of plating MGF-transfected cells exhibited significantly higher proliferation compared to normal control C2C12 cells ($P < 0.001$). Although the rate of proliferation in IGF-I Ea-transfected cells is about twice time as high as the control cells ($P < 0.01$), it is not as high as in MGF-transfected cells. Values shown are the mean ± S.E.M.

duce multi-peptides with distinct biological activities. Examples include pro-insulin [16], pro-EGF [17], pro-TGF (transforming growth factor) [18] and pro-glucagon [19]. Because of alternative splicing of 3′-exons, there are different pro-IGF-I precursors within human tissue including IGF-I Ea [20], IGF-I Eb [21] and IGF-I Ec [14]. It has been suggested that the E peptides of these pro-IGF-I precursors may function as independent growth factors [22]. However, the physiological functions of the E domain had not been studied. This study demonstrated that E peptide of the splice variant, MGF [4,6], is biologically active and has a distinct activity compared to that of mature IGF-I in that it can increase myoblasts proliferation but it totally inhibits the myotubes formation. Also the selective blocking of the IGF-I receptor provides evidence that MGF increases myoblast proliferation via a different signalling pathway.

The growth and regeneration of postnatal and adult skeletal muscle involves mononucleated cells called satellite cells or residual myoblast. In the quiescent state they reside between the basement membrane and plasma membrane of myofibers.

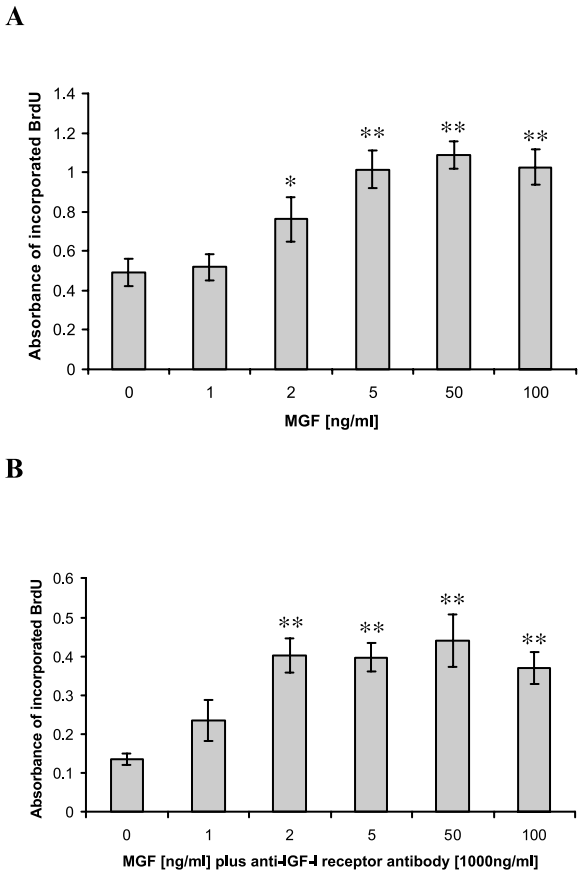

Fig. 5. Cell proliferation data in response to MGF treatment after blocking the IGF-I receptor. Shown in (A) is the dose-independent response of normal C2C12 cell proliferation (indicated by the absorbance of incorporated BrdU) to different concentrations of MGF peptide in serum-free medium (A). Maximum cell proliferation was obtained when the concentration of MGF reached to 5 ng/ml, which was significant between treated and control cells ($P < 0.001$). When anti-IGF-I receptor antibody was included in the medium (B), this dose-independent response was not affected, indicating that the increasing myoblast proliferation function of MGF was not via the IGF-I receptor. Values shown are the mean ± S.E.M.

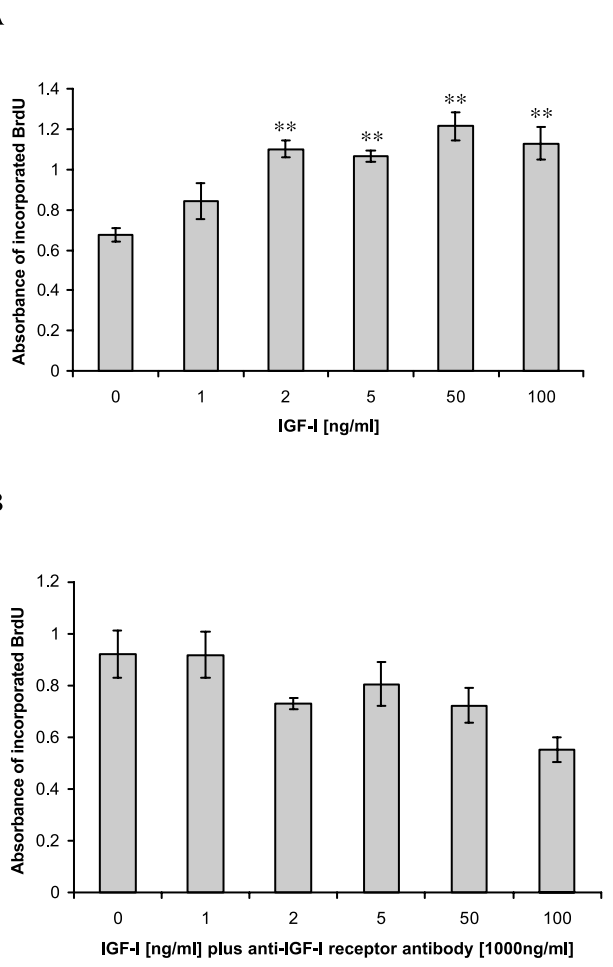

Fig. 6. The data for IGF-I treatment on cell proliferation after blocking the IGF-I receptor. Shown in (A) is the dose-independent response of normal C2C12 cell proliferation (indicated by the absorbance of incorporated BrdU) to different concentrations of mature IGF-I in serum-free medium. When a neutralization anti-IGF-I antibody was included in the medium (B), this dose-independent response disappeared indicating that the myoblast proliferation function of mature IGF-I is indeed via IGF-I receptor. Values shown are the mean ± S.E.M.

They are activated in response to diverse stimuli, including stretching, exercise, injury, and electrical stimulation [23]. Satellite cell activation as measured by specific marker appear to change in association with MGF level of expression [24]. Several growth factors such as fibroblast growth factor, TGF-β and IGF have been implicated to the proliferation, fusion and differentiation of skeletal muscle satellite cell [25]. MGF, one isoform of IGF-I, was shown in this study to stimulate myoblasts proliferation but depress differentiation which appears to play a role in this process, particularly as MGF is expressed before IGF-I following muscle damage as the increase in active in that nuclei is a pre-request for local tissue repair and adaptation. Although the myoblasts used in this study are from a cell line it is most likely that they respond in the same manner to MGF and IGF-I Ea as the residual myoblasts in adult muscle in vivo.

Skeletal muscle regenerative capacity has been shown to decline with age [26,27]. As the satellite cell population within skeletal muscle was not significantly lower in the older comparing to young men and women [28]. This incapacity to

regenerate in aged muscle seems to be due to decreased proliferation ability of satellite cells [29]. Considerably lower expression of the local MGF in older muscles in response to mechanical overload has been associated with the failure to activate satellite cells [24]. The capacity of MGF increasing myoblast proliferation shown in this study helps to explain the muscle loss that occurs during ageing.

In contrast to normal muscle, the mRNA for MGF is not detectable in dystrophic muscles even when subjected to stretch and/or electrical stimulation [30]. Decreased numbers of proliferative cells were found in dystrophic muscle following muscle damage [31]. This indicated that in dystrophic muscles myogenic satellite cells cannot undergo self-renewal, i.e. the rate of proliferation is too low for effective muscle repair. Further studies are in progress to investigate the signalling pathways involved in muscle mass maintenance and repair.

Acknowledgements: We thank Miss Kirsty Minton for her technical help. This work was supported by grants from Wellcome Trust and European Committee in relation to sarcopenia and muscle degeneration in COPD.

References

[1] Tureckova, J., Wilson, E.M., Cappalonga, J.L. and Rotwein, P. (2001) J. Biol. Chem. 276, 39264–39270.
[2] Yang, S.Y., Alnaqeeb, M., Simpson, H. and Goldspink, G. (1997) J. Anat. 190, 613–622.
[3] Coerper, S., Wolf, S., von Kiparski, S., Thomas, S., Zittel, T.T., Ranke, M.B., Hunt, T.K. and Becker, H.D. (2001) Scand. J. Gastroenterol. 36, 921–927.
[4] Yang, S.Y., Alnaqeeb, M., Simpson, H. and Goldspink, G. (1996) J. Musle Res. Cell Motil. 17, 487–495.
[5] Goldspink, G. (1999) J. Anat. 194, 323–334.
[6] McKoy, G., Ashley, W., Mander, J., Yang, S.Y., Williams, N., Russell, B. and Goldspink, G. (1999) J. Physiol. 516, 583–592.
[7] Musaro, A., McCullagh, K., Paul, A., Houghton, L., Dobrowolny, G., Molinaro, M., Barton, E.R., Sweeney, H.L. and Rosenthal, N. (2001) Nat. Genet. 27, 195–200.
[8] Yaffe, D. and Saxel, O. (1977) Nature 270, 725–727.
[9] McMahon, D.K., Anderson, P.A., Nassar, R., Bunting, J.B., Saba, Z., Oakeley, A.E. and Malouf, N.N. (1994) Am. J. Physiol. 266, 795–802.
[10] Palmer, R.M., Thompson, M.G., Knott, R.M., Campbell, G.P., Thom, A. and Morrison, K.S. (1997) Biochim. Biophys. Acta. 1355, 167–176.
[11] Florini, J.R., Ewton, D.Z. and Coolican, S.A. (1996) Endocr. Rev. 17, 481–517.
[12] Cheng, Z.Q., Adi, S., Wu, N.Y., Hsiao, D., Woo, E.J., Filvaroff, E.H., Gustafson, T.A. and Rosenthal, S.M. (2000) J. Endocr. 167, 175–182.
[13] Osses, N. and Brandan, E. (2002) Am. J. Physiol. Cell Physiol. 282, 83–94.
[14] Chew, S.L., Lavender, P., Clark, A.J. and Ross, R.J. (1995) Endocr. 36, 1939–1944.
[15] Li, M., Chan, K., Cai, D., Leung, P., Cheng, C., Lee, K. and Lee, K.K. (2000) Arch. Biochem. Biophys. 384, 263–268.
[16] Ido, Y., Vindigni, A., Chang, K., Stramm, L., Chance, R., Heath, W.F., DiMarchi, R.D., Di Cera, E. and Williamson, J.R. (1997) Science 277, 563–566.
[17] Lakshmanan, J., Salido, E.C., Lam, R., Barajas, L. and Fisher, D.A. (1990) Biochem. Biophys. Res. Commun. 173, 902–911.
[18] Sha, X., Yang, L. and Gentry, L.E. (1991) J. Cell Biol. 114, 827–839.
[19] Bell, G.I., Sanchez-Pescador, R., Laybourn, P.J. and Najarian, R.C. (1983) Nature 304, 368–371.
[20] Jansen, M., van Schaik, F.M., Ricker, A.T., Bullock, B., Woods, D.E., Gabbay, K.H., Sussenbach, J.S. and Van den Brande, J.L. (1983) Nature 306, 609–611.
[21] Rotwein, P. (1986) Proc. Natl. Acad. Sci. USA 83, 77–81.
[22] Siegfried, J.M., Kasprzyk, P.G., Treston, A.M., Mulshine, J.L. and Quinn, K.A. (1992) Proc. Natl. Acad. Sci. USA 89, 8107–8111.
[23] Seale, P., Asakura, A. and Rudnicki, M.A. (2001) Dev. Cell 1, 333–342.
[24] Owino, V., Yang, S.Y. and Goldspink, G. (2001) FEBS Lett. 505, 259–263.
[25] Husmann, I., Soulet, L., Gautron, J., Martelly, I. and Barritault, D. (1996) Cytokine Growth Factor Rev. 7, 249–258.
[26] Sadeh, M. (1988) J. Neurol. Sci. 87, 67–74.
[27] Carlson, B.M., Dedkov, E.I., Borisov, A.B. and Faulkner, J.A. (2001) J. Gerontol. A Biol. Sci. Med. Sci. 56, B224–B233.
[28] Roth, S.M., Martel, G.F., Ivey, F.M., Lemmer, J.T., Metter, E.J., Hurley, B.F. and Rogers, M.A. (2000) Anat. Rec. 260, 351–358.
[29] Chakravarthy, M.V., Davis, B.S. and Booth, F.W. (2000) J. Appl. Physiol. 89, 1365–1379.
[30] Goldspink, G., Yang, S.Y., Skarli, M. and Vrbova, G. (1996) J. Physiol. 495, 162–163.
[31] Heslop, L., Morgan, J.E. and Partridge, T.A. (2000) J. Cell Sci. 113, 2299–2308.