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Expression of IGF-1 Isoforms after Exercise-induced Muscle Damage in Humans: Characterization of the MGF E Peptide Actions *In Vitro*

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Abstract. *Different insulin-like growth factor-1 (IGF-1) isoforms, namely IGF-1Ea, IGF-1Eb and IGF-1Ec (MGF), have been proposed to have various functions in muscle repair and growth. To gain insight into the potentially differential actions of IGF-1 isoforms in the regulation of muscle regeneration, we assessed the time course of their expressions at both mRNA and protein levels after exercise-induced muscle damage in humans. In addition, we characterized mature IGF-1 and synthetic MGF E peptide signalling in C2C12 myoblast-like cells in vitro. Ten healthy male volunteers were subjected to exercise-induced muscle damage and biopsy samples were taken from the exercised muscles before and 6 h, 2, 5 and 16 days post exercise. Muscle damage was documented by specific functional and biochemical responses post exercise. PCR-based analyses of muscle biopsy samples revealed a rapid and transient up-regulation of MGF mRNA expression which was followed by a prolonged increase of IGF-1Ea and IGF-1Eb mRNA expression ($p<0.05$). Patterns similar to those for mRNA expression were detected for MGF and IGF-1Ea expression at the protein level. The action of synthetic MGF E peptide differed from that of mature IGF-1 since its proliferative effect on C2C12 myoblast-like cells was not blocked by an anti-IGF-1 receptor neutralizing antibody and it did not phosphorylate Akt. Therefore, we conclude that the differential expression profile of IGF-1 isoforms in vivo and the possible IGF-1R - independent MGF E peptide signalling in skeletal muscle-like cells in vitro support the notion that tissue-specific mRNA expression of MGF isoform produces mature IGF-1 and MGF E peptides which possibly act as distinct mitogens in skeletal muscle regeneration.*

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Exercise is one of the most powerful stimuli for inducing structural, metabolic and functional re-organization of skeletal muscle. Activity models such as muscle overload and muscle stretch, or their combination as it occurs in eccentric exercise, have been shown to result in muscle damage (1). Exercise-induced muscle damage has been associated with disruption of the normal myofilament structures in sarcomeres, damage to sarcolemma, loss of fibre' integrity and leakage of muscle proteins into the blood, delayed-onset muscle soreness (DOMS) and loss of muscle force (2).

The overexpression of certain growth factors mediates largely skeletal muscle regeneration in response to metabolic or mechanical damage (3). Among those factors, insulin-like growth factor-1 (IGF-1) signalling has been implicated in promotion of chemotaxis of satellite cells, contributing both to their proliferation and differentiation (4-6). Nowadays, there is a growing interest vis-à-vis expression of IGF-1 splice variants [IGF-1Ea, IGF-1Eb and IGF-1Ec or mechano growth factor (MGF)] and their potential role in the regulation of muscle regeneration and hypertrophy following mechanical overloading and/or damage (6-10); however, there is little information regarding the expression of IGF-1 isoforms at the protein level.

Herein we examined the changes of IGF-1 isoforms at both the mRNA and protein levels in human skeletal muscle during the regeneration process following experimentally induced muscle damage, using the eccentric exercise model in young men. In addition, we characterized the potentially different physiological functions of two different IGF-1 peptides *in vitro*, namely mature IGF-1 and a synthetic MGF peptide which comprises the last 24 amino acid sequence of the human E domain of MGF.

Materials and Methods

Ethical approval. A written informed consent was obtained by all the volunteers to participate in this study, which was approved by the Ethics Committee of the National and Kapodistrian University of Athens, and all experimental procedures conformed to the Declaration of Helsinki.

Subjects. Ten healthy men (age 25±1.5 years, height 180.3±1.5 cm, body mass 76.8±2.4 kg, body mass index 23.6±0.5) participated in the study. They were physically active and had not participated in any type of resistance training or regular exercise regime for at least 6 months before the study. These individuals refrained from taking any medications or nutritional supplementations throughout the experimental period. They were also instructed to maintain their habitual diet with no alcohol, while on the day prior to and the day of each biopsy and blood draw they were asked to have similar meals.

Experimental design. The volunteers performed a maximal eccentric exercise protocol of the knee extensor muscles with each leg. One leg served for the assessment of muscle function (*i.e.* the functional assessment leg, FL) and it was biopsied only once in the pre-exercise period. Four post-exercise muscle biopsies (at 6 h, and 2, 5 and 16 days after the exercise protocol) were performed on the contralateral leg (biopsy leg, BL), assessing the post-exercise expression profiles in the exercised muscles. At each time point that required a muscle biopsy, blood samples were also withdrawn from each volunteer.

The functional testing protocol included the assessment of maximal voluntary contractile isokinetic torque of the knee extensors, which was performed on the FL before (PRE) and on days 1, 2, 5, 8, 12, and 16 post-eccentric exercise, but also on BL before and on day 16 post exercise. Thus, muscle biopsy on day 16 post exercise was performed 6 hours after the isokinetic torque testing of the BL. In addition, delayed-onset muscle soreness (DOMS) was measured on both legs at each time point of the testing protocol.

Testing of muscle function. Peak isokinetic torque (PIT) of the knee extensors of each leg was measured using an isokinetic dynamometer (Cybex Norm Lumex, Inc., Ronkonkoma, NY, USA). PIT was measured at an angular velocity of 1 rad/s. Each participant performed two maximal voluntary concentric contractions and the best trial was recorded. The range of motion was from 2.27 rad to 0 rad (0 rad=full extension). A resting period between 60 s and 90 s was allowed between repetitions.

Maximal eccentric exercise protocol. Participants performed an eccentric exercise bout with the knee extensors of each leg on the isokinetic dynamometer; this consisted of 2 sets of 25 maximal, isokinetic eccentric (lengthening) muscle actions with a 5-min break between the sets. Participants were required to maximally resist the forced lengthening of their quadriceps through a range of motion of 2.27 rad, from almost full extension (0.09 rad) to almost full flexion. Each lengthening muscle action was performed at an angular velocity of 0.52 rad/s, lasted ~4 s and was followed by a 15 s rest phase. The exercise protocol lasted for a total of ~22 min for each leg, and the order of limbs (*i.e.* which limb, FL or BL, was exercised first) was randomized across individuals.

Muscle soreness. DOMS was used as an indirect marker of muscle damage and was evaluated on both legs as described in detail elsewhere (7). Briefly, DOMS was evaluated on both legs before any contractions were performed in each measurement session. The perceived pain was recorded on a visual analogue scale that had a continuous line of 100 mm with the left end labelled no 'pain' and the right end labelled 'extremely sore'. Instructions had been given to the participants to rate soreness levels in two ways: (i) during one repetition of flexing and extending the knee joint throughout the entire range of motion and (ii) upon light palpation of the entire knee extensor area (*i.e.* the muscle belly and distal regions of the quadriceps muscle) always by the same investigator, with the thigh at rest. The average of the two values for each participant was used as the criterion score of the day.

Blood sampling and serum measurements. On each muscle biopsy day and before the biopsy procedure, blood samples were withdrawn. Serum was collected, centrifuged and stored frozen (–80˚C). Serum was assayed for creatine kinase (CK) activity, as indirect marker of muscle damage, with automated enzyme reactions (Roche/Hitachi ACN 057, Mannheim, Germany) at 37˚C using a commercially available kit (Roche Diagnostics, Mannheim, Germany). Serum total IGF-1, *i.e.* after removal of IGF binding proteins by an extraction procedure, was determined by a standard sandwich enzyme-linked immunosorbent assay (ELISA) protocol using a commercially available kit (Assay Designs, Michigan, USA) according to the manufacturer's instructions. All samples were run simultaneously, analyzed in triplicate and the results were averaged. According to the manufacturers, the minimal detection limits of the assay used for IGF-1 were 34.2 pg/ml, while the intra- and interassay coefficient of variation (CV) were 3.6% to 8.9% and 3.4% to 10.9% , respectively.

Muscle biopsies and tissue processing. Skeletal muscle biopsies were obtained from the middle portion of the vastus lateralis muscle under local anaesthesia (2% lidocaine; AstraZeneca, London, UK). A 5-mm Bergstrom biopsy needle was inserted at a constant depth using the percutaneous needle biopsy technique. To minimize the potential for interference, biopsy sites were at least 2 cm from previous biopsy sites. The muscle sample (~70-100 mg) obtained from each biopsy was divided into two pieces. The first piece was snap-frozen in liquid nitrogen and then stored at –80˚C until analyzed for RNA and protein content. The second piece was orientated longitudinally and fixed in formaldehyde (10% final concentration) for subsequent immunohistochemical analysis.

RNA extraction and relative quantitative PCR analysis. Each muscle sample was homogenized and total RNA was extracted using Trizol Reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's recommendations. The extracted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and the concentration and purity were determined spectrophotometrically (Genova, Jenway, Essex, UK) by absorption at 260 and 280 nm. The quality and integrity of total RNA were assessed by visual inspection of the electrophoretic pattern of 18S and 28S ribosomal RNA in ethidium bromide-stained 1% agarose gels under ultraviolet (UV) light and electrophoresis of the RNA confirmed that it was intact. The RNA samples were used for the determination of the mRNA of specific IGF-1 isoforms by reverse transcription and relative quantitative RT-PCR procedures. Both the RT and PCR methods used in the present

Table I. *Changes in muscle soreness (DOMS), serum creatine kinase (CK) activity, peak isokinetic torque and serum levels of total IGF-1, 6 h to 16 days after maximal eccentric exercise of knee extensors of both legs, compared to pre-exercise levels (PRE). Values represent the means (±S.E.M), (n=10). FL: Functional assessment leg; BL: biopsy leg. * p<0.05, *** p<0.001, ns: not significant.*

		PRE	6 hrs	Day 1	Day 2	Day 5	Day 8	Day 12	Day 16
DOMS (mm)	FL	0	–	37.15 (±7.26) ***	42.45 (±5.10) ***	13.4 (±3.38) ns	1.35 (±0.73) ns	0	0
	BL	0	–	43.50 (±6.20) ***	50.05 (±6.69) ***	16.8 (±4.34) *	1.75 (±0.72) ns	0	0
CK (IU/l)		144.81 (±12.76)	1719.19 (±264.31) ns	–	2560.54 (±922.19) *	2175.09 (±884.66) *	–	–	295.55 (±58.35) ns
Peak torque (N.m)	FL	208.81 (±3.74)	–	136.68 (±5.61) ***	162.11 (±9.47) ***	183.56 (±10.48) ns	190.77 (±7.16) ns	212.89 (±9.36) ns	212.37 (±8.26) ns
	BL	209.47 (±6.04)	–	–	–	–	–	–	188.73 (±11.08) ns
IGF-1 (pg/ml)		227.38 (±13.58)	218.41 (±17.91) ns	–	209.87 (±18.11) ns	227.77 (±16.18) ns	–	–	222.95 (±14.68) ns

study have been described and extensively validated in previous publications concerning pre- and post-resistance exercise mRNA responses (8, 9).

Primer sets for IGF-1Ea and IGF-1Ec (MGF) were previously used by Bickel *et al*. (8) and by Hameed *et al*. (10), respectively. Primer sequences for the specific *IGF-1Eb* mRNA were the following: ATGTCCTCCTCGCATCTCT, forward primer; CCTCCT TCTGTTCCCCTC, reverse primer. Each set of primers was designed to lie within different exons of the *IGF-1* gene and to detect and amplify only one specific *IGF-1* transcript (namely *IGF-1Ea*, or *IGF-1Eb*, or *MGF*). All target sequences were identified by sequencing analysis to ensure specificity of the primers and to further verify each target *IGF-1* mRNA.

Protein extraction and Western analysis. Total proteins were extracted from the same muscle biopsy sample used for total RNA isolation using the Trizol Reagent protocol. The extracts were analyzed for total protein concentration using the Bradford procedure (Bio-Rad Protein Assay, Hercules, CA, USA). Samples were stored in aliquots at –80˚C until Western blot analysis as previously described (11). The following primary antibodies were used for the immunodetection of MGF and IGF-1Ea: MGF, a rabbit anti-human MGF polyclonal antibody (1:10,000 dilution), which was raised against a synthetic peptide corresponding to the last 24 amino acids of the E domain of human MGF, as has been described elsewhere (11); IGF-1Ea, mouse monoclonal anti-IGF-1 (Clone-M23) (1:1,000 dilution) (MS-1508, NeoMarkers, Fremont, CA, USA; molecular weight of antigen: ~21 kDa). It should be remarked that the bands detected in Western blot analyses by these antibodies represent the (full length) MGF and IGF-1Ea pro-peptides (11).

After the overnight incubation of blots with the primary antibodies, membranes were incubated with a horseradish peroxidase-conjugated secondary anti-rabbit IgG (goat anti-rabbit, 1:2,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-mouse IgG goat anti-mouse, 1:2,000 dilution; Santa Cruz Biotechnology), for 1 h at room temperature. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to correct for potential variation in the protein loading and to normalize the protein measurements on the same immunoblot. Blots were incubated with a mouse monoclonal primary antibody for GAPDH (1:2,000 dilution; Santa Cruz Biotechnology) and with a horseradish peroxidase-conjugated secondary anti-mouse IgG (goat anti-mouse, 1:2,000 dilution; Santa Cruz Biotechnology). Specific band(s) were visualized by exposure of the membrane to x-ray film, after incubation with an enhanced chemiluminecent (ECL) substrate according to the manufacturer's protocol (SuperSignal; Pierce Biotechnology, Rockford, IL, USA). The films were captured under white light in a Kodak EDAS 290 imaging system (Carestream Health, Inc. Rochester, NY, USA) and proteins were quantified by band densitometry using image software (Scientific Imaging Systems, Kodak ID, New Haven, CT, USA).

Immunohistochemical analysis. Formaldehyde-fixed skeletal muscle samples were paraffin wax embedded and processed for paraffin sections. Microtome sections of 3 μm were allowed to adhere to glass slides, dried at 37˚C overnight, dewaxed in xylene and rehydrated in serial dilutions of ethanol. The sections were then incubated with the same primary antibodies used for the Western blot analyses, *i.e*. the polyclonal anti-MGF antibody at a dilution of 1:1,000 in PBS and the monoclonal anti-IGF-1 (1:50 dilution, MS-1508; NeoMarkers)

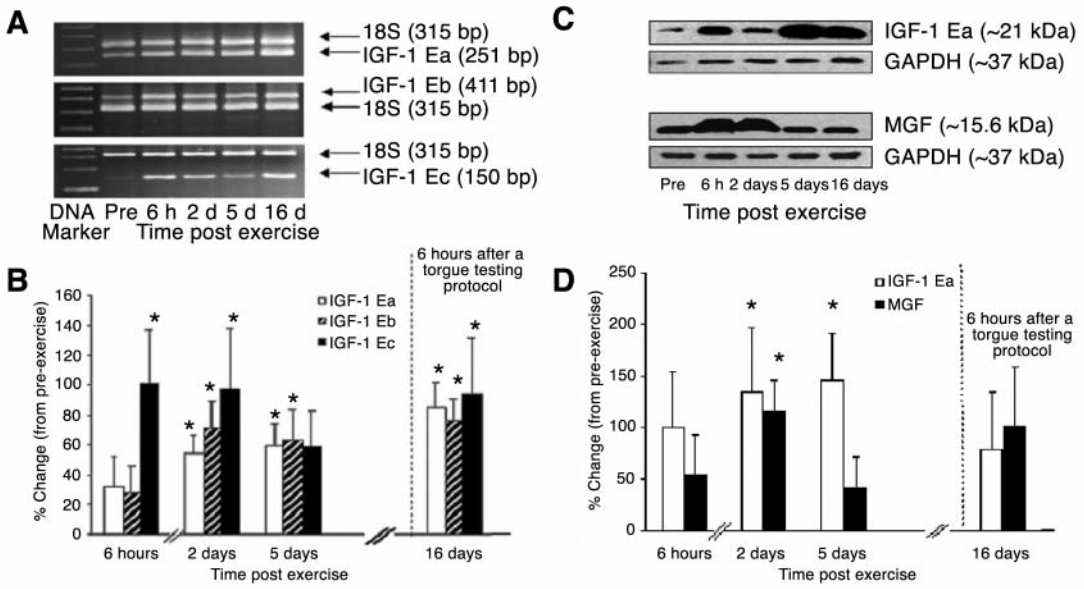

Figure 1. *Transcriptional and translational changes of the different IGF-1 isoforms, i.e. IGF-1 Ea, IGF-1 Eb and IGF-1 Ec (MGF), after muscle-damaging eccentric exercise. A, Representative PCR gel images demonstrate the mRNA expression of the IGF-1 transcripts for an individual volunteer observed before (Pre) and at different time points after muscle-damaging eccentric exercise. B, PCR relative quantification. Values (means±S.E.M.; n=10) were normalized to each corresponding ribosomal 18S and expressed as percentage changes (%) from pre-exercise mRNA levels. C, Representative Western blots of IGF-1Ea and MGF demonstrate the expression of the two distinct IGF-1 isoforms (pro-peptides) for the same individual volunteer observed before (Pre) and at different time points after muscle damaging eccentric exercise. D, Immunoblotting quantification. Values (means±S.E.M.; n=10) were normalized to each corresponding GAPDH on the same immunoblot and expressed as percentage changes (%) from pre-exercise protein levels. *Significantly different from pre-exercise levels ($p<0.05$).*

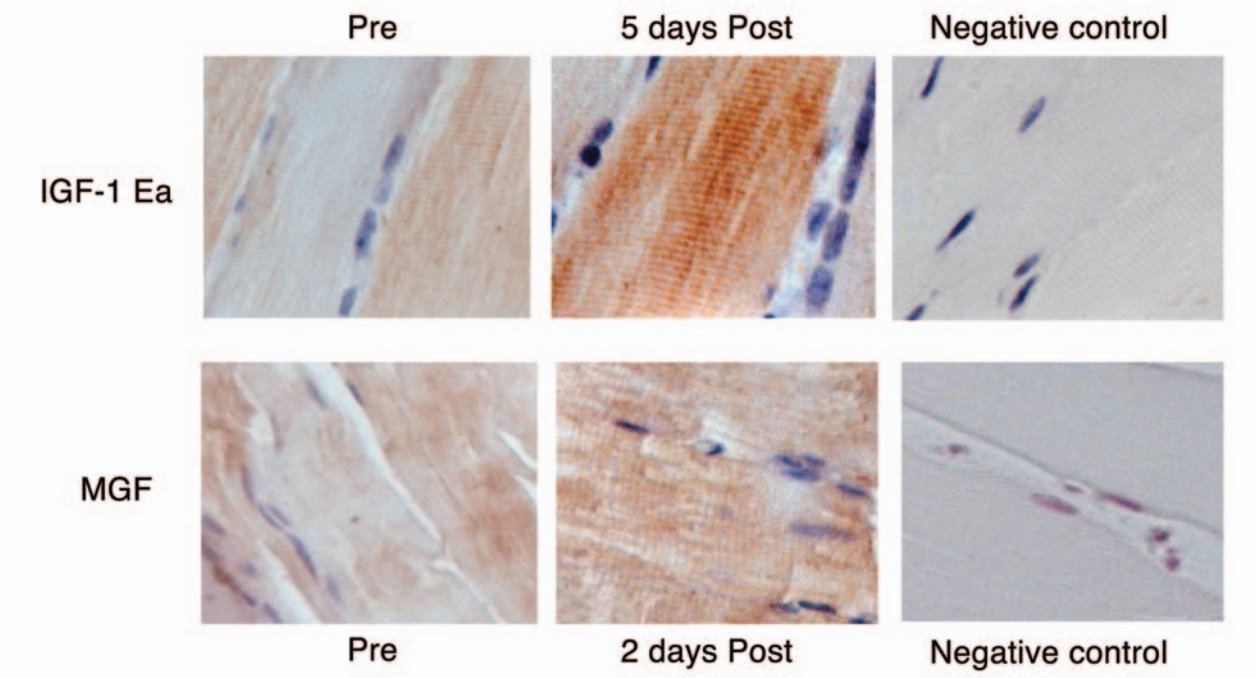

Figure 2. *Shown are representative vastus lateralis muscle samples from pre-exercise (Pre) and post-exercise (Post) biopsies of one individual (the same as in Figure 1) that are stained with anti-IGF-1Ea (upper panel) and anti-MGF antibody (lower panel). Note that the pre-exercise samples appear mildly stained, while the post-exercise samples appear highly stained with both the anti-IGF-1Ea and anti-MGF antibody. Specificity of the immunohistochemical detections was confirmed by the absence of immunoreactivity in the negative control sections; (magnification, ×40).*

overnight at 4˚C. Secondary biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Dako Real EnVision, Glostrup, Denmark) was then added and tissue sections were visualized under light microscopy. Negative control staining procedures were included in all immunohistochemical analyses, as described elsewhere (11).

Cell proliferation assays and signalling pathways. The murine C2C12 skeletal muscle-like cell line was obtained from the American Type Cell Culture (ATCC, Bethesda, MD, USA) and maintained as subconfluent monolayers in culture using Dulbecco's modified Eagle's medium (DMEM/F-12; Cambrex, Walkerville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom, Berlin, Germany), plus 100 U/ml penicillin/streptomycin (Cambrex, Walkerville, MD USA) at 37˚C in a humidified atmosphere with 5% CO_2, with culture media being replaced every 2-3 days. C2C12 cells were plated in 6-well plates at a cell density of 3.5×10^4 cells/well and grown with DMEM/F-12 containing 10% FBS. After 24 h of seeding, the medium was changed to 0.5% FBS for 24 h, in order to reveal the full effect of the growth factors used. A part of the MGF E peptide, corresponding to the last 24 amino acids of the C-terminal of the MGF E-peptide (exons 5 and 6), was synthesized and validated as previously described (11). C2C12 cells were exposed to different concentrations of the synthetic MGF E peptide in a time- and dose-dependent manner, *i.e.* 0.5, 25, 50 ng/ml of synthetic MGF E peptide for 24 and 48 h. Trypan blue exclusion assays were used to measure the number of viable cells as described elsewhere (12). Mature IGF-1 peptide (rhIGF-1, Chemicon international Inc., Temecula, CA, USA) was also used in an identical manner to the synthetic MGF E peptide to compare the physiological functions of these two distinct peptides on C2C12 cells (13). In order to investigate if these two IGF-1 peptides act on myoblasts *via* the same IGF-1R-mediated pathway, C2C12 cells were incubated with a monoclonal anti-IGF-1R neutralizing antibody (R&D Systems, Minneapolis, MN, USA) for 1 h prior to the various treatments with either mature IGF-1 or MGF peptide. The IGF-1R neutralizing antibody was used at a concentration of 10 μg/ml, according to the manufacturer's recommendation.

To further investigate if the mature IGF-1 and the C-terminal of the E domain of the MGF peptide act through different intracellular pathways that could mediate different physiological functions, we analyzed the activation (phosphorylation) of extracellular regulated kinase 1 and 2 (ERK1 and ERK2) and Akt-kinase (Protein kinase B) in C2C12 cells, as described elsewhere (14). Briefly, C2C12 cells were exposed to 50 ng/ml of mature IGF-1 or MGF peptide for 5, 15, 30 and 60 min. Cell extracts were obtained by cell lysis in RIPA buffer and equal amount of cell lysates (20 μg) were heated at 95˚C for 5 min, electrophoresed on SDS–PAGE under denaturing conditions and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA USA). The membranes were probed overnight with primary phosphospecific antibodies against phospho-ERK1/2 (Thr 202/Tyr 204), ERK1/2, phospho-Akt (Ser 473) and Akt (1:1,000 dilution; Cell Signaling, Beverly, MA, USA). The blots then were incubated with a secondary goat antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:2,000 dilution; Santa Cruz Biotechnology) and the bands were visualized as described in detail above.

Statistical analysis. Changes in cell numbers as well as in ratings of DOMS were assessed using two-way analysis of variance (ANOVA) with repeated measures (SPSS v. 11 statistical package, SPSS Inc. Headquarters, Chicago, USA). A one-way ANOVA with repeated measures over time was employed to evaluate changes in peak isokinetic torque as well as transcriptional and translational changes in IGF-1 isoforms and changes in all serum measurements. Where significant F ratios were found for main effects or interaction ($p<0.05$), the means were compared using Tukey's post-hoc tests. All data are presented as mean±standard error of the mean (S.E.M). The level of significance was set at $p<0.05$.

Results

Assessment of eccentric exercise-induced muscle damage. All volunteers reported a significant amount of perceived muscle soreness in both legs, which had higher ratings on days 1 and 2 post exercise and had entirely disappeared by day 8 post exercise. No statistically significant differences were found between the BL and FL in the levels of post exercise muscle soreness (Table I).

The classical biochemical marker of muscle damage, namely CK activity, was elevated post maximal eccentric exercise protocol. Serum CK activity levels were higher and remained elevated up to day 5 post exercise ($p<0.05$; Table I). *Assessment of muscle function following eccentric exercise-induced damage*. As expected, the peak isokinetic torque was significantly changed ($p<0.001$) post exercise compared to pre-exercise levels. No differences in the percentage changes of peak torque were found between legs on day 16 post exercise (Table I).

Assessment of serum IGF-1 changes. The serum levels of IGF-1 did not show any appreciable change throughout the experimental period of measurements (Table I; $P>0.05$).

Expression of IGF-1 isoforms following exercise-induced muscle damage. The IGF-1 isoform mRNAs were differentially altered over time in response to muscle damage-inducing exercise. Compared with the pre-exercise values, the expression of MGF significantly increased, earlier than the expression of the two other IGF-1 splice variants (IGF-1Ea and IGF-1Eb) which exhibited a similar expression profile ($p<0.05$; Figure 1A, B). Interestingly, all specific IGF-1 transcripts were significantly elevated on day 16 post exercise, 6 h after the imposition of the maximal torque assessment protocol on the biopsy leg (Figure 1B).

Consistently with the up-regulation at the transcription level, the protein levels of both MGF and IGF-1Ea pro-peptides also significantly increased in damaged muscle over time in response to eccentric exercise (Figure 1C, D). A single band at the appropriate (expected) molecular weight, *i.e.* ~15.6 kDa (11) and ~21 kDa, was labeled by the anti-MGF and anti-IGF-1 antibodies, respectively. As compared with the pre-exercise values, the expression of MGF showed only an acute increase on day 2 post exercise, while the

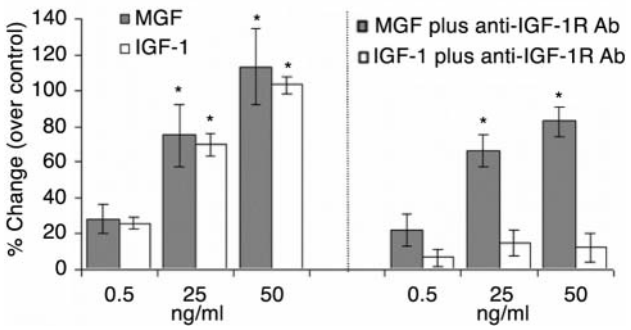

Figure 3. *The effects of 48 h treatment with mature IGF-1 and MGF E peptide on C2C12 cell proliferation (% cell number changes) in a dose-dependent manner, with (right side of the figure) and without neutralization (left side of the figure) of the IGF-1 receptor. Each data point represents the mean of three different wells from three repeated experiments. In all the control cultures, C2C12 cells were treated under the same procedure using PBS (solvent of the treatment factors); in the neutralizing experiments, control wells were treated with anti-IGF-1R antibody (10 μg/ml). Note that anti-IGF-1R antibody blocked IGF-1 effects on C2C12 cells without affecting synthetic MGF E peptide effects. At each dose of the MGF E peptide, there were no differences in changes between the treatments with and without anti-IGF-1R antibody. *Significantly different ($p<0.001$) from results under control conditions.*

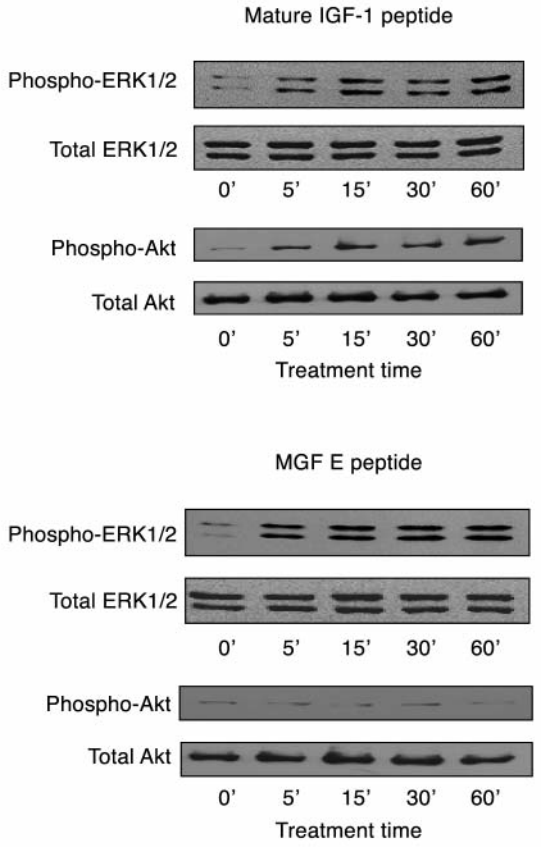

Figure 4. *Effects of mature IGF-1 peptide and synthetic MGF E peptide on C2C12 cell protein content of phosphorylated ERK 1/2 (phospho-ERK 1/2) and Akt (phospho-Akt), in a time-dependent manner. Mature IGF-1 did phosphorylate both the ERK1/2 and Akt, while synthetic MGF E peptide failed to phosphorylate Akt, suggesting that its action is IGF-1R-independent in C2C12 myoblast-like cells.*

expression of IGF-1Ea revealed an increasing and sustained up-regulation which was persistent at day 5 post exercise ($p<0.05$; Figure 1C, D). A similar pattern to that at the transcription level was present for MGF and IGF-1 Ea expression on day 16 post exercise (Figure 1D).

Immunohistochemical analysis. Immunohistochemical analysis revealed the cytoplasmic expression of IGF-1Ea and MGF in skeletal muscle cells of the damaged muscle. Post-exercise muscle samples appeared highly stained with both the anti-IGF-1Ea and anti-MGF antibody, as seen in the representative images in Figure 2.

Characterization of MGF signalling in vitro. Synthetic MGF E peptide was capable of increasing the number of living cells as well as did the mature IGF-1 (Figure 3). Neutralizing antibody against IGF-1R blocked the actions of IGF-1 on C2C12 myoblast-like cells; however, it failed to inhibit synthetic MGF E peptide action, suggesting that MGF action was IGF-1R-independent (Figure 3).

In addition, exogenous mature IGF-1 produced a time-dependent increase in phosphorylation of ERK1/2 and Akt in C2C12 cells (Figure 4). However, synthetic MGF E peptide did not activate Akt phosphorylation (Figure 4). These data suggested further that synthetic MGF E peptide may signal by an IGF-1R-independent pathway in C2C12 cells.

Discussion

All *IGF-1* transcripts yield the same mature IGF-1 peptide, which is derived from the highly conserved exons 3 and 4 of the *IGF-1* gene. These exons are known to code for the domain which is responsible for the binding of the IGF-1R. A post-translational cleavage of IGF-1 precursor polypeptides, by which the signal and the E peptide are removed, results in the same mature peptide but in different E domains (15, 16). Hence, it is tempting to postulate that IGF-1 precursor peptides could be pluripotential, acting jointly through their common mature IGF-1 peptide and, thus *via* IGF-1R signalling pathways, and differentially *via* the autonomous actions of their different E domains, as it has been suggested for Eb (17, 18) and Ec peptides (13, 19, 20).

In studies involving muscle cell lines, it has been documented that activation of the IGF-1R initiates intracellular signalling cascades which are involved in mitogenic and

myogenic responses (21, 22). Two primary signalling pathways have been proposed to be associated with the ligation of the IGF-1R (4, 23). The Raf-MEK-ERK signalling pathway has been shown to increase cell proliferation in muscle cell cultures (4, 22), while the other pathway involves the activation of phosphatidylinositol 3-kinase (PI3K) and portions of this activation signalling cascade appear to be critical for the differentiation of cultured muscle cell lines (24, 25) and for muscle hypertrophy (26-28).

In this study, the eccentric exercise did produce sustained changes in the clinical markers of muscle damage, such as muscle soreness, a deficit in muscle torque generation and increased serum CK activity levels post exercise. The molecular mechanisms involved in the regeneration process following skeletal muscle damage include the release of certain growth factors (3). Among the well-characterized growth factors, IGF-1 is the only one that has been consistently reported to promote both cellular proliferation (*i.e.* mitotic activity) and differentiation (*i.e.* expression of muscle-specific proteins) (4, 21) depending on the time course and intracellular conditions in the post-damage period (29). Furthermore, one of the most attractive aspects regarding IGF-1 is the potentially differential role of its isoforms in muscle regeneration and adaptation processes following muscle overload and damage (30, 31).

Our data did confirm a different expression profile of IGF-1 isoforms both at the mRNA and protein level in humans, which could be consistent with their different physiological functions on exercise-induced skeletal muscle damage. A differential expression of MGF and of IGF-1Ea was also recently observed in C2C12 skeletal muscle cells, in the case of muscle injury (32), or under mechanical signals that simulated those generated in myoblasts during early development of muscle cells (33). The transcriptional and translational responses of the IGF-1 isoforms observed in the present study would suggest that up-regulation of MGF (IGF-1Ec) may initially require a greater degree of transcriptional activity, while increases in IGF-1Ea may be accomplished *via* increased translation of available transcripts as was previously suggested (34). Furthermore, it is known that IGF-1 bioavailability in the extracellular matrix can be increased by hydrolysis of IGF-binding proteins (IGFBPs). Therefore, increasing levels of IGF-1 can be initially produced without requiring the activation of IGF-1Ea transcription as reported in various tissues and systems (6, 35-38). It is presently unknown whether MGF E peptide can bind IGFBPs and thus be regulated extracellularly *via* serine proteinase and metalloproteinase activity (6). In our study, MGF expression increased rapidly during the regeneration process as compared to the two other isoforms (IGF-1Ea and IGF-1Eb). MGF showed maximal expression on day 2 post exercise and declined thereafter. This acute increase of MGF expression following skeletal muscle damage is in consistent with a possible paracrine role of MGF E peptide on cell components of damaged muscle complementary to that of IGF-1 (30, 31). Interestingly, the dual role of IGF-1 on the myogenic program has been previously clarified by its separate effects on proliferation and differentiation (28, 29). Thus, it has been suggested that IGF-1 first acts upon myoblast replication through MAP-kinase signalling (22, 39) and subsequently promotes myogenic differentiation mainly *via* PI3-kinase-Akt signal transduction pathway (25). Our data showed that mature IGF-1 and synthetic MGF E peptide possibly act *via* different receptors in C2C12 myoblast-like cells. The proliferative function of the synthetic MGF E peptide on C2C12 myoblasts coincided with its activation of ERK1/2 (*i.e.* increased phospho-ERK1/2 protein content), along with the lack of Akt activation. This is in accord with previous studies which reported that the activation of Raf-MEK-ERK pathway promotes myoblast proliferation (4, 22, 40) while PI3K-Akt pathway is predominantly linked to myogenic differentiation and to the hypertrophic actions of IGF-1 (24, 25, 28). Hence, it is tempting to postulate that the MGF E domain induced proliferative activation of ERK 1/2 in myoblast-like cells independently of IGF-1R signalling. This is in concert with the notion that the processes of myoblast proliferation and differentiation are mutually exclusive, and there are studies reporting that the activity of one pathway might inactivate portions of the other (40, 41). Therefore, we postulate that the early phase of a mitogenic response following muscle damage involves possibly complementary MGF and IGF-1 actions. It is conceivable that the early and short-term expression of MGF pro-peptide in damaged muscles *in vivo*, combined with its possible IGF-1R-independent action on myoblasts, could reflect an important mechanism implicated in muscle regeneration. The prolonged overexpression of IGF-1Ea and IGF-1Eb isoforms later during the course of the damage repair mechanism would probably serve to generate pure IGF-1R-mediated signalling that includes both ERK1/2 and Akt phosphorylation (27). Our data are similar to those from another study analyzing the role of IGF-1 isoform expression on myoblast proliferation and differentiation (42). Interestingly, this report documented an increased phospho-Akt protein content which coincided with increased protein synthesis, associated with a hypertrophic adaptation of human skeletal muscle 48-72 h following resistance training (43). Moreover, an increased phospho-Akt protein content coincided with the phosphorylation state of p70-S6 kinase (S6K1), an Akt downstream target that promotes protein synthesis, which was documented 40 h after two bouts of resistance exercise spaced 24 or 48 apart (44).

In addition, the transcriptional response of the IGF-1Eb isoform was found to have a similar expression profile to that of the IGF-1Ea isoform, confirming the data of a recent report

(42), while other studies have reported the up-regulation of IGF-1Eb mRNA as a result of strength training (45). Obviously, further studies are required to elucidate the specific action(s) of IGF-1Eb isoform in regenerating skeletal muscle.

Another interesting finding of our study is the remarkably increased levels of all IGF-1 splice variants on day 16 post-eccentric exercise-induced muscle damage and after the imposition of a maximal resistance testing protocol on the repaired muscles (Figure 1A, B). This suggests that the increased mRNA expression, particularly of the MGF splice variant, could reflect an adaptive response to the second bout of mechanical loading, as was previously reported in animal studies (44).

Finally, the increase of the IGF-1 isoform expression in damaged skeletal muscle was not accompanied by an increase of IGF-1 serum levels, confirming the findings reported previously on serum IGF-1 concentrations after eccentric (46) or high-resistance exercise (45). These data support the view that IGF-1 activity in skeletal muscle is independent of changes in systemic growth hormone (GH)-dependent IGF-1 production observed within 60 min after exercise initiation (4).

In conclusion, we have documented a differential expression pattern of IGF-1 isoforms in human skeletal muscle in response to exercise-induced damage independently of changes in systemic IGF-1 production. The differential expression of MGF compared to the two other IGF-1 isoforms, in combination with the different signalling pathway of the synthetic MGF E peptide compared to mature IGF-1 in C2C12 myoblast-like cells, represent evidence of a unique local regulatory process, complementary to IGF-1, which produces an MGF-mediated ERK1/2 phosphorylation independently of IGF-1R signalling. Clearly, additional studies are needed to further elucidate the signalling mechanisms and the potential role of MGF E peptide in skeletal muscle regeneration and hypertrophic processes following exercise-induced damage and overload in humans.

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