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Review

Biological activity of the e domain of the IGF-1Ec as addressed by synthetic peptides

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ABSTRACT

Insulin-like growth factor-1 (IGF-1) is a multipotent growth factor involved in the growth, development and regulation of homeostasis in a tissue-specific manner. Alternative splicing, multiple transcription initiation sites and different polyadelynation signals give rise to diverse mRNA isoforms, such as IGF-1Ea, IGF-1Eb and IGF-1Ec transcripts. There is increasing interest in the expression of the IGF-1 isoforms and their potential distinct biological role. IGF-1Ec results from alternative splicing of exons 4-5-6 and its expression is upregulated in various conditions and pathologies. Recent studies have shown that IGF-1Ec is preferentially increased after injury in skeletal muscle during post-infarctal myocardium remodelling and in cancer tissues and cell lines. A synthetic analogue corresponding to the last 24 aa of the E domain of the IGF-1Ec isoform has been used to elucidate its potential biological role. The aim of the present review is to describe and discuss the putative bioactivity of the E domain of the IGF-1Ec isoform.

Key words: E peptides, Human cells, IGF-1Ec

ALTERNATIVE SPLICING OF THE IGF-1 GENE

Insulin-like growth factor 1 (IGF-1) is a single-chain polypeptide,[1] a multipotent growth factor controlling cell proliferation, differentiation, apoptosis, tissue growth and organ specific functions throughout the body.[2-5] The biological functions of IGF-1 are mediated through binding to type I IGF receptor (IGF-1R), which is a ligand-activated transmembrane tyrosine kinase receptor.[6,7] Once IGF-1R is phosphorylated, its kinase domain can serve as a docking site for several proteins, including Insulin Receptor Substrate Proteins (IRS) and Src homology/collagen (Shc) proteins.[6] These two substrates serve as mediators and docking sites for the propagation of downstream signalling.[8]

IGF-1 is the product of the *igf-1* gene which is located on the long arm of chromosome 12—well conserved among vertebrate species—and extends to a region of approximately 90 kb, composed of six exons (designated as 1, 2, 3, 4, 5 and 6).[9,10] Despite the highly conserved nature of the *igf-1* gene structure, several factors, such as multiple transcription initiation sites,[11-13] alternative splicing[14,15] and different

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polyadenylation signals,[16,17] result in diverse mRNA transcripts[18] that subsequently undergo further post-translational modifications and produce heterogeneous final products.[19]

More specifically, the usage of various transcription initiation sites results in two distinct classes (I & II) of IGF-1 transcripts: Class I transcripts have their promoter on exon 1 (P1), while Class II have it on exon 2 (P2). Both promoters direct transcription at multiple initiation sites and lack typical control elements (e.g. "TATA" box, or "CCAAT" box).[20,21] Alternative splicing at both the 5' and 3'-end further increases the complexity of *igf-1* gene regulation. When transcription is initiated, it undergoes two alternative splicing modifications: at the 5'-end, the untranslated regions within either exon 1 or exon 2 are removed and spliced to exon 3, and at the 3'-end, exon 4 is spliced to either exon 5, which classifies this splice variant as IGF-1Eb,[22] or insertion of exon 6, which classifies it as IGF-1Ea.[17,23] A third variant has been identified in humans[15] and recently in *Cervus Elaphus*[24] containing both exon 5 and exon 6, and it is similar to the IGF-1Eb isoform found in rodents. The transcription of exons 3 and 4 does not undergo any modifications and remains constant.[25] Transcription initiation at P1 gives rise to transcripts highly expressed in extrahepatic tissues (e.g. muscle),[26,27] while initiation at P2 produces transcripts commonly found in the liver.[11,28] The possibility that the two promoters (P1 and P2) are not mutually exclusive and certain stimuli or hormones could induce even more production of the locally expressed isoform(s) cannot be ruled out (Figure 1).

In addition, as a result of different polyadenylation sites, a large number of IGF-1 pre-mRNAs are produced in humans, ranging from 1 to 7.5 Kb in size:[29] IGF-1Ea pre-mRNA transcripts (exons 1-2-3-4-6) that are produced primarily in liver and other tissues,[30] IGF-1Eb pre-mRNA (exons 1-2-3-4-5) produced in liver,[22] skeletal muscle[31] and a variety of cell lines[32] and IGF-1Ec (exons 1-2-3-4 49 bp from exon 5 and then exon 6).[15]

Moreover, further modifications, at a post-transcriptional level, by miRNAs may exist.[33] MiRNas are non-coding RNAs, 22 nucleotides in length, that regulate gene expression at the post-transcription level and are found in plants and animals.[34] By binding to the 3'-UTR (untranslated region) of a gene, they repress the target transcript and consequently its translation, coordinating gene expression and protein translation. Eleven of the top 50 conserved miRNA sites have been identified in the IGF-1 3'-UTR.[35] Evidence of *igf-1* gene expression regulation by miRNA has been found: in cardiac and skeletal muscle physiological and pathological conditions,[36] in IGF-1 antiapoptotic actions,[37,38] in myocardial microvascular endothelial cells of the type 2 diabetic Goto-Kakizaki rat[39] and during the growth of teleost tilapia.[40]

The mRNA transcripts encode a number of IGF-1 precursor proteins, which in their turn undergo post-translational modifications.[10,41,42] Post-translational cleavage of the IGF-1 precursor protein produces the signal peptide, the mature IGF-1 and the E domain-related peptides.[41] The signal peptides are produced by alternative splicing of exons 1, 2 and 3 of IGF-1 prohormone. The presence of multiple initiation sites in exons 1 and 2 and their location relative to translation initiation codons give rise to multiple signal peptides of variable length. Usage of the two transcription initiation sites of exon 1 (Class I), upstream of the Met-48 translation initiation codon, gives rise to a 48 amino acids signal peptide (21 aa from exon 1 and 27aa from exon 3), while the downstream transcription initiation site relative to Met-48 in exon 1 gives rise to a 25 amino acids signal peptide (25 aa from exon 3).[43] Transcription initiation sites of exon 2 (Class II) are located upstream of the Met-32 translation initiation codon and give rise to a 32 amino acid signal peptide (5 aa from exon 2 and 27 aa from exon 3).[10,43]

Exons 3 and 4 encode the mature IGF-1 peptide (25 and 45 aa, respectively),[19,44] which is highly conserved.[43] IGF-1 contains four domains: B, C, A, D. The B domain is associated with IGF binding proteins[45] and the activation of IGF-1R.[46] Furthermore, two alternative products of the mature IGF-1 have been identified in the human brain: a truncated IGF-1 form (-3N:IGF-1) that lacks the first 3 amino acids of the mature peptide, and the tripeptide glycyl-prolyl-glutamate (GPE) that corresponds to the amino terminal end of the B domain of the mature peptide.[47,48]

Finally, by alternative splicing of IGF-1 at the

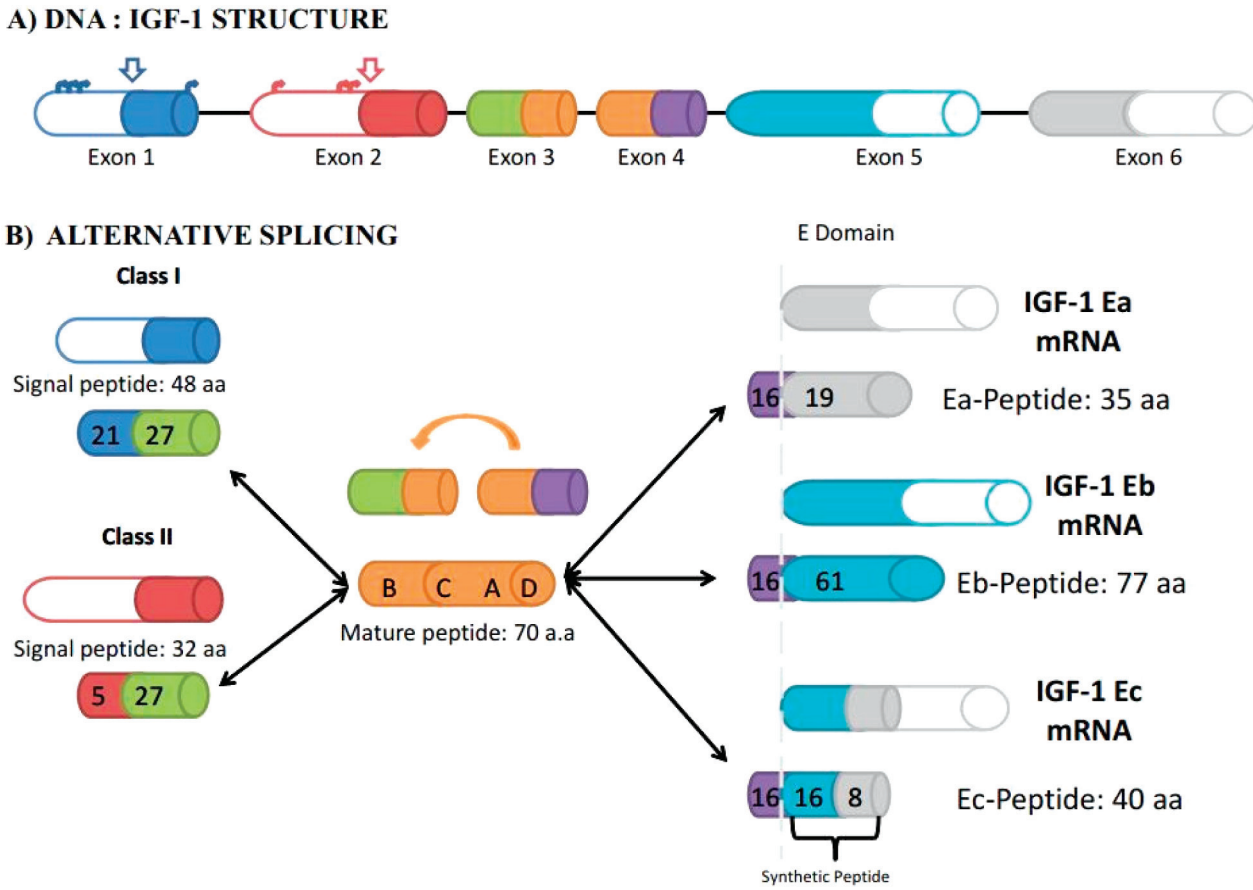

Figure 1. Human IGF-1 isoforms. A) IGF-1 gene structure with its 6 exons. Coloured regions represent the regions that encode IGF-1 precursor peptide. In exons 1 and 2, various transcription initiation sites (solid arrows) are present that can give rise to heterogeneous signal peptides of different amino acid sequences. The translation initiation sites are also presented (hollow arrows). B) Alternative splicing of the IGF-1 pre-mRNA and the three isoforms IGF-1Ea, IGF-1Eb and IGF-1Ec are presented with their number of amino acids. Diverse E peptides are produced by alternative splicing.

3'-end, a variety of IGF-1 E peptides are produced. In humans, three mRNAs encode three different E-peptides (Figure 1). Splicing of exon 4 to exon 6 leads to an mRNA sequence that encodes a 35 amino acid E-peptide, Ea. Its first 16 amino acids (common to all IGF-1 E-peptides) are encoded by exon 4 and the remaining 19 aa by exon 6.[17,42] Eb-peptide derives from splicing alternation of exon 4 to exon 5 and it contains 16 amino acids encoded by exon 4 and 61 amino acids from exon 5, resulting in a 77 aa peptide. This isoform of the IGF-1 precursor appears to have a nuclear and nucleolar localization.[49] A third isoform (Ec-peptide), an exon 4-5-6 variant, contains a total of 40 amino acids: 16 amino acids from exon 4, 16 from exon 5 and 8 from exon 6, sharing 73% homology with the rat Eb-peptide.[15] This particular isoform was initially termed Mechano Growth Factor (MGF) due to its upregulation after skeletal muscle stretch or damage.[50] It is noteworthy that the existence of an N-glycosylation site in the Ea-peptide (absent in Ec) possibly modifies the potential biological role of the Ea peptide.[19,51]

IGF-1E ISOFORM EXPRESSION IN MECHANOSENSITIVE CELLS

Mechanocytes (fibroblasts, osteoblasts, smooth, cardiac and skeletal muscles cells) respond to mechanical/external stimuli which can regulate gene expression and affect the splicing events of IGF-1 pre-mRNA towards a particular isoform.[52] In resting skeletal muscle, all three IGF-1 isoforms (Ea,

Eb and Ec) are expressed,[31,53] although their expression levels vary based on gender and age.[31,54-56] The stimulation of muscle via a mechanical or electrical loading upregulates both IGF-1Ea and IGF-1Eb in rodents.[50,57] In humans, particularly in young men subjected to resistance exercise, documentation has been made of an upregulation of IGF-1Ec mRNA (from 2% to 864%) within 2.5 hours post-exercise, however without significant changes in IGF-1Ea mRNA expression, while this effect was attenuated in older subjects.[31] In addition, a study on levator ani muscle showed that IGF-1Ec and IGF-1Ea mRNA levels were increased 100- to 1000-fold in pregnant women after vaginal delivery.[55]

In rat myocardium, the IGF-1Eb mRNA expression, which corresponds to human IGF-1Ec, was preferentially stimulated at 4 and 8 weeks post-infarction at both mRNA and protein level.[58] Cyclic stretching of osteoblasts induced a higher expression of IGF-1Ec and IGF-1, while IGF-1Ec was undetectable in the controls cells and under static-stretching.[59]

Theoretically, the upregulation of IGF-1Ec mRNA results in the production of IGF-1Ec precursor protein, which, in its turn, can be processed into two bioactive peptides: mature IGF-1 and E peptide (Ec peptide). E peptide has been proposed to have a dual role, activating quiescent satellite cells which can proliferate them, providing a niche of myogenic precursor cells (MPCs) and supporting muscle repair.[60] At the point when the IGF-1Ec mRNA decline, the IGF-1Ea transcript increases, thus supporting myoblast differentiation.[60] In a recent study, rodent Eb peptide did enhance myoblast proliferation, while IGF-1Ea suppressed myoblast differentiation.[61] However, specific silencing of either IGF-1Ea or IGF-1Eb in cultured mouse myoblasts suggested that both Ea and Eb-derived peptides may be necessary for normal myoblast differentiation.[62]

IGF-1 E INVOLVEMENT IN HUMAN DISEASES

The biological role of the IGF-1 isoforms and peptides and the mechanism(s) that regulate their expression *in vivo* and *in vitro* remain unclear. Additionally to the studies that have investigated the expression pattern of the particular transcript(s) in skeletal muscle,[53,55,60] there is growing interest in their potential role as part of the pathophysiology of various human diseases. A differential expression of the IGF-1 isoforms has been reported, dependent on the stage and type of disease: for example, upregulation of the IGF-1Ec isoform has been detected in HPV positive women with cancer abnormalities,[63] in colorectal cancer,[64] endometriosis,[65] prostate cancer[66] and MG-63 human osteoblast-like osteosarcoma cells.[67]

BIOACTIVITY OF SYNTHETIC IGF-1 E DOMAIN PEPTIDES

One of the first attempts to examine the biological activity of the IGF-1 E peptides, by using synthetic analogues, was made by Siegfried et al.[32] Based on the human amino acid sequence of IGF-1Eb peptide and the existence of a putative proteolytic cleavage sequence (Gly-Lys-Lys-Lys), a peptide corresponding to 103-124 aa of IGF-1Eb was identified as having a possible biological role. A synthetic analogue to the predicted peptide was synthesized (termed Y-23-R); in addition, two alternative forms of the peptide were synthesized: a free-acid derivative (Y-23-R-OH) and an amidated (Y-23-R-NH2). The peptides had a dose-dependent growth promoting activity on normal and malignant human bronchial epithelial cells, and in particular the amidated peptide analogue binds to high-affinity receptors, while its actions (binding and proliferation) were not inhibited by the presence of either recombinant Insulin, IGF-1 or an antibody antagonist of the IGF-1R. Immunoblot analysis of human lung tumour cells extracts (using an antibody raised against Y-23-R-NH2) detected a protein of approximately 5 kDa. The authors suggested that in species where an IGF-1 mRNA homologue to the human IGF-1Eb domain has not yet been described, an alternative mRNA must be produced with a sequence similar to the mid-portion of the human IGF-1Eb E domain, and that it may mediate its effects through a specific, high affinity receptor, conserved in many species.[32]

After the identification and characterization of the IGF-1Ec, independent research groups have developed synthetic analogues for the IGF-1 E peptides that correspond to the C-terminal 24 amino acids of the IGF-1 E peptide.[32,58,61,65-68,77,78]

Studies by Kuo and Chen,[68,69] using a recombinant

Ea-4 peptide of the rainbow trout (*oncorhynchus mykiss*) pro-IGF-1, showed that the particular peptide reduced significantly the anchorage-independent growth of human neuroblastoma cells (SK-N-F1) as well as morphological differentiation of those cells. Similar biological activities were observed with the use of a human synthetic Eb peptide but not with Ea peptide.[68,69] Further investigation of the effects of the two peptides showed that the activation of ERK1/2 is increased in response to those two peptides, an indication of MAPK signalling cascade involvement.[78] The authors suggested that the E peptide(s) of pro-IGF-1 could have a role in regulating cell growth and differentiation in neuroblastoma cells. They further examined the binding properties of the two peptides to intact neuroblastoma cells (SK-N-F1) and membrane preparations.[69] The peptides share common binding sites, distinct from those of IGF-1 and insulin, binding to them with high affinity. They concluded that the action of the IGF-1 E peptide(s) is mediated through the interaction with conserved and specific putative membrane receptors on neuroblastoma cells.

The first study in which a synthetic peptide was used to investigate the biological functions of IGF-1Ec was by Yang and Goldspink.[70] They synthesized a predicted E peptide based on the IGF-1Ec sequence (see Table 1) and exposed C2C12 mouse myoblasts to various concentrations of either the synthetic E peptide or the mature IGF-1 peptide. They showed that the E peptide of the IGF-1Ec splice variant exerts biological activity, distinct from the activity of the mature IGF-1, and is capable of increasing myoblast proliferation while inhibiting myotubes formation. In addition, they selectively blocked the IGF-1 receptor by a specific anti-IGF-1 receptor antibody in the presence of the synthetic E peptide. It was found that the function of the E peptide was not inhibited by blocking the receptor, providing evidence that its proliferating actions are not mediated via the IGF-1R, but possibly involves a different pathway.

Mills et al[71] based on analysis of the IGF-1Ec human gene, synthesized a synthetic peptide similar to the one used by Yang and Goldspink,[70] seeking to verify whether it could possibly improve the success of human myogenic precursor cells (hMPC) transplantation. They used hMPCs obtained from a postmortem biopsy as their *in vitro* model to test cell proliferation enhancement under treatment with different doses of the synthetic peptide (Table 1) and IGF-1 (50 ng/ml). The addition of the two factors significantly increased the number of cells counted after 48 hours; their promitogenic capacity was still present after 96 hours. Maximum proliferation rates were obtained under 25 and 50 ng/ml of the synthetic peptide, while it delayed hMPCs differentiation in a dose-dependent manner. In the same model, they examined the ability of the synthetic peptide to exert its effects through the IGF1R by treating the hMPCs with an antibody against the human IGF-1R. The mitogenic activity of the synthetic peptide was not affected by the anti-IGF-1R antibody, suggesting that the peptide could enhance hMPCs proliferation possibly through another receptor. Moreover, since *in vivo* intramuscular or systemic administration of the synthetic peptide at 25 ng/ml significantly promoted engraftment of hMPCs in mice, the authors proposed that the synthetic peptide could be used as an agent to increase hMPCs transplantation success in patients with Duchenne Muscular Dystrophy (DMD). In a follow-up study,[72] they examined its effects on migration of hMPCs. Using both *in vitro* and *in vivo* migration assays, the pro-migratory effect of the synthetic peptide at various concentrations was examined (Table 1), as well as with and without the addition of human IGF-1 (50 ng/ml *in vitro* and 50 ng *in vivo*). They reported that the addition of synthetic peptides at lower concentrations (15 ng/ml) exerted significant but lower pro-migratory activity compared to higher concentrations *in vitro*. Higher concentrations (50 and 100 ng/ml) enhanced the motility of human cells, while optimum motility was observed at a concentration of 25 ng/ml. The authors speculated the existence of a turning point between 25 and 50 ng/ml where downregulation of the pro-migratory pathway is observed. *In vivo* administration of the synthetic peptide at 25 ng induced human myogenic cells to migrate further than the administration of 50 ng. Co-administration of IGF-1 and synthetic peptide at 25 ng did not show an increase in migration compared with the separate administration of those factors. The use of an antibody against the IGF-1R did not diminish the enhanced migration of hMPCs by the synthetic peptide. Moreover, at optimal concentrations, the peptide demonstrated enhanced expression of urokinase-type plasminogen

Table 1. Synopsis of the effects of the synthetic peptides on various *in vitro* and *in vivo* models

Researcher	AA Sequence	Treatment Duration	Optimum Concentration*3	Culture Conditions	*In vitro*/*in vivo* model
Yang & Goldspink (2002)	*1 (Human)	24 hours proliferation	Dose-dependent manner 1-100 ng/ml 5 ng/ml	Serum free media	C2C12 mouse myoblasts
Dluzniewska (2005)	*2 (Human)	Continuously present in the medium	25 µg	Injection left carotid artery	Male Mongolian gerbils
			100 ng/ml		Organotypic hippocampal cultures of neurodegeneration
Mills et al (2007)	*1 (Human)	48 hours proliferation 30 days period	Dose-dependent manner 15-100 ng/ml 25-50 ng/ml	Normal proliferation medium	Human MPCs
			Dose-dependent manner 1-10 µg 5-10 µg	Intramuscular injection	Mice
		14 hours migration assay	Dose-dependent manner 15-100 ng/ml 25 ng/ml	Serum free media	Human MPCs
			25 and 50 ng/ml	Polyethylene microtude technique	Mice
Carpenter (2008)	*2 (Human)		200 nM	Circumflex branch of the coronary artery	Sheep
Philippou et al (2009)	*4 (Human)	24 & 48 hours proliferation	Dose-dependent manner 0.5-50 ng/ml 50 ng/ml	0.5% FBS	C2C12 mouse myoblasts
Stavropoulou et al (2009)		48 hours proliferation	Dose-dependent manner 5-50 ng/ml 50 ng/ml	0.5% FBS	H9C2 rat myocardial cells
Milingos et al (2011)		24 & 48 hours proliferation	Dose-dependent manner 5-50 ng/ml 50 ng/ml	0.5% FBS	KLE Human endometrial adenocarcinoma cells
Philippou et al (2011)		24 & 48 hours proliferation	Dose-dependent manner 0.5-50 ng/ml 50 ng/ml	0.5% FBS	Human osteoblast-like osteosarcoma cells
Ates et al (2007)	*2 (Human)	48 hours proliferation	10 ng/ml	Culture media with 2% FCS	Human primary muscle cells Human skeletal muscle myoblasts

Table 1. (continue) Synopsis of the effects of the synthetic peptides on various *in vitro* and *in vivo* models

Researcher	AA Sequence	Treatment Duration	Optimum Concentration*3	Culture Conditions	*In vitro*/*in vivo* model
Queasada et al (2009)	*2 (Human)	24 and 48 hours proliferation	Dose-dependent manner 10^{-9} to 10^{-5} g/ml	Serum free media	SH-SY5Y cells Human neuroblastoma
		7 days	5 and 20 µg	Subcutaneous injection	Long-Evans male rats
Collins et al (2010)	*5 (Human)	Migration 20 hours	Dose-dependent manner 1-100 ng/ml 30 ng/ml	Serum free media	Human mesenchymal stem cells isolated from bone marrow aspirates
		Proliferation 4 days	No effect		
Kandalla et al (2011)	*2 (Human)	Proliferation every 3 days treatment Counted at day 7	Dose-dependent manner 3-100 ng/ml 100 ng/ml	Serum free media	Primary human muscle cells
		Differentiation 7 days	Dose-dependent manner 10, 50 and 100 ng/ml		
Brisson & Barton (2012)	*6 (Rodent)	KIRA assay 15, 30, 60 minutes	IGF-1 (2, 10nM)+ Ea (10, 100 nM) Or/+ Eb (100 nM)	Serum free media	Mouse fibroblasts (P6)
		Proliferation	Dose-dependent manner Ea 1-100 nM Eb 1-100 nM 10 nM		C2C12 mouse myoblasts
		Migration 5 hours	Ea 100 nM Eb 100 nM		
		Differentiation	Ea 100 nM Eb 100 nM		

*1 NH_2-YQPPSTNKNTKSQRRKGSTFEEHK-COOH
*2 NH_2-YQPPSTNKNTKSQR (-d)R (-d)KGSTFEEHK-NH2
*3 When the authors indicated that a maximum response (e.g. proliferation, migration, etc) was observed
*4 YQPPSTNKNTKSQRRKGSTFEERK
*5 NH_2-YQPPSTNKNTSQ (l-R)(l-R)KGSTFEERK-COOH (naturally occurring)
NH_2-YQPPSTNKNTSQ (d-R)(d-R)KGSTFEERK-amidated (D-form)
NH_2-YQPPSTNKNTSQ (d-R)(d-R)KGATFEERK-amidated (S substituted by A)
NH_2- EFSTKENQGTPSQHTNK (d-R) PYK (d-R) ks-amidated (Scramble)
*6 Ea HTDMPKTQKEVHLKNTSRGSAGNKTYRM
Eb HTDMPKTQKSPSLSTNKKTKLQRRRKGSTFEEHK
Scramble RFLHRTEKRATGTKDSNVQSKHTSMTKQPKY
FBS: Fetal Bovine Serum, FCS: Fetal Calf Serum.

activator (u-PA), its receptor (u-PAR) and matrix metalloproteinase-7 (MMP-7) while reducing plasminogen activator inhibitor-1 (PAI-1) activity, with no effect on gelatinases MMP-2 and -9. The above factors compose a system that plays a role in the general control of matrix degradation,[73] suggesting that the combination of the aforementioned effects may favour cell migration.

Ates et al[74] also used a synthetic peptide (Table 1) to study the effects of IGF-1Ec E peptide on human primary cell cultures from normal, dystrophic and motor neurone disease (ALS) muscles and compared them with the effects of recombinant mature IGF-1 peptide, as well as with the effects of the combination of both peptides. The IGF-1Ec E peptide increased the number of myogenic progenitor cells in normal muscle primary cultures, but in the presence of both IGF-1 and the synthetic E peptide, the effect of the E peptide was abolished. Similar actions of the E peptide were observed in muscle progenitor cells derived from muscles with congenital muscle dystrophy (CMD), Fascio-Scapulo-Humeral Dystrophy (FSHD) and ALS, thus leading to the conclusion that the E domain activates the muscle progenitor cells while, on the other hand, IGF-1 induces the cells into the fusion pathway and removes them from the stem cell pool. The use of an antibody to block the IGF-1R indicated that the action of the E domain has a unique signalling that does not involve the IGF-1R. Moreover, they found that in the presence of the IGF-1Ec E peptide (synthetic E peptide), creatine phosphokinase (CPK) —a marker of differentiated cells— was not induced, suggesting that the E peptide may prevent terminal differentiation.

Using an *in vivo* gerbil model of transient brain ischemia, Dluzniewska et al[75] demonstrated that the IGF-1Ec E peptide (Table 1) provided protection to the vulnerable neurons. Additionally, an *in vitro* organotypic hippocampal culture model of neurogeneration was used to further investigate the role of the IGF-1Ec E peptide and IGF-1 as neuroprotective agents. The results established that both molecules have the same effectiveness, but the synthetic peptide had a longer-lasting effect. While there was no competitive effect between them, their mode of action was independent, due to the fact that synthetic peptide action was not arrested by the presence of an IGF-1R inhibitor.

Another study[76] examined whether a synthetic peptide of the IGF-1Ec E domain reduces the severity of injury to sheep myocardium after infarct and whether it would improve cardiac function. A comparison of the effects of a synthetic analogue of the IGF-1Ec splice variant (mature and E), mature IGF-1 domain (70 amino acids) and the IGF-1Ec E peptide on post-infraction cardiac function was made. The IGF-1Ec E peptide used was similar to that of Dluzniewska[75] at an optimum concentration of 200 nM. The major conclusions were that the IGF-1Ec E peptide has a greater effect compared to mature IGF-1 or synthetic analogue of the IGF-1Ec splice variant (mature and E), it protects myocardium against ischemia and it is able to reduce the loss of cardiac function after myocardial infarction.

A study by Queasada et al[77] was the first to show that IGF-1Ec E peptide protects Dopamine (DA) neurons both *in vitro* and *in vivo* (Table 1). In cultured SH-SY5Y human neuroblastoma cells, the peptide upregulated the expression of a small stress protein involved in heme catabolism, i.e. heme oxygenase-1 (HO-1), and protected the cells against apoptosis and cell loss induced by DA cell specific neurotoxins: 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP^+) and rotenone. In addition, it inhibited the release of mitochondrial apoptotic-inducing factors, induced by 6-OHDA, MPP^+, and rotenone into the cytoplasm and maintained the mitochondrial membrane potential. Moreover, inhibition of HO-1 by a chemical agent (protoporphyrin-IX) blocked the neuroprotective actions of the peptide against the neutotoxin agents, while its actions were independent of the Akt signalling pathway, since the peptide did not activate Akt and inhibition of Akt did not block its protective action on SH-SY5Y cells. Peripheral or central administration of IGF-1Ec E peptide in 6- OHDA-lesioned rats had a protective role against the development of contralateral forelimb under-utilization, reduced ipsilateral nigral DA cell body loss and attenuated tyrosine hydroxylase fiber loss in the ipsilateral stratum, through a mechanism independent of IGF-1R activation. Peripheral administration of IGF-1Ec E peptide upregulated HO-1 expression in striatal and midbrain tissue.

Our research team has extensively studied the effects of a synthetic peptide corresponding to the last 24 amino acids of the human sequence of the E domain of the IGF-1Ec isoform (Table 1) in a range of *in vitro* models: C2C12 murine skeletal muscle-like cells,[53] H9C2 rat myocardial cells,[58] KLE human endometrial adenocarcinoma cells,[65] human normal prostate epithelial cells (HPrEC) and prostate cancer (PC-3 and LNCaP) cells[66] and MG-63 human osteoblast-like osteosarcoma cells.[67] We have further confirmed previous data of other reports in respect of the proliferative actions of the IGF-1Ec E peptide by specifically showing a IGF-1R-, insulin receptor (IR)- and hybrid receptor IGF-1R/IR-independent signalling of the synthetic Ec peptide i.e. the activation of ERK 1/2 along with the lack of Akt activation. It is known that the Raf-MEK-ERK pathway promotes myoblast proliferation,[78] while the PI3K-Akt pathway is linked to myogenic differentiation.[79]

In our studies, the optimum dose and time at which the maximum phospho-ERK 1/2 activation is elicited have been determined at 50 ng/ml within the first 15 minutes after exposure with the synthetic peptide. Moreover, IGF-1R activity was inhibited by using two different techniques, siRNA Knock Out and a specific antibody inhibitor, neither of which altered the effects induced by the synthetic Ec peptide. It has been postulated that the activation of ERK 1/2 in all the above cell lines is independent of the IGF-1R pathway and is possibly mediated via a unique receptor for the E domain of the IGF-1Ec isoform.

CONTROVERSIES

Muscle mass and strength loss is a common problem during aging, and, since the expression of IGF-1Ec isoform has been attributed to mechanical stimulation of the muscle and has been thought to be diminished by aging,[56,80] Kandalla et al[81] investigated the behaviour of satellite cell populations isolated from young and older individuals with respect to cell proliferation, differentiation and hypertrophy after exogenous treatment with IGF-1Ec E peptide and IGF-1. Treatment with the IGF-1Ec E peptide increased the proliferative capacity of human myoblasts isolated from neonatal and young muscle, but no effect was observed on human myoblasts isolated from old adult muscle. This could be attributed to the fact that IGF-1Ec E peptide delays the onset of senescence of neonatal and young adult satellite cells but not those derived from old adult muscle. In all cell cultures, hypertrophy associated with a significant decrease in the percentage of the reserve cells was observed, which was independent of increasing proliferation. The major conclusion was that the synthetic peptide could produce similar effects to IGF-1 by inducing an increase in the fusion index and in myosin heavy chain (MyHC) protein synthesis, and it was proposed that IGF-1Ec E peptide, similarly to IGF-1, could possibly deregulate an equilibrium between "reserve cells" and fusion-committed cells by recruiting these reserve cells into myotubes.

Mesenchymal stem cells (MSCs) are beneficial for tissue regeneration due to the fact that they are readily available, can self-renew and have the potential to differentiate into multiple cell types.[82] A study by Collins et al[83] examined the effects of synthetic peptides on cell proliferation and migration of human MSCs isolated from bone marrow. Of particular interest was the choice of synthetic peptides that were used: in addition to the biologically active IGF-1Ec E peptide peptide (Table 1), they synthesized a biologically inactive peptide by substituting a serine from a PKA site with alanine, 18 amino acids from the N-terminus and a "scramble" peptide that contained the 24 amino acids of the IGF-1Ec E peptide. Migration of the hMSCs was optimal at a concentration of 30 ng/ml and reached its peak after 20 h in response to biologically active synthetic peptide, compared to the other two peptides and the control. The chemotactic action of IGF-1Ec E peptide was attenuated both in the scrambling peptide and in the peptide with a substitution of the serine with alanine at position 18. The authors proposed that the migration effect depends on the presence of serine, which forms a PKA phosphorylation site, and that the E domain possibly has a role in mediating intracellular signalling found exclusively on the IGF-1Ec splice variant, since the serine near the C-terminus of the E peptide is not present in other IGF-1 isoforms. Proliferation of the stem cell population was only affected by IGF-1 and not by any of the other peptides. IGF-1 proliferating actions were observed only in small cells (5-9 μm) but not in larger cells (13 μm), suggesting that IGF-1 has

the capacity to increase a proportion of cells capable of differentiating into other cell types that would be useful for tissue regeneration.

A recent study has put forward an alternative hypothesis regarding the bioactivity of the E peptides, proposing that their role is, to an extent, auxiliary to the action of IGF-1.[61] The authors compared the biological actions of Ea and Eb synthetic peptides corresponding to the rodent sequence (Table 1) in an *in vitro* model of muscle formation that included cell signalling, proliferation, migration and differentiation, using the C2C12 mouse myoblast cell line. In addition, a scramble peptide was generated as a negative control. Both peptides enhanced MAPK signalling in a dose- and time-dependent manner. Maximum P-ERK 1/2 activation was elicited at 1μM in15 minutes and then disappeared by 30 minutes, and at 100 nM after 15 minutes, and remained elevated for at least 30 minutes, for Ea and Eb, respectively. To examine whether the synthetic peptides actions were dependent on the IGF-1R, they used a small molecule, NVPAEW541, that inhibits the tyrosine kinase activity of the IGF-1R in combination with the Ea or Eb peptide and IGF-1. They found that in the presence of the inhibitor, the ablation in P-ERK1/2 response was similar in Ea, Eb and IGF-1 treatments, suggesting that a functional IGF-1R is required for E peptide-induced phosphorylation. With the use of a kinase receptor activation assay (KIRA), performed on IGF-1R over-expressing mouse fibroblasts (P6) cells, they determined that the combined exposure of IGF-1 and either Ea or Eb peptide significantly increased the activation of IGF-1R compared to IGF-1 alone. Moreover, it was shown that the peptides do not activate the receptor directly, but they amplify IGF-1R activation in an IGF-1-dependent manner and, more specifically, they increase the bioavailability of the receptor for its ligand by increasing cell surface IGF-1R. To evaluate the effects of the peptides on cell proliferation and migration, C2C12 cells were treated with various concentrations of Ea and Eb peptides, in the presence of NVP inhibitor or PD, a MEK inhibitor. Proliferation was significantly increased only at 10 nM and 100 nM for Eb and treatment with optimum concentration of Eb increased proliferation, but its action was inhibited in the presence of PD or NVP. Thus, it was suggested that Eb peptide-induced proliferation requires MAPK signalling and a functional IGF-1R. Migration assay showed that only Eb peptide enhanced cell migration. PD inhibition (a MEK inhibitor) decreased Eb-induced migration effect but without reaching significance, indicating that cell migration must involve an additional pathway to MAPK. On the other hand, NVP inhibition significantly decreased cell migration, suggesting that IGF-1R is involved in the increase of migration by Eb. In the presence of Ea, both inhibitors decreased migration significantly. In addition, Ea peptide diminished the later stages of cell differentiation and maturation (causing significantly less Myh3 expression). Eb peptide did not inhibit cell differentiation, and this effect was attributed to its short half-life in the culture media. The authors found that, by incubating the synthetic peptides in 10% FBS growth media and taking aliquots at various time points for immunoblotting, Ea remained stable for 24 hours, while Eb appeared as a doublet at 30 minutes and was a smaller size than the initial peptide after 2 hours of incubation.

The conflicting results between the aforementioned studies regarding the biological actions of the IGF-1 E peptides can be attributed to various factors, such as the synthetic peptide(s) sequence used, modifications of the peptides, the concentration that was used, the duration of the cell treatment with the peptides, the variations in the cell culture conditions and the choice of inhibitors for the IGF-1R (Table 1). Moreover, fetal bovine serum (FBS) and fetal calf serum (FCS) contain IGF-binding proteins (IGFBPs) that bind IGF-1 and could inhibit its action on the cells, while the high protein concentration of serum could preserve the low molecular weight synthetic peptide and hinder its action.

The most popular choice for IGF-1 inhibition is the use of an IGF-1R neutralizing antibody that binds to its IGF-1 recognition site on the extracellular domain and blocks IGF-1 from binding and activating the receptor.[32,58,65,67,70-72,74-79] It should be mentioned that there are some disadvantages to using an IGF-1R neutralizing antibody; thus, despite the fact that it has a high specificity for IGF-1R, it can also activate downregulation of the IR due to co-downregulation of IRs in IR:IGF1R dimmers, or through endocytosis of the IR in close proximity

to IGF1R.[84] On top of that, it can lead to receptor internalization and degradation[61] and consequently to a change in IGF-1R localization. An alternative method is the inhibition of the tyrosine kinase domain of the IGF-1R. The fact that tyrosine kinase domains of IGF-1R and of IR share 85% homology and that the ATP binding cleft is 100% conserved would result in a lower specificity. In particular, a study by Garcva-Echeverrva[85] showed that NVP-AEW541 had analogous IC50 (i.e. half maximal inhibitory concentration, a measure of a compound's effectiveness in inhibiting a biological or biochemical function)[86] for IGF-1R and IR kinase domains *in vitro*. Nevertheless, a 27-fold higher affinity for the IGF-1R was observed in assays measuring phosphorylation of the receptor as an end point[91] and a lower affinity was evidenced for other tyrosine kinases, indicating a fairly specific molecule.[87] Inhibition of the IGF-1R tyrosine kinase was related to inhibition of the PI3K and MAPK signalling pathways after IGF-1 stimulation of cell cultures.[87] As already mentioned, these signalling pathways are linked to myogenic differentiation and myoblast migration, respectively.[78,79]

Noteworthy at this point is the fact that the peptides used in various studies (Table 1) were based on the human sequence and contained two rather than three arginines. Also, in order to increase peptide stability, instead of the L-form that is naturally expressed, the D-form was used. Moreover, in some cases the C-terminal of the peptide was chemically modified with the addition of a polyethelene glycol (PEG) group to overcome the blood brain barrier (BBB). The particular choices for the synthesis of the peptide raise a series of questions. It has been reported that D-arginine has pharmacological actions even in humans and can be an active molecule in mammals.[88.] PEGylation of a peptide produces alterations in the physiochemical properties and, despite the fact that it can increase their systemic retention, it may alter the binding affinity of the moiety to cell receptors;[89] it has also been reported that PEGylation of therapeutic proteins improves their efficacy by extending their half-lives and reducing concentration fluctuations.[90]

Metzger et al[91] developed an IGF-1 variant by addition of a PEG group to lysine at position 68 (PEG-IGF-1). The authors suggested that PEG-IGF-1 *in vitro* decreased the affinity for both IGF-1 and insulin receptor and slowed down the association to IGF-1 binding proteins, sustaining PEG-IGF1's anabolic activity. *In vivo* the PEGylated variant increases half-life and recruitment of IGF-1 binding proteins. Possibly a PEGylated IGF-1Ec E peptide could have similar effects and regulate its affinity for IGF-1 and insulin receptors. Thus, it could be speculated that the divergence of IGF-1 and the IGF-1Ec E peptides was due either to different biological activities or to modification alterations of the peptide that have not been clarified yet.

The use of a synthetic peptide corresponding to the human sequence on cell lines of non-human origin could not be a representative model to clarify the biological activity of the peptide. A comparative genomic study by Wallis[10] on the IGF-1 precursor sequences (signal peptide, E domains) among various mammals showed that the N-terminal region of the IGF-1 signal peptide is strongly conserved, suggesting specific function associated with nucleotide rather than protein sequence. There is a strong conservation between the Ea domain of the pro-IGF-1 and the N-terminal end of the pro-IGF-II. In regard to the Eb and Ec domains of pro-IGF-1, high variability is observed. The author commented that variation in the sequence cannot be considered as a burden on biological activity, but indicates that the activity will vary extensively among species and that testing human-derived sequences in animal models must be interpreted with extreme caution.

CONCLUSION - FUTURE PERSPECTIVES

Our knowledge of the biological mechanisms and effects of the IGF-1 E peptides is still limited. There are indications that: a) the peptides could possibly be translocated to the nucleus,[77] b) protein kinase C (PKC) activity is required to activate the peptide,[83] c) it could modulate IGF-1 signalling through the IGF-1R,[61] d) it controls IGF-1 bioavailability by preventing systemic circulation, tethering IGF-1 to the site of synthesis.[92] Another issue that has to be clarified is whether an *in vivo* peptide product equivalent to the synthetic one exists. Although full-length pro-IGF-1Ec has been identified in skeletal muscle tissue,[53] the identification of the 24 amino acid peptide has not been confirmed, despite the development of specific

polyclonal[93] and monoclonal[94] antibodies. The IGF-1 molecules aggregate at pH close to their isolectric point.[95] If the E domain peptides are involved in such aggregations, their presence in tissues could be masked. Buchanan et al,[96] using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) and reverse-phase HPLC methods, identified a 34 amino acid (3.95 kDa) peptide derived from the E peptide of pro-IGF-2 from cultured murine β cells (βTC6-F7). Possibly the use of similar techniques could provide evidence for the existence of Ec peptides *in vivo.*

Matheny et al[98] pinpointed several issues regarding the detachment of E domain from the mature IGF-1 peptide after expression of the full (Ec) precursor peptide. Pro-IGF-1 processing is mediated through the action of subtilisin-like proprotein convertase (SPC) family. SPC family relies on a multiple-basic-residue motif to direct cleavage at the scis-sile bond, with preference for Arg at the P_1 position. Studies of the role of SPC family members in pro-IGF-1Ea processing revealed that they regulate cleavage at Arg residue which could lead to the removal of the E domain from the mature IGF-1, but failed to identify possible cleavage at the boundary between exons 4-6. However, a response to the review by E. Cortes mentions that there is a putative ectopeptidase site just upstream the E peptide, thus detachment could be possible.

The detection and characterization of a novel receptor by which the IGF-I Ec peptide exerts its effects has not been identified as yet. At present, only one study[103] claimed that in osteoblasts after mechanical stimulation, the IGF-1Ec E peptide and its receptor are localized to cell nuclear, acting as messengers that transfer the information to the nuclei, to regulate cell proliferation.

Given the multiple functions of the IGF-1Ec E peptide, further investigation into their biological role is imperative, as it may provide crucial information about the mechanisms involved.

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