Regulatory Peptides 152 (2009) 48–53

Contents lists available at ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

Inhibition of compensatory renal growth by the N-terminus of a sheep-derived peptide

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ARTICLE INFO

Article history:
Received 29 April 2008
Received in revised form 25 October 2008
Accepted 27 October 2008
Available online 8 November 2008

Keywords:
Inhibitor
Peptide
Rat
Kidney
Compensatory renal growth
Sheep

ABSTRACT

The N-terminal sequence of a novel sheep-derived peptide with growth inhibitory activity has been obtained. The N-terminal fragment was chemically synthesised and designated EPL001. The kidney was chosen as the first mammalian system in which to study EPL001 since kidney growth can be accurately quantified following a surgical reduction in renal mass. Cell proliferation was measured in mouse collecting duct kidney (MCDK) cells stimulated with insulin-like growth factor I (IGF-I). Compensatory renal growth (CRG) was induced in Wistar rats and either EPL001 or an EPL001 antibody delivered by continuous renal tissue infusion. Mouse monoclonal antibodies to EPL001 were generated for immunoneutralisation, rabbit polyclonal antibodies were generated for immunohistochemistry. EPL001 had no apparent effect on IGF-I stimulated cell proliferation in MCDK cells *in vitro*, yet provoked a dose-dependent inhibition of CRG *in vivo*. An EPL001 antibody potentiated CRG, in the absence of exogenous EPL001, consistent with an inhibitory role in kidney growth for an endogenous peptide containing the EPL001 sequence. Tubular staining for epitopes to the EPL001 sequence was detected in normal human kidney sections and enhanced in renal cell carcinoma. Results support the presence of growth inhibitory activity in the N-terminus of a sheep-derived peptide with evidence for both its presence and endogenous activity in the kidney. Attempts to further characterise its structure and activity are ongoing.

1. Introduction

The concept that organ size may be controlled through the local inhibition of organ growth was proposed in the 'chalone theory' of the 1960s [1], chalones being organ-specific endogenous inhibitors of mitosis [2]. Many endogenous growth inhibitors have now been described including the tumour suppressor PTEN, which inhibits the chico/PI3-kinase signalling pathway [3], and the suppressor of cytokine signalling (SOCS-2), which inhibits GH-induced JAK2 phosphorylation and is stimulated by oestrogen [4]. During a search for endogenous inhibitors of tissue growth, biological activity was detected in fractions of sheep serum [5] containing a peptide of about 7.5 kDa and the N-terminal sequence of this peptide was obtained. The N-terminal sequence, comprising 14 amino acid residues, was synthesised and designated EPL001. EPL001 has recently been shown to increase both lifespan and fecundity in *C. elegans*, when added to the aqueous medium in which this nematode can be maintained [6]. This conjunction of properties was surprising given that the usual biological trade-off for more offspring is a shorter life [7].

The kidney was chosen as the first mammalian system in which to study the influence of EPL001 on organ growth. The evaluation of endogenous growth inhibition requires the use of experimental models of stimulated growth. In the kidney, growth can simply be provoked by the surgical reduction of renal mass. Although the liver is perhaps better known as a surgical model of organ growth, the use of the kidney possesses the advantages of reduced surgical trauma, more accurate quantification and simpler drug delivery systems. Evidence for the involvement of growth hormone [8], insulin-like growth factor I (IGF-I) [9], epidermal growth factor (EGF) [10] and vascular endothelial growth factor (VEGF) [11] in compensatory renal growth (CRG) have all previously been proposed with transforming growth factor β (TGFβ) mediating cellular hypertrophy [12]. Interventional studies would suggest a major role for IGF-I, in particular in the rat models employed in the present study [13,14]. The concept that endogenous growth inhibitors may play a role in compensatory growth was originally proposed for the liver [2]. Both positive and negative control elements are known to be up-regulated in the first few hours following nephrectomy in the rat [15].

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0167-0115/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.regpep.2008.10.011

In the present study, EPL001 was chemically synthesised and its growth inhibitory activity examined *in vitro* using mouse collecting duct cells stimulated with insulin-like growth factor 1 (IGF-I). *In vivo*, renal growth was stimulated in the rat following a reduction in renal mass and EPL001 delivered directly into kidney tissue by continuous infusion. Previous studies in which we have successfully used the technique of renal tissue infusion include the response of epithelial meschymal transdifferentiation to γ-interferon [16] and matrix protein deposition to inhibitors of the enzyme transglutaminase [17]. Monoclonal antibodies to EPL001, raised in the mouse, were also delivered into kidney tissue by continuous infusion following a reduction in renal mass in an attempt to immunoneutralise the biological activity of any endogenous analogue of the N-terminal fragment. Polyclonal antibodies to EPL001, raised in the rabbit, were employed to detect the presence of epitopes to EPL001 in the human kidney.

2. Materials and methods

2.1. Peptides

The 14 amino acid peptide Met-Lys-Pro-Leu-Thr-Gly-Lys-Val-Lys-Glu-Phe-Asn-Asn-Ile (purity 98%, molecular weight 1621) was generated from l-isomer amino acids by solid phase peptide synthesis (Model 9050, Milligen) and designated EPL001. This amino acid sequence (patent applied for) had previously been identified as the N-terminus of a peptide (circa 7.5 kDa) derived from sheep serum during a search for endogenous mammalian tissue growth inhibitors, the biological activity of which had previously been detected by organ weight reduction in the rat *in vivo* [5]. EPL001 was dissolved in sterile 0.9% saline. A second 14 amino acid peptide Lys-Gly-Asn-Glu-Val-Met-Lys-Leu-Phe-Pro-Thr-Ile-Asn-Glu was synthesised as a control peptide, designated EPL010 and also dissolved in sterile 0.9% saline. Recombinant human IGF-I was a gift from Chiron, California, USA.

2.2. Cell culture

Mouse collecting duct kidney (MCDK) cells (expressing a T antigen under the control of a temperature-sensitive and γ-interferon inducible promoter) were maintained at 33 °C in DMEM/F12 media supplemented with 5% Nuserum and recombinant γ-interferon. Cells were serum starved for 24 h at 37 °C, then stimulated with insulin-like growth factor 1 (IGF-I) 50 ng/ml. After 3 h, either EPL001 or EPL010 peptides were added and cells incubated for a further 45 h while maintaining the IGF-I concentration at 50 ng/ml. Within a single experiment, responses to each peptide concentration were performed in quadruplicate and the average obtained. The experiment was then repeated on 5 occasions. Cell proliferation was assessed using the Aqueous One Solution Cell Proliferation Assay (Promega, UK). The assay is based on the production of NADPH by dehydrogenase enzymes in metabolically active cells. The reduction of a tetrazolium substrate into a coloured formazan product was measured on 96-well plates at 490 nm. The colour generated is proportional to the cell number.

2.3. Compensatory Renal Growth (CRG)

Male Wistar rats (250–300 g, Harlan UK) were allowed free access to water and food (Teklad Global 18% protein rodent diet, Harlan UK). Anaesthesia was induced by isoflurane 5% in oxygen and maintained on isoflurane 2.5% in oxygen.

For unilateral nephrectomy (UNx), the left kidney was exposed through a flank incision and decapsulated. A perforated tissue infusion cannula was inserted from pole to pole and secured using a biological glue (Histoacryl, Braun). The cannula was attached to an osmotic infusion pump (Alzet 2002), which was then placed in a subcutaneous pocket [16]. The osmotic infusion pumps were pre-loaded with peptide and allowed to equilibrate for 4 h at 37 °C prior to implantation. Flank incisions of the muscle pan were closed by continuous suturing, while skin wounds were closed with discontinuous mattress stitches.

For 5/6 subtotal nephrectomy (SNx), in addition to the procedures described for UNx, ligatures were placed around the upper and lower poles of the left kidney. The poles were then removed using a surgical blade (the excision technique) prior to insertion of the perforated cannula through kidney tissue. SNx was undertaken to amplify the growth response and osmotic pumps were pre-loaded with antibody and allowed to equilibrate for 4 h at 37 °C prior to implantation.

Three days later the contra-lateral right kidney was exposed via a flank incision under isoflurane anaesthesia, the renal hilus ligated and the right kidney removed. After 11 days, the left kidney was removed under isoflurane anaesthesia, weighed, sectioned into four and stored in liquid nitrogen. Kidney tissue was dried to constant weight (180 °C) to determine tissue water content. Pilot studies ($n=6$) showed the wet weight of the left kidney (LK, 1.26±0.03 g) to be the same as the wet weight of the right kidney (RK, 1.26±0.04 g). This allowed the right kidney to be used as a contralateral control for the calculation of CRG employing the following formulae (a) (LK/RK)×100 following UNx and (b) (LK/RK-poles)×100 for SNx. A 10% kidney homogenate was prepared in a HEPES buffer containing proteolytic enzyme inhibitors. The homogenate was assayed for its protein [18] and DNA [19] content.

2.4. Immunohistochemistry

Kidney tissue present in a human array (SuperBioChips Laboratories, Seoul, Korea) was employed containing both normal (AA8) and tumour (BB4) sections. Epitopes were revealed by microwaving in 0.01 M citrate buffer (pH 6) and immunohistochemical staining undertaken using the Dako Autostainer Universal Staining System (Dako A/S, Denmark). Briefly, sections were treated with peroxidase blocking reagent (Envision System, Dako) (15 min) followed by CAS block (Zymed) for non-specific binding (30 min). Sections were incubated with a rabbit polyclonal antibody to EPL001 at a dilution of 1/200 (1 h, room temperature). The secondary antibody, an anti-rabbit immunoglobulin conjugated to a peroxidase labelled polymer (Envision system, Dako), was incubated (15 min, room temperature) followed by

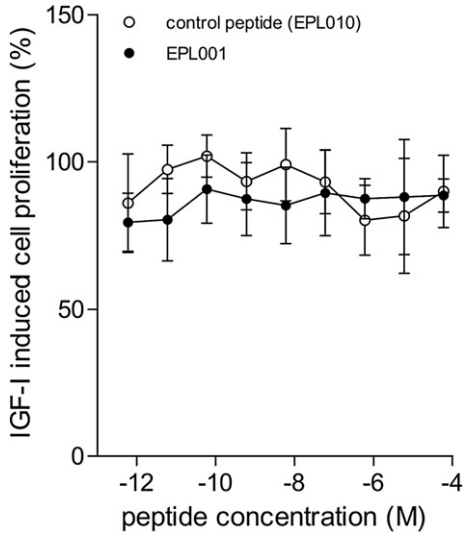

Fig. 1. Effect of N-terminal fragment EPL001 on mouse collecting duct kidney cell proliferation induced by IGF-I 50 ng/ml. The response obtained to IGF-I in the absence of test peptide was given a value of 100% within each of 5 experiments where an average was obtained from 4 repeated measurements. Another 14 amino acid peptide (EPL010) was employed as the control. Vertical bars indicate sem.