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Expression and functional analyses of liver expressed antimicrobial peptide-2 (LEAP-2) variant forms in human tissues

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ABSTRACT

The antimicrobial peptide Liver Expressed Antimicrobial Peptide-2 (LEAP-2) is proposed to function as part of the vertebrate innate immune system. However, the highly conserved nature of the LEAP-2 peptide primary structure among vertebrates suggests more fundamental physiological roles. RT-PCR analyses confirmed expression of LEAP-2 mRNA variants in human gastro-intestinal (GI) epithelial tissues and THP-1 monocytes. Three cDNA products indicative of at least three different spliced transcripts were observed. Translation of the cDNA sequences supported synthesis of transcripts encoding the secreted LEAP-2 peptide and two variants lacking signal sequences suggesting intracellular localisation. The synthesis and cytoplasmic localisation of LEAP-2 peptides in epithelia was supported by immunohistochemical analyses. Functional data suggested that LEAP-2 is not involved in the physiological response of GI epithelia to iron, nor is it mitogenic for epithelial cells or chemotactic for THP-1 monocytes. However, changes in the LEAP-2 transcript patterns associated with the challenge of THP-1 monocytes with lipopolysaccharide (100 ng/ml) were supportive of the peptides having multiple roles in the innate immune response.

1. Introduction

Epithelia comprise cell layers that, in addition to essential absorptive and secretory functions, act as physical and biological barriers that protect the host against microbial assault. As part of their defence mechanisms epithelial cells synthesise and secrete a variety of small (<10 kDa) cationic antimicrobial peptides (CAMPs), including the well characterized defensins and cathelicidins, which not only kill potential pathogens through disruption of their microbial membranes, but also promote bacterial clearance through the enhancement, inhibition or complementation of cellular functions such as chemotaxis, apoptosis, gene transcription and cytokine production [1].

While the information relating to the activities of CAMPs in the post-genomic era has increased dramatically, that related to Liver Expressed Antimicrobial Peptide-2 (LEAP-2), the sole representative of a unique four cysteine (Cys) peptide family, remains less well reported. Since its discovery in 2003, LEAP-2, a secreted 4 kDa peptide characterized by two intracellular di-sulphide bridges, has been acknowledged as a CAMP functioning as part of the vertebrate innate immune system [2,3]. Despite its name LEAP-2, encoded by a gene consisting of three exons and two introns, is actually expressed by a plethora of vertebrate epithelial tissues [2,3].

Genomic studies have predicted LEAP-2 peptides to be synthesised by a number of species including the mouse, monkey, cow and chicken [2,3]. In addition all the mammalian peptide primary structures show almost 100% amino acid (aa) homology. Between species conservation of classical CAMPs is atypical as the primary sequences of traditionally recognised CAMP molecules reflect generally the adaptation of the host species to its specific endemic microbes. This is illustrated particularly well by the defensins which in vertebrates have evolved in response to microbial challenges through gene duplication, exon shuffling and the positive selection of amino acid mutations [4,5].

This conservation of amino acid sequence suggests that LEAP-2 has, in addition to its antimicrobial activities (AMA), other functions that are of fundamental physiological importance to mammals. The observation that physiologically important molecules possess AMA is not unique. For example hepcidin, formerly known as LEAP-1, was identified originally as a CAMP but actually plays a key role in mammalian iron homeostasis [6,7]. Similarly the angiogenins, discovered nearly two decades ago, were characterized initially as having roles in vasculogenesis and tumour cell growth, but

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have been shown to exhibit bacteriocidal and fungicidal activity, suggesting an important role in systemic innate immunity [8].

This study aimed to confirm the presence of multiple LEAP-2 transcripts in epithelial and immune cells and to investigate whether the functions of LEAP-2 extend beyond microbial killing. To explore potential physiological and immunological properties of the LEAP-2 peptides, tissues and *in vitro* cell lines, modelling gastro-intestinal and liver epithelia, and cell monocytes were employed.

2. Materials and methods

2.1. Cell lines

The culture and seeding of Caco-2 cells on Transwell (Costar) filter inserts were as previously described [9]. HepG2 cells were cultured in six well plastic plates in 5% CO_2 at 37 °C in growth medium consisting of Eagle's Minimal Essential Medium containing 1% 200 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin solution (Invitrogen) and 10% foetal calf serum.

For the iron (Fe^{2+}) challenge experiments the growth media of the Caco-2 cells, cultured as polarised monolayers, and the HepG2 cells, cultured as monolayers, was replaced with medium containing 100 μM Fe SO_4 for time periods of up to four hours. After the appropriate incubation RNA was extracted from the cells for semi-quantitative analyses of Hepcidin (LEAP-1) and LEAP-2 expression.

THP-1 promonocytic cells (a kind gift of Dr. S. Ali, Newcastle University) were cultured in plastic flasks (Corning, UK) at 5% CO_2 and 37 °C in RPM1-1640 medium (Sigma, Poole, UK) supplemented with 1% 200 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin solution (Invitrogen) and 10% foetal calf serum (Sigma). THP-1 pro-monocytes were converted to THP-1 monocytes by incubation with 100 nM 1α,25-dihydroxy-vitamin D_3 (Merck Chemicals, Nottingham, UK) for 48 h. Further differentiation to THP-1 macrophages was performed by subsequent incubation with 50 ng/ml phorbol myristate acetate (PMA) for 48 h. Dendritic cells were derived from primary monocytes as follows. The primary monocytes were obtained from buffy coat fractions of human blood (national Blood Service, Newcastle, UK). The peripheral blood mononuclear cell population (PBMC) was extracted from the buffy coat fraction using Histopaque-1077 (Sigma). PBMCs were cultured in complete RPM1-1640 medium for 24 h at 37 °C with 5% CO_2. After 24 h the non-adherent cell population was removed by repeated washes in RPM1-1640 medium to reveal adherent primary monocytes. Primary monocytes were converted to dendritic cells by incubation in RPMI medium supplemented with 10 ng/ml granulocyte–macrophage stimulating factor (GMCSF) and 50 ng/ml interleukin-4 for 7 days, changing the medium every other day. Differentiation was confirmed by morphological changes and analysis of CD1a, CD11C, CD14, HLA-DR and CD83 by FACS.

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

Total RNA was isolated from the cell lines using the SV Total RNA Isolation System (Promega) and subjected to reverse transcription (RT) using random hexamer primers. To amplify LEAP-2, RT-PCR was performed as previously described using a primer pair designed to exon 1: F-5′CAAGATGTGGCACCTCAAAC3′ and exon 3: R-5′GCATTGTCGGAGGTGACTG3′ and which detected all known variants of LEAP-2 [10]. For each RNA sample a RT negative control was generated in which reverse transcriptase was omitted. PCR conditions included 15 min at 95 °C, 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, followed by a final extension step of 10 min at 72 °C. The cDNA products were separated on 1% agarose ethidium bromide gels and visualised using UV light. All cDNA products were verified by sequencing. For relative quantification of template and sample RNA concentrations all reactions were measured in the linear exponential phase, 15 cycles for the 18S ribosomal RNA primer pair and 30 cycles for each antimicrobial primer pair. Product optical density was determined on an AlphaImager 1200 gel documentation and analysis system (Flowgen). The Hepcidin and 18S primers were as previously described [10].

2.3. Western analyses

HCT-8 cells were lysed in 0.1% Triton X-100. Samples were separated on an SDS-12.5% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Hybond P; Amersham). The membrane was incubated overnight at 4 °C in a blocking solution of phosphate-buffered saline (PBS), 0.1% (vol/vol) Tween 20, and 5% (wt/vol) milk powder containing a 1:5000 dilution of anti-LEAP-2 antibody (Phoenix Pharmaceuticals, Germany). The membrane was washed in PBS-Tween 20 (0.1%) and incubated with a secondary anti-rabbit IgG antibody at room temperature for a further 2 h. Following further washing, as above, a chemiluminescence detection reaction was performed by using ECL Plus (Amersham), and the membrane was exposed to X-ray film according to the manufacturer's recommendations.

2.4. Immunofluorescent staining

7 μm frozen sections of the respective tissue were fixed in 2% paraformaldehyde, permeabilised using 0.1% Triton X-100 in PBS, and incubated in 10% goat serum to block any non-specific protein binding. Sections were incubated overnight at 4 °C with 1:500 dilution of anti-LEAP-2 antibody (Phoenix Pharmaceuticals). After overnight incubation, any unbound primary antibody was removed by washing in (phosphate-buffered saline) PBS. For each staining protocol negative controls in which the primary antibody was omitted were performed. The secondary antibody goat anti-rabbit TRITC, (Chemicon), diluted 1:50 in PBS, was incubated for 1 h at room temperature and any unbound material removed by washing. The slides were air-dried for 5 min, mounted with Vectashield (Vector Laboratories, Burlingame, USA), and imaged on a Leica TCS LASER scanning confocal microscope.

2.5. Chemotaxis and cell proliferation

The chemotactic potential of LEAP-2 was assessed using the method of Ali et al. [11]. Essentially synthetic mature LEAP-2 peptide (Phoenix Parmaceuticals) was diluted to the appropriate concentrations (1–500nM) in 1 ml medium, each dilution added to the lower compartments of a 24 microchemotaxis chamber and 3 μm pore membrane inserts added (Corning). After a 30 min incubation at 37 °C, the upper compartment of the filter was loaded with 500 μl of THP-1 cells (1×10^6) stimulated overnight with IFNγ (300 U/ml). After 90 min at 37 °C the filters were removed and placed in a 24 well plate containing methanol. After 2 h at −20 °C the methanol was decanted off, the filters washed with distilled water, 0.5 ml of Mayer's haemotoxylin added to each filter and left for 30 min at room temperature. After removal of the stain, the filters were washed with 1× SCOTTS water and incubated at room temperature for 5 min before washing with tap water and then air-drying. Once dry the filters were mounted onto glass slides in DPX and visualised under high power field (HPF). Chemotaxis was assessed by counting the mean number of migrant cells per HPF. Negative controls contained only medium in the lower chamber or THP-1 cells in both chambers. The positive control used 10 ng/ml FMLP (Formyl Met-Leu-Phe) an activator of chemotaxis.