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Inhibition effect of enteropeptidase on RANKL–RANK signalling by cleavage of RANK

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ABSTRACT

Enteropeptidase can cleave trypsinogen on the sequence of Asp-Asp-Asp-Asp-Lys and plays an important role in food digestion. The RANKL–RANK signalling pathway plays a pivotal role in bone remodelling. In this study, we reported that enteropeptidase can inhibit the RANKL–RANK signalling pathway through the cleavage of RANK. A surrogate peptide blocking assay indicated that enteropeptidase could specifically cleave RANK on the sequence NEEDK. Osteoclast differentiation assay and NF-κB activity assay confirmed that enteropeptidase could inhibit osteoclastogenesis in vitro through the cleavage of RANK. This is the first study to prove that the RANKL–RANK signalling pathway can be inhibited by cleavage of RANK instead of targeting RANKL.

Structured summary of protein interactions:
EP cleaves **hRANK** by cleavage assay (View interaction)
EP cleaves **mRANK** by cleavage assay (View interaction)

1. Introduction

Enteropeptidase (EP), a type II transmembrane serine protease localised to the brush border of the duodenal and jejunal mucosa, is synthesised as a zymogen (proenteropeptidase), which requires activation by duodenase [1] or trypsin. Trypsinogen is specifically cleaved after the sequence of Asp-Asp-Asp-Asp-Lys, which is highly conserved in vertebrates. This cleavage results in trypsin-dependent activation of other pancreatic zymogens, such as chymotrypsinogen, proelastase, procarboxypeptidase and prolipase in the lumen of the gut [2,3]. Diarrhoea, oedema, vomiting, hypoproteinaemia, anaemia, and failure to gain weight are common symptoms of patients with congenital EP deficiency during early infancy [4,5]. Conversely, duodeno-pancreatic reflux of proteolytically active EP can cause acute pancreatitis [6].

EP is a two-chain polypeptide consisting of an N-terminal ~120 kDa heavy chain disulphide linked to a C-terminal ~47 kDa light chain including a chymotrypsin-like serine protease domain [7]. The membrane association of the enzyme is not required for substrate recognition since trypsinogen activation was not impaired when the transmembrane domain of EP was deleted. The catalytic capacity of the light chain in the absence of the heavy chain decreased by approximately 500-fold compared to the complete EP, suggesting that the heavy chain is necessary for optimal cleavage. However, the light chain of EP was sufficient for the normal recognition of substrate target sequences [8]. In 1998, Lu and Sadler summarised the features of substrates required by EP [7]: the substrates of it should

Abbreviations: EP, enteropeptidase; BMM, bone marrow-derived macrophage; RANKL, receptor of activator of NF-κB ligand; RANK, receptor of activator of NF-κB; TNFRSF18, tumour necrosis factor receptor superfamily 18; M-CSF, macrophage-colony stimulating factor; TRAP, tartrate-resistant acid phosphatase

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contain Lys/Arg residue at position P1 and four Glu/Asp residues at positions P2–P5, but more variation is shown at position P5. Receptor of activator of NF-κB (RANK) is expressed on mammary gland cells, osteoclast precursors, intestinal epithelial cells and some cancer cells. There is peptide motif with the sequence Asn-Glu-Glu-Asp-Lys (NEEDK) (Fig. 1) in the sequence of RANK, which indicates that RANK may be recognised and cleaved by EP. Since RANK plays a pivotal role in the differentiation of osteoclasts, we predicted that EP could inhibit the differentiation of osteoclasts by cleaving RANK expressed at the surface of osteoclast precursors. There are several inhibitors which could block RANKL–RANK signalling, such as denosumab [9], an antibody drug approved by the FDA, OPG-Fc [10], RANK-Fc [11], and peptides that simulate the interaction between RANKL and RANK [12,13]. However, all these strategies were based on the blockade of RANKL. By cleavage of RANK EP would be the first inhibitor for RANK–RANKL signalling by targeting RANK instead of RANKL.

The aim of this study was to confirm the potential ability of EP to inhibit osteoclast differentiation by the cleavage of RANK. In this study, RANK could be efficiently cleaved by EP, this was demonstrated by bands shift on SDS–PAGE. The inhibition effect of osteoclast development was demonstrated in a model of osteoclast differentiation with bone marrow-derived macrophages (BMMs) in the presence of EP.

2. Materials and methods

2.1. Cell lines, reagents and protein purification

The Raw264.7 cell line was purchased from American Type Culture Collection and propagated in culture according to the manufacturer's protocol. BMMs were isolated from the bone marrow, which was obtained by flushing femurs and tibiae from 7-wk-old BALB/c mice as previously reported [14]. Human EP was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TRAP kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-RANK (Ab10498) antibody was purchased from Abcam (Hong Kong, China). The rat anti-mouse RANKL mAb (IK22.5) was the gift from Dr. Hisaya Akiba (Juntendo University, JP). Phenylmethanesulfonyl fluoride (PMSF) was purchased from MP Biomedical (Santa Ana, California, USA). Macrophage-colony stimulating factor (M-CSF) was purchased from eBioscience (San Diego, CA, USA). The Cell Counting Kit was purchased from Dojindo Laboratories (Kumamoto, Japan).The NF-κB luciferase plasmid was the gift from professor Xin Ye (Institute of Microbiology, CAS, China). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA).The SuperSignal West Pico Trial Kit was purchased from Thermo (Rockford, USA). The purifications of recombinant human RANK, mouse RANK and mouse RANKL were performed as previously reported [14,15]. TNFRSF18 was prepared by our laboratory using standard E coli expression system.

2.2. The synthesis of a surrogate peptide

A surrogate peptide of RANK, DTWNEEDKCLL was synthesised on 4-MeBHA-resin using manual 'in situ neutralisation' Boc chemistry protocols for stepwise SPPS[16]. Boc amino acids were used with the following side chain protection: Asp(OcHxl), Asn(Xan), Glu(OcHxl), Cys(4-CH3Bzl), Lys(2C1-Z), Thr(Bzl), Glu(OcHex), Trp(CHO). Other amino acids were used side chain unprotected. The synthesis was carried out on a 0.25 mmol scale. Prior to HF deprotection and cleavage, the CHO group of the residue Trp was removed from the resin-bound peptide segment by treatment with 10% v/v piperidine plus 5% v/v water in DMF for 2 h at 0 °C. After removal of the N-αBocgroup, the peptide was cleaved from the resin and simultaneously deprotected by treatment at 0 °C for 1 h with anhydrous HF containing 5%v/v p-cresol as scavenger. After removal of HF by evaporation under reduced pressure, the crude peptide was precipitated and washed with diethyl ether, then dissolved in 50% aqueous acetonitrile containing 0.1% TFA and lyophilised. Analytical RP-HPLC was performed on a Shimadzu LC-15C system with a C-18 silica column (4.6 × 250 mm, Venusil ASB150 Å, 5 μm, Bonna-Agela Technologies, China) at flow rate of 1 ml/min. The peptide was eluted from the column using a gradient of acetonitrile/0.08% TFA (solvent A) versus water/0.1% TFA (solvent B). Preparative HPLC of the crude peptide after SPPS was performed on Shimadzu LC-15C system on aVenusil ASB C-18 (250 × 10 mm, 150 Å, 5 μm, Bonna-Agela Technologies, China) column.

2.3. RANK cleavage assay

Recombinant mouse, human RANK, and the mixture of recombinant mouse RANK and RANKL at different ratios were incubated with different concentrations of EP at 37 °C for six hours. Recombinant mouse RANK and EP were incubated with PMSF at 37 °C for six hours. TNFRSF18 was treated at the same conditions. Different concentrations of the surrogate peptide were added into the mixture of mouse RANK and EP then incubated at 37 °C for six hours. All the samples were detected by SDS–PAGE. The recombinant mouse RANK was incubated with or without EP at 37 °C for six hours, and the samples were detected by Western blotting. All proteins, proteases and peptide above were dissolved in PBS.

2.4. Differentiation of BMMs

BMMs were cultured in α-MEM media containing 10% foetal bovine serum (FBS) and 25 ng/ml M-CSF with different concentrations of EP at a density of 1×10^5 cells per well in a 24-well culture plate. After adding EP for 1 day, 20 ng/ml mouse RANKL was added to each well except for the control and the EP alone group. The media with fresh growth factors was changed every two days. Ten days later, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Surrogate peptide

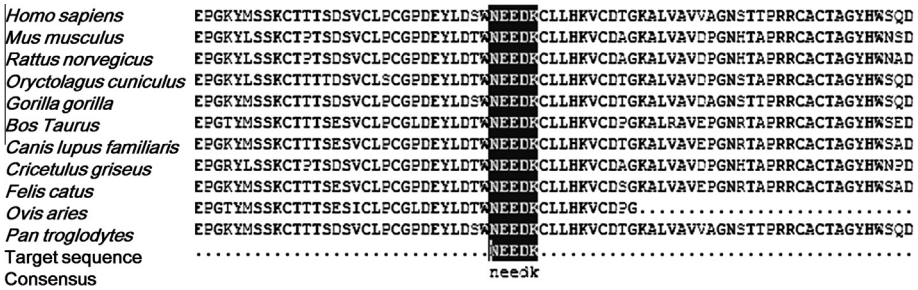

Fig. 1. Sequence alignment of the extracellular domain of RANKs from different species.

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