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Knockdown of legumain inhibits cleavage of annexin A2 in the mouse kidney

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ABSTRACT

Legumain (EC 3.4.22.34) is an asparaginyl endopeptidase. Strong legumain activity was observed in the mouse kidney, and legumain was highly expressed in tumors. We previously reported that bovine kidney annexin A2 was co-purified with legumain and that legumain cleaved the N-terminal region of annexin A2 at an Asn residue *in vitro*. In this study, to determine whether annexin A2 is cleaved by legumain *in vivo*, siRNA-lipoplex targeting mouse legumain was injected into mouse tail veins. Mouse kidneys were then isolated and the effect of knockdown of legumain expression on annexin A2 cleavage was examined. The results showed that both legumain mRNA and protein expression levels were decreased in the siRNA-treated mouse kidneys and that legumain activity toward a synthetic substrate, Z-Ala-Ala-Asn-MCA, was decreased by about 40% in the kidney but not in the liver or spleen. Furthermore, cleavage of annexin A2 at the N-terminal region was decreased in the mouse kidney that had been treated with the legumain siRNA-lipoplex. These results suggest that legumain siRNA was delivered to the kidney by using LipoTrust and that the reduced legumain expression inhibited legumain-induced degradation of annexin A2 *in vivo*.

1. Introduction

Legumain (EC 3.4.22.34) is an asparaginyl endopeptidase belonging to the cysteine peptidase C13 family [1]. Legumain activity has been detected in a number of mammalian tissues, including the kidney, placenta, spleen, liver and testis, and the highest level of activity was detected in the kidney [2]. We previously reported that legumain is mainly expressed in proximal tubules of the rat kidney [3]. We further reported that legumain might have an important role in remodeling of the extracellular matrix through degradation of fibronectin in renal proximal tubular cells [4]. It has recently been suggested that legumain plays an important role in tumor growth/metastasis, carotid artery-atherosclerosis development [5–10], hemophagocytic syndrome [11] and formation of human unstable carotid plaque [12].

Annexin A2, also named annexin II, calpactin 1, lipocortin II, chromobindin 8 and placental anticoagulant protein IV, was co-purified with legumain from the bovine kidney, and co-purified annexin A2 lacked the N-terminal region [3]. The N-terminal region of annexin A2 contains both p11 subunit-binding sites required for hetero-tetramer formation of annexin A2 and phosphorylation sites of $pp60^{src}$ and protein kinase C (PKC) [13]. Annexin A2 is abundantly expressed in the receptor-recycling compartments of rat liver hepatocytes [14] and is required for aquaporin 2 (AQP2) trafficking in renal cells [15,16]. Furthermore, it has been shown that the N-terminal region of annexin A2 has a role in endosomal membrane fusion [17] and that phosphorylation of Tyr-23 is essential for proper endosomal association and for annexin A2 function [18].

Cationic liposomes have been used in drug delivery systems, and LipoTrust, a novel cationic liposome, has been used as an efficient delivery system *in vivo* [19,20]. In this study, to examine annexin A2 degradation by legumain *in vivo*, siRNA-lipoplex targeting mouse legumain was injected into the mouse tail veins. The results showed that both the expression levels of legumain mRNA and protein and the legumain activity toward a synthetic substrate were decreased and that degradation of annexin A2 at an N-terminal Asn residue was inhibited in legumain siRNA-lipoplex-treated mouse kidneys, indicating an important role of legumain in annexin A2 functions.

Abbreviations: Z, benzyloxycarbonyl; MCA, methylcumarinamide; siRNA-lipoplex, siRNA-cationic liposome complex; NEM, *N*-ethyl maleimide; 2-ME, 2-mercaptoetanol; PBS, phosphate buffered saline.

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2. Materials and methods

2.1. Materials

Sequences of siRNA targeting mouse legumain were designed by Hokkaido System Science (Sapporo, Japan) using the B-Algo™ algorithm. The sense and antisense strands of siRNAs and LipoTrust™ *EX* Oligo were also chemically synthesized by Hokkaido System Science. NIH3T3 cells were purchased from American Tissue Culture Collection (Manassas, VA, USA). RNA*later* and RNeasy Mini were purchased from Qiagen (Venlo, Netherlands). SuperScript III, Bioanalyzer capillary chip and Z-Ala-Ala-Asn-MCA were obtained from Invitrogen (St. Louis, MO, USA), Agilent Technologies (Santa Clara, CA, USA) and Peptide Institute (Osaka, Japan), respectively. Recombinant human annexin A2 and human legumain were purchased from ProSpec-Tany TechnoGene (Ness Ziona, Israel) and R&D Systems (Minneapolis, USA), respectively. Balbc mice were purchased from Sankyo Lab. (Tokyo, Japan). All other chemicals were of analytical grade and purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Preparation of anti-annexin A2 antibodies

Annexin A2 purified from the bovine kidney was used as an immunogen to establish rabbit anti-annexin A2 polyclonal antisera. Briefly, one hundred 50 μg of purified annexin A2 dissolved in PBS was first injected into rabbits and then injected five times as booster injections at 10-day intervals. Antigen solutions were mixed with equal volumes of complete adjuvant and with incomplete adjuvant and were used for the initial and subsequent injections, respectively. An IgG fraction of an anti-annexin A2 antibody was purified from antisera using a protein A-Sepharose column.

2.3. Western blotting and antibodies

To examine the degradation levels of annexin A2 and expression levels of legumain in the mouse kidney, proteins were extracted from cultured cells or from the mouse kidney in a solution containing 50 mM sodium citrate buffer pH 5.0. Proteins were then separated on a 12.5% polyacrylamide gel and subjected to Western blotting with respective antibodies. Proteins on the membrane were reacted with an IRDye 800- (Rockland, Philadelphia, PA, USA) or Alexa Fluor 680-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) and visualized by using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE, USA). Antibodies used were anti-legumain (1:500, Santa Cruz, CA, USA) and anti-annexin II (1:1000) antibodies.

2.4. Inhibition of annexin A2 degradation in bovine kidney homogenates

To examine annexin A2 degradation by legumain under an acidic condition, 8 μg of bovine kidney homogenates was incubated in a solution containing 50 mM sodium citrate (pH 5.0) and 2 mM 2-mercaptoethanol in the presence or absence of 1 mM *N*-ethylmaleimide for 30 min at 37 °C, separated on a 12.5% SDS–polyacrylamide gel, and subjected to Western blotting with an anti-annexin A2 antibody.

2.5. Preparation of siRNAs

Three siRNAs targeting mouse legumain (B-Bridge, Cat#: SMF27A-1537-1, SMF27A-1537-2 and SMF27A-1537-3) were used. Nucleotide sequences of the siRNAs used were as follows: LGMN (SMF27A-1537-1; siRNA1): sense, 5′-GGGCAAAGGGUCUGGAAAATT-3′ and antisense, 5′-UUUUCCAGACCCUUUGCCCTT-3′; LGMN (SMF27A-1537-2; siRNA2): sense, 5′-GGGAAACUGCUGAGAGACATT-3′ and antisense, 5′-UGUCUCUCAGCAGUUUCCCTT-3′; and LGMN (SMF27A-1537-3; siRNA3): sense, 5′-GGGAAGGAAUCGUCUGAGATT-3′ and antisense, 5′-UCUCAGACGAUUCCUUCCCTT-3′. Nucleotide sequences of the negative control siRNA were as follows: sense, 5′-CGUACGCGGAAUACUUCGATT-3′ and antisense, 5′-UCGAAGUAUUCCGCGUACGTT-3′.

2.6. Formation of transfection complex

siRNAs were transfected into NIH3T3 cells by using LipoTrust™ *EX* Oligo (Hokkaido System Science). A vial of LipoTrust was reconstituted of 1 ml of nuclease-free water, and 10 μl of LipoTrust solution was mixed with 100 pmol of siRNA. After incubation of the mixture for 20 min at room temperature, legumain siRNA-lipoplex was formed. For *in vivo* study, a vial of LipoTrust was reconstituted of 1 ml of nuclease-free water containing 1.6, 3.2 and 6.4 nmol siRNAs. After incubation of the mixture for 20 min at room temperature, legumain siRNA-lipoplex was formed.

2.7. Characterization of legumain siRNA-lipoplex

The size dispersion of legumain siRNA-lipoplex was determined by N4 Plus (Beckman Coulter, Miami, FL, USA) and the zeta potential was measured using a Zeta Potential Analyzer Ver.3.47 (Brookhaven Instruments Corp., NY, USA).

2.8. Cell culture and knockdown of legumain expression in vitro

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were transfected with Legumain siRNA-lipoplex. 48 hours after transfection, cells were collected and their total RNAs were extracted.

2.9. Knockdown of mouse legumain expression in vivo

C57BL/6 mice were purchased from Sankyo Lab. (Tokyo, Japan). Eighty microliters of each of the legumain siRNA-lipoplex solutions (1.6 and 3.2 and 6.4 nmol/ml siRNA) was injected into mouse tail veins. Lipoplex solution containing negative control siRNA was also injected. At 48 h after injection, mouse kidneys were extracted and their total RNAs and proteins were extracted. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Hokkaido University (permit number: 08-0484).

2.10. Quantification of mRNA level by reverse transcription polymerase chain reaction

Tissues were dissected and quickly frozen in an RNAlater solution. Total RNAs were prepared from approximately 20 mg of the kidney using an RNeasy mini kit, and quality of RNA was examined using Bioanalyzer. Reverse transcription was carried out in a mixture containing 500 ng of total RNAs, Superscript III and specific primers under the conditions of 96 °C for 15 min, 32 cycles of 96 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min by using a QuantiTect SYBER Green kit (Qiagen) and Opticon II real-time PCR analyzer (Bio-Rad, Hercules, CA, USA). β-Actin (ACTB) mRNA was also amplified and used as an internal control.