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Enzyme-linked immunosorbent serum assays (ELISAs) for rat and human N-terminal pro-peptide of collagen type I (PINP) — Assessment of corresponding epitopes

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ABSTRACT

Objectives: The present study describes two newly developed N-terminal pro-peptides of collagen type I (PINP) competitive enzyme-linked immunosorbent assays (ELISAs) for the assessment of corresponding PINP epitopes in the rat- and human species.

Methods: Monoclonal antibodies were raised against corresponding rat and human PINP sequences and competitive assays were developed for each species. They were evaluated in relevant pre-clinical or clinical studies.

Results: The antibody characterizations indicated that PINP indeed was recognized. Technical robust assays were obtained. Rat PINP and tALP showed similar patterns in the gold standard osteoporosis rat ovariectomized (OVX) model. No liver contribution was observed in the liver fibrosis rat bile duct ligation model (BDL). In an osteoporosis study, the human serum PINP levels were significantly decreased after ibandronate treatment compared to placebo.

Conclusions: The two corresponding PINP assays were specific and these bone turnover markers may improve translational science for the evaluation for bone-related diseases.

Introduction

Osteoporosis is one of the major bone-related diseases of the Western world and the associated fragility fractures in postmenopausal women remain a major healthcare burden [1]. Currently, the prevalence of osteoporosis in industrialized countries is approximately 40% in women in their 60s, and 70% in women in their 80s [1]. A key factor of the pathogenesis of osteoporosis is an imbalance between the function of two key players of bone turnover, namely the bone resorbing osteoclasts and the bone-forming osteoblasts. Estrogen deficiency arising after the menopause leads to acceleration of bone turnover, with the rate of bone resorption exceeding the rate of bone formation. This in turn leads to a net negative calcium balance and consequent demineralisation of bone [2,3].

The rate of bone resorption and formation can be estimated by immunoassays measuring the serum concentration or urinary excretion of different target molecules produced specifically during these cellular processes [4]. Over the past decade, a wide array of such immunoassays has been launched for *in vitro*, *ex vivo*, and *in vivo* investigations. Their systematic validation led to an appreciation of their utility in biomedical research into the pathogenesis of osteoporosis, improving clinical diagnosis, and the evaluation of novel treatment modalities [3]. The most common markers of bone resorption and formation are those measuring peptide fragments derived from collagen type I, such as the C-telopeptide of collagen type I (CTX-I) and N-telopeptide of collagen type I (NTX); N-terminal pro-peptide of collagen type I (PINP) and crosslinks of collagen type I D-pyridinolines (DPYR) [5–11].

The US Food and Drug Administration (FDA) has stated that "Additional biomarkers and additional surrogate markers are needed to guide product development" [3]. Accurate, early, prediction of the efficacy of drugs under development might not only avoid wasting pharmaceutical company funding of research, but also prevent patients being exposed to experimental drugs that are unlikely to be effective. Most drugs are tested first in animals, and if these studies indicate an acceptable risk–benefit ratio, are subsequently trialed in humans in a shift that is referred to as translational science. Thus it may be interesting to investigate whether biomarkers of efficacy in animals may also be predictive in humans. Biochemical markers detecting the same epitopes produced by both animals and humans during certain disease processes may provide such an opportunity.

We describe two new markers of the N-terminal pro-peptide of type I collagen (PINP) produced by rats and humans, using monoclonal

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antibodies. We present assays specifically designed to detect corresponding epitopes produced by both rats and humans (Fig. 1). The monoclonal antibodies we discovered were characterized and adapted to ELISA development.

Materials and methods

Reagents

All reagents were standard high-quality chemicals from companies such as Merck (Whitehouse Station, NJ, USA) and Sigma Aldrich (St. Louis, MO, USA). The synthetic peptides used for monoclonal antibody production were purchased from the Chinese Peptide Company, Beijing, China.

Monoclonal antibodies

4–6 week old Balb/C mice were immunized subcutaneously in the abdomen with 200 μL emulsified antigen 50 μg/immunization using Freund's incomplete adjuvant (Rat: OVA-C-PEEYVSPDAEVIG); (Human: KLH-CGG-PDGSESPTDQETT). Alignment for PINP for the two species was performed using the NLP CLUSTALW software at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html. Consecutive immunizations were performed at two week intervals, until stable elevated sera titer levels were reached. At each bleeding, the serum titer was investigated and the mouse with the highest titer was selected for fusion. The selected mice were boosted intravenously with 50 μg rat or human PINP, respectively, in 100 μL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion.

Fusion and antibody screening

The fusion procedure has been described elsewhere [12]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were cloned using a semi-solid medium method and plated into 96-well microtiter plates for further growth. Here limited-dilution was used to obtain monoclonal growth. Supernatants were screened using an indirect ELISA; biotinylated peptide Biotin-K-PEEYVSPDAEVIG-VEG (rat PINP) or Biotin-K-PDGSESPTDQETT-QV (human PINP) was used as catcher peptides on streptavidin coated microtitre plates respectively. The specificities of clones were further tested towards free peptide PEEYVSPDAEVIG (rat PINP) or PDGSESPTDQETT-QV (human PINP) respectively together with a non-sense peptide.

Characterization of clones

Native reactivity and peptide affinities of the monoclonal antibodies were assessed by displacement of rat or human third trimester amniotic fluid and serum in preliminary ELISA assays using 10 ng/mL biotinylated peptide coater on streptavidin coated microtitre plates and the supernatant from growing monoclonal hybridoma. Human amniotic fluid was obtained from 30 women undergoing elective lower segment Caesarean section at the Beijing obstetrics gynecology hospital over a 2 month period. 100–200 ml amniotic fluid was collected directly after incision and the fluid was stored at −20 °C until use. The local Ethical board had approved the study and all women provided written consent prior to the collection. Rat amniotic fluid was drawn from the uterus of pregnant Wistar rats two days prior to the expected birth. The isotyping of the monoclonal antibodies was performed using the Clonotyping System-HRP kit, cat.5300-05 (Southern Biotech, Birmingham, AL, USA). Human PINP clones were additionally tested for cross-reactivity toward the corresponding N-terminal pro-peptide of collagen type II (PIINP; TASGQPGPKGQKGE) and N-terminal pro-peptide of collagen type III (PIIINP; CPQPPTAPTRPPNG) due to high homology between PI/II/IIINP. Western blot analyses were carried out of selected clones. Rat or human amniotic fluid was mixed with 2× denaturing loading buffer, heated and loaded on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and run for 2 h at 20 mA per gel. The protein bands were blotted onto polyvinylidene fluoride (PVDF) membrane in a blotting chamber 1 h at 60 mA per gel. The membrane was blocked in blocking buffer (5% skimmed milk in phosphate buffered saline buffer) overnight at 4 °C and incubated 1 h with hybridoma culture supernatant diluted 1:200 in blocking buffer shaking at RT. Normal mouse IgG antibody cat.015-000-003 (Jackson Immunoresearch, West Grove, PA, USA) was used as a primary antibody as control for specificity and secondary antibody alone to control the background. The membrane was incubated 1 h shaking with secondary horse radish peroxidase (HRP) labeled antibody, cat.31432 (Thermo Scientific, Waltham, MA, USA) diluted 1:5000 in blocking buffer. Between all steps the membrane was washed 3×5 min in tris buffered saline (TBS) buffer. Bands were visualized using chemiluminescence detection kit (ECL) (Abcam, Cambridge, UK) and developed on film for 2 min (Kodak, Perkin Elmer, Waltham, MA, USA). The selected clones were purified using Protein G columns according to the instructions of the manufacturer (GE Healthcare Life Science, Little Chalfont, Buckinghamshire, UK).

Rat and human PINP ELISA protocol

We labeled the selected monoclonal antibodies NB46-3G1 (rat PINP) and NB15-5A7 (Human PINP) with horse radish peroxidase (HRP) using

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CLUSTAL W (1.82) multiple sequence alignment (Grey = PINP) *** Helix structure

sp|P02452|CO1A1_HUMAN      MFSFVDLRLLLLLAATALLTHGQEEGQVEGQDEDIPPITCVQNGLRYHDR
sp|P02454|CO1A1_RAT        MFSFVDLRLLLLLGATALLTHGQE---------DIPEVSCIHNGLRVPNG
                           *************.**********         *** ::*::****  :

sp|P02452|CO1A1_HUMAN        'WKPEPCRICVCDNGKVLCDDVICDETKNCPGAEVPEGECCPVCPDGSE
sp|P02454|CO1A1_RAT        ETWKPDVCLICICHNGTAVCDGVLCKEDLDCPNPQKREGECCPFCPEEYV
                           :.***: * **:*.**..:**.*:*.*  :**..:  ******.**:

sp|P02452|CO1A1_HUMAN      SPTDQETTGVEGPKGDTGPRGPRGPAGPPGRDGIPGQPGLPGPPGPPGPP
sp|P02454|CO1A1_RAT        SP-DAEVIGVEGPKGDPGPQGPRGPVGPPGQDGIPGQPGLPGPPGPPGPP
                           ** * *. ********.**:*****.****:********************

sp|P02452|CO1A1_HUMAN      GPPGLGGNFAPQLSYGYDEKSTGGISVPGPMGPSGPRGLPGPPGAPGPQG
sp|P02454|CO1A1_RAT        GPLGLGGNFASQMSYGYDEKSAG-VSVPGPMGPSGPRGLPGPPGAPGPQG
                           ** *******.*:********:* :************************
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Fig. 1. Position of the corresponding rat PINP (—) and human PINP (−−−−−) sequences within the alpha 1 chain of the N-terminal pro-peptide of collagen type I. The alignment was performed using the NLP CLUSTALW software.