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Combination of PTH (1-34) with anti-IL17 prevents bone loss by inhibiting IL-17/N-cadherin mediated disruption of PTHR1/LRP-6 interaction☆

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ABSTRACT

Combinations of anabolic and anti-resorptive agents have potential to improve bone density more than either agent alone. In this study, we determine the combining effect of anti-IL17 antibody and PTH (1-34) in mitigation of ovariectomy induced bone loss. Ovariectomized BALB/c female mice were treated with anti-IL17 and iPTH monotherapies and their combination. Combination of iPTH and anti-IL17 has synergistic effect in the restoration of skeletal and immune parameters compared to mono-therapies. Immunofluorescence analysis shows decreased expression of PTHR1 in iPTH + anti-IL17 treated bone sections. Our studies show that IL-17 up regulates N-cadherin which disrupts PTHR1/LRP-6 interaction thereby inhibiting wnt signaling and promoting bone loss. Our studies advocate use of iPTH and anti-IL17 combination therapy for post-menopausal osteoporosis.

1. Introduction

Osteoporosis is a disease characterized by depletion of bone mass and structural deterioration of bone tissue leading to enhanced risk of fractures [1]. Osteoporosis is more prevalent in women than in men, with approximately two hundred million patients worldwide [2]. Osteoporosis occurs after menopause due to deficiency in sex steroid estrogen [3]. Estrogen level decreases in both women and men with age, the decline being more robust in women. Under normal circumstances, healthy skeleton is continuously replaced with new bone by a process called remodeling which occurs by the coordinated action of osteoblasts and osteoclasts [3]. At menopause, however, the rate of new bone formation is decreased with a net gain in bone resorption [3]. This leads to loss in bone mass and osteoporosis. Current osteoporosis treatments include anti-resorptive agents like bisphosphonates, Denosumab and selective estrogen receptor modulators (SERMs) [4]. Among the bone anabolic treatments, teriparatide (PTH) has been approved by FDA while agents like Romosozumab (anti-sclerostin antibody) are under clinical trials [4]. However, due to the coupling of bone formation and resorption, it has been found, that the drugs that decrease bone resorption also decrease bone formation [5]. Simultaneously, drugs enhancing the bone formation end up increasing the bone resorption [5]. It has thus been hypothesized that the combination of a bone anabolic agent like PTH with an anti-catabolic agent may stimulate new bone formation and suppress bone resorption [6].

It is believed that combining an anabolic agent with an anti-resorptive agent has the potential to improve bone density and bone strength more than either agent alone. A large number of small clinical trials have been carried out to evaluate the combination of PTH (1-34)/teriparatide or PTH (1-84)/Natpara with a number of anti-resorptives like raloxifene, bisphosphonates (alendronate, zoledronic acid), estrogen/hormone replacement therapy or Denosumab [7]. However, various clinical and preclinical studies of combination therapy have given highly variable results [8–12]. Most of these studies evaluated the effect on bone mineral density (BMD) which varied according to the timing of introduction of anabolic agents (before, after or simultaneously) and the anti-resorptive agent used. It was observed that the combination of PTH with bisphosphonates or Denosumab led to superior hip BMD compared to monotherapy alone [12]. However, in majority of these studies there was no increment in spine BMD when combination therapy was compared with monotherapy. Moreover, none of these studies were powered to assess the effect on fracture risk.

Abbreviations: PTH, parathyroid hormone; RANKL, receptor activated nuclear factor kappa B ligand; TNF-α, tumor necrosis factor; CTX-1, C-terminal telopeptide; P1NP, procollagen type-1 N terminal propeptide.

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Studies by Tokuyama et al. have compared the individual and combining effects of anti-RANKL antibody and teriparatide in ovariectomized (Ovx) mice [13]. They observed that compared with antibody group, antibody + PTH group exhibited increased femoral BMD and cortical bone volume. Bone turnover was also suppressed to same degree in combination group as with antibody monotherapy [13]. Apart from RANKL, IL-17 and TNF-α are also important mediators of bone loss [14]. Previous study in our laboratory compared the osteoprotective efficacy of neutralizing antibodies against RANKL, IL-17 and TNF-α. Neutralizing IL-17 antibody was found to have superior immunoprotective and osteoprotective effects compared to anti-TNFα and anti-RANKL antibodies [15]. Additionally, studies have shown that combined blockade of TNF-α and IL-17 was more effective than single blockade in suppressing tissue destruction associated with rheumatoid arthritis. Report suggests that bi-specific anti-TNFα/IL-17 antibodies may have superior efficacy in the treatment of arthritis [16]. All these observations prompted us to study the individual and combining effect of anti-IL17 antibody and PTH (1-34) and compare these results with mono-therapies in mitigation of Ovx induced bone loss model. Moreover, we also propose a possible mechanism by which anti-IL17 antibody might be mitigating the supportive role of IL-17 cytokine in the catabolic activity of PTH via PTHR1.

2. Materials and methods

2.1. Reagents and chemicals

Cell culture chemicals and primers were purchased from sigma Aldrich (St. Louis, MO). PTH (1, 84) and IL-17A were purchased from R and D diagnostics (Minneapolis, MN, USA). Brefeldin-A, PMA and Ionomycin was purchased from sigma Aldrich (St. Louis, Missouri, United States). FACS antibodies $CD4^+$-APC, IL-17A-PE, CBA TH1/TH2/TH-17 kit and FACS tubes from BD biosciences (San Diego, CA, USA). LEAF™ Purified anti-mouse IL-17A antibody were purchased from Biolegend San Diego, CA (USA). Antibodies were administered subcutaneously bi weekly for 4 weeks.

Hisep for PBMCs isolation was purchased from Himedia (Mumbai, India). cDNA synthesis kit was purchased from Invitrogen (Carlsbad CA, USA). LRP-6, N-cadherin, SOST antibodies and Immunoprecipitation kit was purchased from Abcam (Cambridge, UK). PTHR1 antibodies were purchased from Biolegend (San Diego, CA, USA). $CD4^+$ microbeads and LS columns for $CD4^+$ T cells isolation were purchased from Miltenyi Biotec (Singapore).

2.2. In vivo studies

Adult balb/c female mice 8–9 weeks' old were used for in vivo studies. The study was conducted in accordance with current legislation on animal experiments and was approved by institutional ethics committee, Central Drug Research Institute (CPSCEA registration no. 34/1999, dated November 3, 1999, extended to 2015, approval reference no. IAEC/2013/93/renew 02, dated December 03, 2014). All the animals were housed at 25 °C, 12-hour light and 12-hour dark cycles. Normal chow diet and water were provided ad libitum. Mice were divided in seven groups with 8 mice per group. Mice were ovariectomized and left for one month. After one-month time period treatment was started for 4 weeks in Ovx mice. Groups in experiments were sham operated (ovary intact) mice, ovariectomized (Ovx) mice, Ovx mice + iPTH (40 μg/kg/day) [17], Ovx + anti-17A (100 ng/mice) twice a week, Ovx + iPTH (40 μg/kg/day) + anti-IL-17A (100 ng/mice) twice a week in Ovx mice. Neutralizing antibodies were administered subcutaneously bi weekly for 4 weeks. PBMC was isolated from bone marrow of long bones by using Hisep. PBMCs were used for FACS staining and $CD4^+$ T cell isolation. Femur and tibia bones were kept for microCT analysis and histological studies.

2.3. PBMC isolation

PBMC cells were labelled with anti-CD4, anti-IL-17A (APC-conjugated anti-mouse CD4, and PE conjugated anti-mouse IL-17A). Specificity of immunostaining was ascertained by the background fluorescence of cells incubated with Ig isotype controls. Fluorescence data from at least 10,000 cells were collected from each sample. Immunostaining was done as per as manufacturer's instructions. In brief, single cell suspension of the BM was prepared in PBS. Cells were counted using haemocytometer and were re-suspended in FACS buffer. 10^6 cells/500 μl PBS and antibody was added as 10 μl/10^6 cells and further incubated for 45 min at room temperature. After incubation cells were washed twice with PBS and transferred to FACS tubes for analysis. FACS Caliber and FACS Aria (BD Biosciences Mississauga, ON, CA) were used to quantify the percentage of CD4 + IL-17A +. FACS staining was also performed for quantification of PTHR1 in osteoblasts cells.

2.4. Intracellular staining

Intracellular staining was performed as per manufacturer's instructions for IL-17A cytokine. For Staining IL-17A, PBMCs were isolated from spleenocytes and these cells were stimulated for 6 h with 10 ng/ml PMA, 250 ng/ml ionomycin and 10 μg/ml brefeldin A. After this cells were harvested, fixed and permeabilized by using Leucoperm Reagent A (Fixation Reagent) and Leucoperm Reagent B (permeabilizing reagent). Permeabilization was followed by intracellular staining of IL-17A cytokine with PE labelled IL-17 antibody [18]. Specificity of immunostaining was ascertained by back ground fluorescence of the cells incubated by Isotype control of IgG. After intracellular staining, cells were washed twice with PBS and transferred in FACS tube for analysis. FACS calibur and FACS Aria (BD Biosciences Mississauga, ON, CA) were used to quantify % of IL-17A positive cells. PBMCs were gated on the basis of FSC vs SSC. After this lymphocytes population was gated and in gated population APC vs PE positive population was measured.

2.5. Apoptosis assay

Osteoblasts apoptosis was measured by AnnexinV-PI staining. Osteoblasts were deprived of serum 6 h. Osteoblasts were treated for 24 h by PTH and IL-17A in different sets and cells were analysed for apoptosis as per as instructions given in manual.

2.6. CBA array for cytokines

Cytometric Bead Array (CBA) flex from BD biosciences was used for the measurement of various levels of cytokines in different in vivo groups. Levels of IL-6, TNF-α, IFN-γ, and IL-17A were measured in serum samples using Fluorescent bead based technology according to Manufacturer's instruction (BD biosciences, San Diego, USA). Fluorescent signals were read and analysed on FACS Aria Flow cytometer (BD biosciences, San Diego, USA) with help of BD FCAP Array v.1.0.1 software BD Biosciences.

2.7. RNA isolation

Total RNA was isolated from $CD4^+$ T cells, osteoblasts cells in all in vitro and in vivo groups using Trizol (Invitrogen Carlsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA with Revert AidTM H Minus first strand cDNA synthesis kit (Fermentas, Mumbai, India). SYBER green chemistry was used for quantitative determination of mRNAs of BMP-2, OCN, MCL-1, Caspase-3, PTHR1 and housekeeping gene GAPDH. Primers for different genes were designed using universal probe library. Sequences of primers are provided in Table 1. For real time cDNA was amplified with light cycler 480 (Roche Diagnostics Pvt. Ltd., Basel, Switzerland) to allow for quantitative detection of product in a 20 μl reaction volume. The temperature of reaction was 95 °C for