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Antibody-based inhibition of circulating DLK1 protects from estrogen deficiency-induced bone loss in mice

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ABSTRACT

Soluble delta-like 1 homolog (DLK1) is a circulating protein that belongs to the Notch/Serrate/delta family, which regulates many differentiation processes including osteogenesis and adipogenesis. We have previously demonstrated an inhibitory effect of DLK1 on bone mass via stimulation of bone resorption and inhibition of bone formation. Further, serum DLK1 levels are elevated and positively correlated to bone turnover markers in estrogen (*E*)-deficient rodents and women. In this report, we examined whether inhibition of serum DLK1 activity using a neutralizing monoclonal antibody protects from E deficiency-associated bone loss in mice. Thus, we generated mouse monoclonal anti-mouse DLK1 antibodies (MAb DLK1) that enabled us to reduce and also quantitate the levels of bioavailable serum DLK1 in vivo. Ovariectomized (ovx) mice were injected intraperitoneally twice weekly with MAb DLK1 over a period of one month. DEXA-, microCT scanning, and bone histomorphometric analyses were performed. Compared to controls, MAb DLK1 treated ovx mice were protected against ovx-induced bone loss, as revealed by significantly increased total bone mass (BMD) due to increased trabecular bone volume fraction (BV/TV) and inhibition of bone resorption. No significant changes were observed in total fat mass or in the number of bone marrow adipocytes. These results support the potential use of anti-DLK1 antibody therapy as a novel intervention to protect from E deficiency associated bone loss.

1. Introduction

Osteoporosis is a metabolic bone disorder characterized by reduced bone mass, strength, and microarchitecture deterioration leading to increased risk of fracture [1]. Postmenopausal estrogen (E) deficiency is a major risk factor for bone loss and osteoporotic fractures [2]. Recently, a number of antibody-based biological therapies have been employed for treatment of osteoporosis [3] e.g. neutralizing monoclonal antibodies against receptor activator of NF-κB ligand (RANK) (Denosumab®) [4] sclerostin (Romosozumab®) [5] or Dickkopf-related protein 1 (DKK1) [6]. Clinical outcome measures showed that biological therapy is a feasible strategy for the prevention and treatment of osteoporosis.

Delta-like 1 homolog (DLK1) is a membrane-bound protein that is proteolytically cleaved by the ADAM17/TACE enzyme to form the active soluble circulating protein (sDLK1) which have six epidermal growth factor (EGF)-like repeats and belongs to the Notch/Serrate/delta family, (for review, see [7]). The function of membrane bound DLK1 versus the soluble form remains to be determined definitively. However, DLK1 was shown to function mainly as a circulating active protein to regulate a variety of physiological processes including adipogenesis [8,9], osteoblastogenesis [7] and hematopoiesis [10]. Several signaling pathways have been reported to mediate the biological function of DLK1 in these differentiation processes, including a non-canonical interaction

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between DLK1 and Notch family receptors [11]; an interaction between DLK1 and insulin growth factor binding protein 1 (IGFBP-1) [12]; an interaction between DLK1 and fibronectin that lead to an activation of integrin and MEK/ERK signaling [13] and blocking of Akt signal activation [14].

We have identified soluble DLK1 as a novel factor that functions in a paracrine/endocrine fashion to inhibit osteoblast differentiation and bone formation [15] and stimulate bone resorption [16]. Furthermore, we identified soluble DLK1 as a possible mediator of *E*-deficiency associated-bone loss as its serum levels are significantly elevated following *E*-deficiency in both rodents [16] and humans [17] and these levels were positively associated with biochemical markers of bone turnover under E-deficiency condition [16]. In addition, the elevated serum levels of DLK1 in postmenopausal women were normalized upon E-replacement therapy [17]. In this study, we aimed to examine whether the inhibition of DLK1 using a neutralizing monoclonal antibody is an effective strategy to protect from E deficiency-induced bone loss in mice. For this purpose, we generated a highly specific mouse monoclonal anti-mouse DLK1 antibody (MAb DLK1) that neutralized the inhibitory effects of serum DLK1 on osteogenesis. In vivo injection of MAb DLK1 in ovx mice inhibited bone resorption and maintained bone mass.

2. Materials and methods

2.1. Generation of monoclonal DLK1 antibody

2.1.1. Cell cultures and reagents

The mouse NIH3T3 fibroblast-, 3T3-L1 preadipocyte, C2C12 myoblast- and human HT1080 epithelial cell lines were obtained from the American Type Culture Collection. Mouse stromal cells ST2 were obtained from Leibniz Institute DSMZ (ACC 333, Braunschweig, Germany). Primary osteoprogenitor cells (OB) were isolated from the calvarias of neonatal (3–4 days old) pups and subjected to sequential collagenase II digestion at 37 °C as described [18]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, CA, USA) with 10–20% fetal bovine serum (FBS) or 10% calf serum (3T3-L1 cells) and 100 mg/ml of streptomycin (Gibco) and 100 U/ml of penicillin (Gibco). For osteoblast differentiation, the same culture medium was supplemented with 50 mg/ml of vitamin C (Sigma-Aldrich ApS, Brondby, Denmark), and 10 mM β-glycerol-phosphate (Sigma), and medium was changed every three days.

2.1.2. Western blot analysis

Twenty μg of protein were separated on 8% to 12% NuPAGE® Novex® Bis-Tris gel systems (Invitrogen, Taastrup, Denmark) and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked and probed with MAb DLK1 (2 μg/ml) and incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Aarhus, Denmark). Proteins were visualized by ECL chemiluminescence (Thermo Fisher Scientific, Roskilde, Denmark).

2.1.3. Immunohisto-/cytochemistry

For immunohistochemistry, E12.5 mouse embryos were snap-frozen in Tissue Tek. Four to 5 μm cryosections were then fixed in 4% NBF prior to blocking in 2% BSA/TBS. Sections were incubated with MAbs diluted to 2 μg/ml followed by Alexa 488-conjugated donkey anti-IgG (1:200, Molecular Probes). Nuclei were stained with DAPI (Vectashield, Vector Labs). Images were captured using a Leica DMI4000B Cool Fluo Package instrument equipped with a Leica DFC340 FX Digital Camera. Exposure (camera settings) and picture processing (minor adjustments of contrast/brightness in Adobe Photoshop) were applied equally to sample sections and controls. For immunofluorescent staining, cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS and stained with MAb DLK1 using Alexa Fluor® 488-conjugated mouse anti-mouse IgG (H + L) as secondary antibody (Cell signaling). Fluorescent images were analyzed by Operetta high content imaging system (Perkin Elmer, Germany) at 20× magnification.

2.1.4. Alkaline phosphatase (ALP) activity assay

Each sample was measured in 6 replicates in 96-well plates. Cells induced with osteogenic medium were stained with FITC fluorescent ALP live stain dye according to the manufacturer's instructions (ThermoFisher Scientific, Denmark) and counter stained with DAPI (Sigma-Aldrich ApS) for nuclear staining. Fluorescent images were analyzed by Operetta high content imaging system (Perkin Elmer, Germany) using Harmony® Software (Perkin Elmer) and ALP activity was represented after normalization to the cell count.

2.1.5. Purification and biotinylation of mouse DLK1

Soluble mouse DLK1 (mDLK1) was purified from mouse amniotic fluid or from conditioned 3T3-L1 medium by immuno-specific affinity chromatography (monospecific rabbit anti-mDLK1 coupled sepharose) and further concentrated using cation exchange (Resource Q column, GE Healthcare). For immunizations, mDLK1 was dialyzed against PBS and sterile filtered through a low-protein binding 0.22 μm filter. For the inhibition/epitope specificity assay, mDLK1 was biotinylated by addition of 1/6 (*w*/w) biotinsuccimid ester (BNHS; 10 mg/ml in dimethylsulfoxide). Unreacted BNHS was removed by buffer exchange to PBS.

2.1.6. Immunization of mice

Three adult female *Dlk1*$^{-/-}$ mice were each injected subcutaneously with 25 μg purified mouse DLK1 mixed with $Al(OH)_3$ and incomplete Freund's adjuvant (IFA). DLK1-deficient (*Dlk1*$^{-/-}$) mice were obtained from J. Laborda (University of Castilla–La Mancha, Ciudad Real, Spain) [19]. The immunizations were repeated further two times, 14 days apart and by replacing IFA with PBS for intraperitoneal injections. One week after the third immunization, a blood sample from each mouse was tested for circulating antibodies against mDLK1 using direct- (antigen) ELISA, cyto-ELISA and flow cytometry (see below). Normal mouse serum (Chemicon) was used as a non-immune reference serum in all screening assays. Two mice that revealed a positive polyclonal signal in all three assays were selected for a final boost i.p. with 25 μg mDLK1 in PBS, three days prior to fusion.

2.1.7. Fusion and cloning

Using PEG 4000, spleen cells from the responding mice were fused with the myeloma cell line SP2/0-Ag14 as described by Köhler and Milstein (1975) [20] and modified by Reading (1982) [21]. Hybridoma supernatants from 96-well plates were screened using cyto-/antigen ELISAs and later in the cloning process, also by flow cytometry. Selected hybridomas were cloned 3–5 times using the standard limiting dilution procedure [21]. In total, 19 clones were further propagated in RPMI/10% FCS/1%PS and stored frozen at −80 °C in FCS-containing 10% DMSO while the antibody containing supernatants were kept at 4 °C until further analyses.

2.1.8. Screening of hybridoma supernatants

Hybridoma supernatants were screened throughout the cloning process using direct (antigen, Ag) ELISA and cyto-ELISAs (positive screening on 3T3-L1 cells and negative on C2C12 cells). An intermediate screen was also performed on live C2C12 and 3T3L1 cells by flow cytometry (see below). For direct ELISAs, purified mouse DLK1 (0.5–1.0 μg/ml) was coated onto 96-well Maxisorp flat bottom microtiter plates. For cyto-ELISAs, 3T3-L1 and C2C12 cells were cultured in 96-well plates and fixed with 4% NBF at sub-confluence, as previously described [22]. Hybridoma supernatants were then transferred to the wells and plates incubated overnight. After an additional incubation with horseradish peroxidase conjugated rabbit-anti mouse immunoglobulins (P260, Dako Denmark), plates were developed using *O*-phenylenediamine (Kem-En-Tec Diagnostics A/S)/H_2O_2 and read at 490 nm on a Labsystems iEMS Reader.