Bone 98 (2017) 38–46

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

The role of the progressive ankylosis protein (ANK) in adipogenic/osteogenic fate decision of precursor cells

Takeshi Minashima [1], Martin Quirno, You Jin Lee, Thorsten Kirsch *

Musculoskeletal Research Center, Department of Orthopaedic Surgery, New York University School of Medicine, NY, New York, United States

ARTICLE INFO

Article history:
Received 21 December 2016
Revised 6 March 2017
Accepted 7 March 2017
Available online 08 March 2017

Keywords:
Adipogenesis
Bone fracture healing
Osteogenesis
Progressive ankylosis protein (ANK)
Wnt/β-catenin signaling

ABSTRACT

The progressive ankylosis protein (ANK) is a transmembrane protein that transports intracellular pyrophosphate (PPi) to the extracellular milieu. In this study we show increased fatty degeneration of the bone marrow of adult *ank/ank* mice, which lack a functional ANK protein. In addition, isolated bone marrow stromal cells (BMSCs) isolated from *ank/ank* mice showed a decreased proliferation rate and osteogenic differentiation potential, and an increased adipogenic differentiation potential compared to BMSCs isolated from wild type (WT) littermates. Wnt signaling pathway PCR array analysis revealed that Wnt ligands, Wnt receptors and Wnt signaling proteins that stimulate osteoblast differentiation were expressed at markedly lower levels in *ank/ank* BMSCs than in WT BMSCs. Lack of ANK function also resulted in impaired bone fracture healing, as indicated by a smaller callus formed and delayed bone formation in the callus site. Whereas 5 weeks after fracture, the fractured bone in WT mice was further remodeled and restored to original shape, the fractured bone in *ank/ank* mice was not fully restored and remodeled to original shape. In conclusion, our study provides evidence that ANK plays a critical role in the adipogenic/osteogenic fate decision of adult mesenchymal precursor cells. ANK functions in precursor cells are required for osteogenic differentiation of these cells during adult bone homeostasis and repair, whereas lack of ANK functions favors adipogenic differentiation.

1. Introduction

ANK is a transmembrane protein that transports intracellular pyrophosphate (PPi) to the extracellular milieu [1]. The progressive ankylosis allele (*ank*) is a spontaneous recessive mutation that causes the lack of a functional progressive ankylosis protein (ANK) in *ank/ank* mice [1]. We have previously shown that *ank/ank* mice show an osteopenic phenotype [2]. Several studies have now established that ANK is an important regulator of extracellular PPi homeostasis in skeletal tissues [1–5]. Previous findings from our laboratory have suggested that extracellular PPi directly affects osteogenic differentiation [2]. Other studies have suggested that ANK may play a role of mediating ATP efflux in musculoskeletal cells [6]. ATP and its hydrolysis products have been shown to affect osteoblast differentiation and mineralization [7–12]. Therefore, it is possible that ANK is an important regulator of osteoblast differentiation. However, it is not clear how ANK affects osteogenic differentiation of BMSCs.

Wnt/β-catenin signaling has been shown to induce osteogenic differentiation in osteoprogenitor cells [13]. Consequently, activating mutations in Wnt/β-catenin signaling pathway in humans causes high bone-mass phenotype such as van Buchem disease, whereas inactivating mutations cause osteopenic diseases such as osteoporosis-pseudoglioma syndrome [14]. In addition, loss of Wnt/β-catenin signaling is mainly responsible for the loss of osteogenic potential of aging bone marrow stromal cells (BMSCs), the osteogenic precursor cells in the bone marrow [15,16]. These cells have the potential to differentiate into adipocytes, chondrocytes and osteoblasts [17]. As a consequence of loss of Wnt/β-catenin signaling, aging BMSCs decrease their ability to differentiate into osteoblasts and increase their ability to differentiate into adipocytes [18–20]. Little, however, is known about upstream factors that regulate Wnt/β-catenin signaling in aging BMSCs.

Abbreviations: ADM, adipogenic differentiation medium; ALP, alkaline phosphatase; ANK, progressive ankylosis protein; *ank*, progressive ankylosis gene; AP2, adipocyte protein 2; APC, adenomatous polyposis coli; CTNNB1, beta-catenin; BMSCs, bone marrow stromal cells; BV, bone tissue volume in callus; BV/TCV, bone tissue volume fraction of the total callus volume; C, callus; CAT, cartilaginous tissue; C/EBPβ, CCAAT-enhancer-binding protein beta; CKK-8, cell counting kit-8; CSNK1A1, casein kinase I isoform alpha; CT, cycle value; DMEM, Dulbecco's Modified Eagle Medium; Dvl1, disheveled segment polarity protein 1; GM, growth medium; LEF1, lymphoid enhancer-binding factor 1; n.d., not detectable; OC, osteocalcin; ODM, osteogenic differentiation medium; PCR, polymerase chain reaction; Pi, inorganic phosphate; PPARγ, peroxisome proliferator-activated receptor gamma; PPi, pyrophosphate; S1P, sphingosine-1 phosphate; SPHK1, sphingosine kinase 1; TCF 7, transcription factor 7; TCV, total callus volume; WT, wild type.

* Corresponding author at: Musculoskeletal Research Center, Department of Orthopaedic Surgery, NYU School of Medicine, 301 East 17th Street, Suite 1500, United States.
E-mail address: thorsten.kirsch@nyumc.org (T. Kirsch).
[1] Present address: Department of Physiology and Regenerative Medicine, Kinki University School of Medicine, Osaka, Japan.

http://dx.doi.org/10.1016/j.bone.2017.03.003
8756-3282/

Since Wnt/β-catenin signaling in osteoprogenitor cells induces their differentiation, Wnt/β-catenin signaling is also essential during bone healing [21]. The declining Wnt/β-catenin signaling in aging osteogenic precursor cells has been suggested as a main reason for delayed fracture healing in elderly. In fact, a recent study has shown that treatment of a bone fracture with liposomal Wnt3a resulted in markedly faster and more robust bone healing with earlier onset of mineralization and new osteoid deposition than happens normally in young animals [15].

ank/*ank* mice have a limited life span of 4 to 6 months [22]. Previously we have shown that adult *ank*/*ank* BMSCs show reduced osteogenic differentiation [2], suggesting that these cells rapidly lose their osteogenic differentiation potential. The goal of this study was to determine how lack of ANK function affects the osteogenic differentiation potential of adult BMSCs. Specifically, we asked the question of whether ANK directly affects Wnt/β-catenin signaling and ultimately osteogenic differentiation of adult BMSCs. To accomplish this goal we analyzed the trabecular bone and bone marrow structure in adult *ank*/*ank* mice and WT littermates and analyzed the adipogenic and osteogenic differentiation potentials of BMSCs isolated from *ank*/*ank* mice and WT littermates. In addition, we analyzed how lack of ANK function affects bone fracture healing in mice.

2. Materials and methods

2.1. Animals

The *ank*/*ank* breeding colony used was originally on a hybrid background derived from crossing a C3H and C57BL/6 hybrid male with BALB/c female. Heterozygote breeders (C3FeB6 A/A$^{w\text{-}J}$-Ank$^{ank/J}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). Heterozygote breeders were used to generate and study *ank*/*ank* and wild-type (WT) littermates, with genotypes analyzed by polymerase chain reaction (PCR) as previously described [1].

To analyze bone fracture healing, we utilized a well-established femur fracture model in 6-week-old *ank*/*ank* mice and WT littermates [23]. Briefly, a medial parapatellar incision was used to expose the femoral condyles. A hole was drilled into the femoral intramedullary canal at the intracondylar notch using a 30-gauge needle. A 0.01-inch diameter stainless steel pin was inserted into the intramedullary canal to stabilize the impending fracture. A closed diaphyseal femoral fracture was created using a blunt impact force in a three-point bending technique. Pain was managed postoperatively with subcutaneous doses of buprenorphine (0.05 mg/kg). Mice were euthanized at 2 weeks and 5 weeks post fracture. Surgery was performed on approximately equivalent numbers of male and female mice. Each group consisted on 5 mice. Protocols were approved by the Institutional Animal Care and Use Committee at New York University School of Medicine in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Histology

Hindlimbs from 2 month-old and 4-month-old female and male *ank*/*ank* mice and WT littermates were used. We analyzed the hindlimbs of 4 animals in each group (*ank*/*ank* and WT). Dissected femurs and tibias from *ank*/*ank* mice and WT littermates were fixed in 4% paraformaldehyde, decalcified in 0.2 M EDTA pH 7.4 for 14 d, and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin. For immunohistochemical staining, sections were pretreated with bovine testicular hyaluronidase (2 mg/ml; Sigma) for 30 min at 37 °C, blocked with goat serum for 20 min at room temperature, and incubated over night with a primary antibody specific for perilipin A (ab3526, Abcam, Cambridge, MA) followed by biotinylated secondary antibodies for 30 min at room temperature. After washing, sections were incubated with a streptavidin-peroxidase conjugate for 10 min at room temperature followed by a solution containing diaminobenzidine (chromogen) and 0.03% hydrogen peroxide for 5 min at room temperature. Control sections were incubated with nonimmune rabbit serum. Specimens were viewed under Evos FL Auto Cell Imaging System (Thermo Fisher Scientific). Quantitative analysis of the total area of the fat vacuoles in the bone marrow of the tibia was performed on sections from 2-month old and 4-month old tibiae from *ank*/*ank* mice ($n = 3$) and wild type littermates ($n = 3$). The analysis was performed in an area 100 μm to 700 μm distal to the growth plate using the Evos FL Auto Cell Imaging System software. In addition, the number of perilipin immune-positive cells in this area was counted using the Evos FL Auto Cell Imaging System software.

For histological evaluation of bone fracture healing, femurs were excised, the needle was removed, and the samples were fixed and decalcified as described above. Five-micrometer sections were cut and stained with hematoxylin and eosin or Alcian blue.

2.3. MicroCT evaluation

Specimens were fixed in 4% paraformaldehyde and analyzed by high-resolution microCT using a Skyscan 1172 (Bruker, Billerica, MA). To analyze the degree of bone fracture healing, the following parameters were determined: total callus volume (TCV), bone tissue volume in the callus (BV), bone tissue volume fraction of the total callus volume (BV/TCV).

2.4. Plain radiography

Serial radiographs were taken directly after the creation of the fracture and 2 weeks after fracture surgery using the digital radiographic function of the IVIS Lumina III In Vivo Imaging System (PerkinElmer, Shelton, CA).

2.5. Cell cultures

Bone marrow stromal cells (BMSCs) were isolated from femurs of 8-week-old female and male *ank*/*ank* mice or WT littermates and cultured at 25×10^6 cells per 10-cm in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal calf serum as described previously [24–26]. To obtain 25×10^6 cells for one experiment, we used 4 hindlimbs from 2 *ank*/*ank* mice and 4 hindlimbs from 2 WT littermates. For osteogenic differentiation, BMSCs were cultured in osteogenic differentiation medium (□MEM supplemented with 10% fetal calf serum, 5 mM β-glycerophosphate, 50 μg/ml ascorbic acid, 10^{-7} M dexamethasone) [2,26,27]. After 7, 14, and 21 d of culture, cells were stained for alkaline phosphatase (ALP) activity using Alkaline Phosphatase Magenta Immunohistochemical Substrate Solution (Sigma). To determine mineralization in these cultures, alizarin red S staining was performed on day 14 and day 21 as described previously [28]. For adipogenic differentiation, BMSCs were cultured in adipogenic differentiation medium (DMEM supplemented with 10% fetal calf serum, 0.5 mM 3-isobutyl-1-methylxanthine, 5 μg/ml insulin, and 1 μM dexamethasone) as described previously [26]. Oil red staining was performed after 7, 14, and 21 d of culture.

For Wnt PCR array using the RT2 Profiler PCR array for the Wnt signaling pathway (Qiagen) we cultured *ank*/*ank* and WT BMSCs after these cells have reached confluency for 2 d in osteogenic differentiation medium.

2.6. Luciferase reporter assays

For luciferase assays to determine β-catenin activity, cells were co-transfected with a firefly TCF/LEF-specific luciferase reporter vector (TOPFlash Reporter; EMD Millipore, Billerica, MA) and a constitutively expressed Renilla luciferase reporter, which served as an internal control for normalizing transfection efficiencies and monitoring cell