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Bone 36 (2005) 215–223

BONE

www.elsevier.com/locate/bone

Loss of the transcription factor p45 NF-E2 results in a developmental arrest of megakaryocyte differentiation and the onset of a high bone mass phenotype

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Received 29 April 2004; revised 24 September 2004; accepted 27 September 2004

Abstract

NF-E2 is a transcription factor required for megakaryocyte differentiation. The phenotype of mice deficient in p45 NF-E2 has been characterized by increased numbers of immature megakaryocytes and the absence of functional platelets. These mice also exhibited a high bone mass phenotype with up to a 6-fold increase in trabecular bone volume and a 3- to 5-fold increase in the bone formation rate. Our data indicated that both osteoblast and osteoclast numbers were increased in vivo with a 4- to 10-fold increase in osteoblast number/tissue area and approximately a 5-fold increase in osteoclast number/tissue area. Serum osteocalcin levels were also increased in NF-E2-deficient mice, corroborating the histomorphometric data and confirming that the osteoblasts were functional. Urinary cross-links levels were measured to confirm osteoclast activity. Interestingly, the increased bone was observed only in bony sites of hematopoiesis, and was not seen in flat bones such as calvariae. We showed that cells of the osteoblast lineage do not express NF-E2 mRNA. The increased bone phenotype was adoptively transferred into irradiated wild-type mice using spleen cells from NF-E2-deficient mice. These observations suggest that a megakaryocyte–osteoblast interaction occurs which is anabolic for bone.

Keywords: p45 NF-E2; Megakaryocytes; Osteoblasts; Transcription factors; Anabolic

Introduction

Bone marrow (BM) is the source of osteogenic, hematopoietic, and immune cells. In the osteogenic lineage, osteoblasts arise from uncommitted mesenchymal stem cells and pass through a series of discrete stages of maturation resulting in mature osteoblasts secreting osteoid. In contrast, osteoclasts arise from hematopoietic precursors. Other cell lineages are also present in BM and are supported by the stromal cell system. The close juxtaposition of these cells makes BM the focus for many of the regulatory interactions required for homeostatic development of bone. Changes in these regulatory interactions may result in altered bone formation or resorption, providing clues as to how skeletal homeostasis is maintained. Recent data indicate that hematopoietic cells can influence the differentiation of osteogenic cells and vice versa [1]. It has been suggested that one such cell, the megakaryocyte (MK), is unique by being the only cell, other than osteoblasts and odontoblasts, to express the matrix protein osteocalcin (OC) [2]. In addition to OC, MK or platelets also secrete other matrix proteins including osteonectin, bone sialoprotein, and osteopontin [3–6]. These data imply that MK could, under the appropriate circumstances, contribute to bone formation by the secretion of these proteins.

The molecular dissection of MK differentiation has been greatly facilitated by the identification of transcription factors required for the cell's successful advancement from stage to stage. Loss of these specific factors precludes the cells from continued maturation, and results in the accumulation of cells at the latest stage of differentiation prior to the arrest. The selective loss of p45 NF-E2 transcription factor, which was

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doi:10.1016/j.bone.2004.09.024

originally thought to be required exclusively for erythroid lineage development, has now been shown to play a critical role in MK differentiation [7].

NF-E2 is a heterodimeric nuclear protein comprised of two polypeptide chains, a hematopoietic-specific 45-kDa subunit and a p18 subunit that is expressed in many cell types; both proteins belong to the leucine zipper family of transcription factors [8,9]. Expression of p45 is restricted to erythroid precursors, MK, mast cells, and multipotential progenitors. Mice lacking p45 NF-E2 (NF-E2) exhibited profound thrombocytopenia resulting from a maturational arrest of mature MK with <5% of the number of platelets detectable in wild-type peripheral blood [10]. MK number was increased 2- to 5-fold in the BM and spleen of adult NF-E2−/− mice, but was only slightly increased in peripheral blood. The cells had a polyploid DNA content, and expressed many lineage-restricted markers, including GPIIb and Mpl. They responded to exogenous thrombopoietin (Mpl-ligand or TPO), the major growth factor for MK, with a marked proliferation, but there was no detectable increase in platelet production in vivo. The mutant mice had normal levels of TPO [11]. Ultrastructurally, the MK had extensive demarcation membranes; however, essentially no delimitation of platelet fields could be observed [7].

Importantly, here, we show that NF-E2-deficient mice developed strikingly increased trabecular and cortical bone mass, with increased bone formation, increased numbers of osteoblasts, and increased numbers of osteoclasts. In this investigation, we analyzed the site-specific bone phenotype in NF-E2-deficient mice by histological and histochemical analysis of bone. We analyzed the biochemical properties of both the bone and blood in NF-E2-deficient mice compared to wild-type controls. We examined cells of the osteoblast lineage for NF-E2 expression and we showed that the NF-E2 bone phenotype could be adoptively transferred into irradiated wild-type mice using spleen cells from NF-E2-deficient mice.

Materials and methods

Mice

Generation and breeding of chimeric mice was described previously [12,13]. Briefly, to inactivate the p45 NF-E2 gene, a PGK-neo cassette (NeoR) was inserted into the unique *Sal*l site upstream of the bZip encoding region [10]. NF-E2−/− mice were maintained on the inbred 129/Sv genetic background.

Histomorphometry

To label bone mineralization fronts, mice were injected with 30 mg/kg of calcein 8 days and 24 h prior to sacrifice. Tibiae, calvariae, and sternum were removed, stripped of soft and connective tissue, fixed in 10% buffered formalin at 4°C, and then transferred 4 h later to 70% acetone. Bone samples were dehydrated, infiltrated with methylmethacrylate, and prepared for sectioning. Non-decalcified, 4-μm sections were stained with toluidine blue. Histomorphometric parameters, described previously [14], were analyzed by Osteomeasure software (Osteometrics, Atlanta, GA). All tibial measurements were taken in a standard area just below the growth plate. Measurements were done on trabecular bone only, and did not include cortical or endosteal bone surfaces. The initial tracing included marrow osteoid; therefore, parameters such as bone volume (BV) include mineralized bone as well as osteoid. Dynamic histomorphometric measurements were performed on 8 μm sections that were mounted with Fluoromount Mountant (BDH Laboratories Supplies, Poole, England) and visualized under epifluorescence. Sternum (cross-sections) and calvarial (cross-sections approaching from the frontal plane) measurements were performed on the total bone. Significant differences between groups analyzed histomorphometrically were calculated by the Student's t test for $P < 0.05$.

Histochemical and immunohistochemical staining

Histochemical staining procedures for Hematoxylin and Eosin (H&E), toluidine blue, Von Kossa, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRACP) have been previously described [15–17].

Cell isolation and culture

Murine calvarial cells were prepared as previously described [18]. In brief, calvariae from less than 48-h-old p45 NF-E2−/− and littermate control mice were pretreated with ethylenediaminetetraacetate (EDTA) in phosphate-buffered saline (PBS) for 30 min. The calvariae were then subjected to sequential collagenase digestion (Worthington Biomedical Corp., Lakewood, NJ). Cells were collected, following 15-min incubation each, in collagenase. Fractions 3–5 were used as the starting population.

Primary cortical adult bone cells (ABC) were prepared from mice using a modification of the technique of Robey and Termine [19]. The tibia and femora were cleared of soft tissue and periosteum. After removing the BM cells by flushing the marrow channel with medium, the cortical shafts were mechanically reamed using dental files. The tibia and femora were placed into 2-ml conical vials and minced into fine chips. The chips were then incubated twice with 250 U/ml CLS-2 bacterial collagenase for 40 min at 37°C to remove adherent cells. The chips were washed and cultured in 100-mm culture dishes in Eagle's minimal essential medium (EMEM). Cells grew out of the chips and formed a subconfluent monolayer in 10 days. These cells did not secrete IL-1 (hematopoietic cell marker) and CD31 (endothelial cell marker) could not be detected by fluorescent activated cell sorting (FACS), which suggested that