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Apolipoprotein E plays crucial roles in maintaining bone mass by promoting osteoblast differentiation via ERK1/2 pathway and by suppressing osteoclast differentiation via c-Fos, NFATc1, and NF-κB pathway

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ABSTRACT

Apolipoprotein E (ApoE) plays crucial roles not only in lipid metabolism but also in bone metabolism. Specifically ApoE4, one of major ApoE isoforms, has been demonstrated to be associated with increased risk of developing osteoporosis compared to another major isoform ApoE3. However, the detailed mechanism of how the different ApoE isoforms affect bone metabolism remains unclear. Micro-CT analyses of distal femora demonstrated severely decreased bone mass in 48-week-old female homozygous ApoE-knockout (ApoE-KO) mice compared to age- and gender-matched wild type C57BL/6 J (WT) mice. Physiological levels of either ApoE3 or ApoE4 protein (1–20 μg/ml) significantly increased the expression of osteoblast-related genes and alkaline phosphatase (ALP) activity of primary calvarial osteoblasts by inhibiting extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in a dose-dependent manner, and ApoE3 showed greater osteoblastic induction compared to ApoE4. Furthermore, both ApoE3 and ApoE4 protein inhibited osteoclastogenesis and the expression of osteoclast-related genes of mouse bone marrow derived macrophages (BMDM) via down regulation of c-Fos, nuclear factor of activated T-cells 1 (NFATc1) and nuclear factor-kappa B (NF-κB) pathway. Moreover, ApoE3 showed greater inhibition of c-Fos, dendritic cell-specific transmembrane protein (DC-STAMP), and Cathepsin K gene expression compared to ApoE4. Collectively, ApoE plays crucial roles in preserving bone mass, suggesting that targeting ApoE and its isoforms as a promising treatment candidate of both osteoporosis and hyperlipidemia.

1. Introduction

Both osteoporosis and arteriosclerosis are serious complications in the aging society [1,2], and low bone mass is associated with the progression of arteriosclerosis [3,4]. Both osteoporosis and arteriosclerosis are associated with elevated levels of various inflammatory cytokines, such as receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) [5–8], although detailed common mechanisms remain unclear. Apolipoprotein E (ApoE) is a 34 kDa

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glycoprotein which is mainly produced by the liver and macrophages, and recognized as a crucial mediator of hyperlipidemia and atherosclerosis [9], as ApoE-knockout (ApoE-KO) mice showed severe lipidosis and atherosclerosis [10]. ApoE binds to hepatic lipoprotein receptors such as low-density lipoprotein receptor (LDLR), LDLR related protein 1 (LRP1), and LRP8 to mediate the cellular uptake of lipoproteins from circulation [11–13]. Human ApoE consists of three common alleles (ε2, ε3 and ε4) and six different genotypes (ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4), which leads to the difference in concentration of each isoform (ApoE2, E3, and E4) in the serum [13,14]. The frequency of each allele is about 60–80% in Apoε3, 10–15% in Apoε4, and 5–10% in Apoε2 among Caucasians [12]. The difference between isoforms causes variable affinities to lipoprotein receptors, plasma lipid levels [15,16], and incidence rate of various metabolic diseases. For example, Apoε2 is associated with type III hyperlipoproteinemia [12], and Apoε4 is associated with atherosclerosis and Alzheimer's disease [17].

Concerning the relation with ApoE isoforms and bone metabolism, previous reports demonstrated that Apoε4 carriers exhibit lower bone mineral density (BMD) and higher fracture risk than Apoε3 carriers [12,18,19]. However, there are few basic studies addressing the effects of ApoE isoforms on bone metabolism, partially because murine ApoE does not possess these isoforms.

Recent reports demonstrated that aged ApoE-KO mice displayed reduced bone formation and low bone mass [20], and high-fat diet induced bone loss was observed in ApoE-KO mice compared to wild type (WT) mice [21]. However, other reports demonstrated that young ApoE-KO mice showed higher bone mass compared to young WT mice [20,22]. Collectively, ApoE may play an important role in bone metabolism, although detailed mechanisms remain controversial. As far as we know, previous research focused mainly on the minor isoform ApoE4 [23], and no reports which directly compared the effects of ApoE4 and ApoE3 on bone metabolism, especially *in vitro*. In this study, we focused on confirming the differences in bone parameters between aged homozygous ApoE-KO mice and WT mice, and also investigating the differential effects of ApoE3 and E4 on osteoblastogenesis and osteoclastogenesis.

2. Materials and methods

2.1. Animal

Homozygous ApoE-KO mice (n = 8) on a C57BL/6 J background were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and WT C57BL/6 J (n = 8) mice were obtained from Charles River Laboratories (Osaka, Japan). Experiments were performed using age- and gender-matched ApoE-KO mice and WT mice which were fed with a normal-fat diet and water from birth to 48 weeks in a temperature- and humidity-controlled facility with a 12 h light/dark cycle. Mice were anaesthetized with an intraperitoneal injection of midazolam, medetomidine and butorphanol for micro-computed tomography (μCT) analyses and then femora were harvested following euthanization as previously described [24]. All experimental protocols were approved by the Ethics Review Committee for animal Experimentation of Osaka University, Graduate School of Medicine.

2.2. Micro-CT

μCT scanning (Rigaku Mechatronics, Tokyo, Japan) was performed to evaluate distal femur of mice (500 μm proximal to the growth plate). Scanned images were analyzed using Tri/3D Bon software (Ratoc System Engineering Co., Ltd., Tokyo, Japan) for the parameters including bone volume fraction (BV/TV: bone volume/tissue volume), trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular spacing (Tb. Sp), cortical bone ratio (Cv/Av: cortical volume/all volume), and mean cortical bone thickness (Ct. Th).

2.3. Immunohistochemistry

Sections were incubated with anti-osteocalcin antibody (Takara bio, Shiga, Japan) according to the manufacturer's protocol. Next day, the sections were incubated with secondary antibody (Vectastain Elite ABC kit Rabbit IgG: Vector Laboratories, Inc., San Diego, CA, USA) for 30 min at room temperature followed by color development using 3, 3′-Diaminobenzidine tetrahydrochloride (DAB) (Dako, Tokyo, Japan). Finally, the sections were counterstained with hematoxylin.

2.4. Serum assay

Serum concentration of osteocalcin (Takara Bio) and CTX-1 (CUSABIO, Hubei, China) were examined by ELISA kit according to the manufacture's protocol.

2.5. Cell culture

Murine primary osteoblasts were enzymatically isolated from the calvaria of 3-day-old C57BL6/J mice and then cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA) and 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37 °C in a humidified atmosphere of 5% CO_2. Once primary osteoblasts were expanded, cells were re-seeded at 1×10^6 cells per well in 12-well plates. Medium was replaced with osteoblast culture medium containing 50 μg/ml ascorbic acid (Sigma-Aldrich) and 10 mM β-glycerophosphate (Calbiochem, San Diego, CA, USA). At 80% confluency, cells were treated with either ApoE3 (R&D Systems, Minneapolis, MN, USA) or ApoE4 (PeproTech, Rocky Hill, NJ, USA) recombinant protein (vehicle, 1, 5 and 20 μg/ml) for 5 days.

Murine primary osteoclasts were differentiated from mouse bone marrow (BM) cells. Specifically, BM cells isolated from femora and tibiae of C57BL/6 J mice were cultured in α-MEM with 5 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D Systems) overnight at 37 °C in 5% CO_2 as previously described [25]. Adherent cells collected by washing twice with phosphate buffered saline (PBS) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were seeded at 1×10^6 cells per well in 12-well plates or 5×10^5 cells per well in 48-well plates. Osteoclast differentiation was induced with 10 ng/ml M-CSF and 50 ng/ml RANKL (R&D Systems). The following day, either ApoE3 or ApoE4 protein (vehicle, 1, 5 and 20 μg/ml) were added into the culture and then cells were incubated for 5 days.

2.6. Extraction of first-strand complementary DNA (cDNA) and quantitative real-time PCR analysis

RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany), and cDNA was reverse-transcribed from total RNA (1 μg) using SuperScript III First-Strand Synthesis System (Life Technologies, Tokyo, Japan).

Real-time PCR was then performed using a Step One Plus Real-Time PCR System (Life Technologies) and Fast SYBR Green Master Mix (Life Technologies). Gene expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of PCR primers (forward and reverse, respectively) were described below: GAPDH, 5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-TGTAGACCATGTAGTTGAGGTCA-3’; Alkaline phosphatase (ALP), 5′-AATCGGAACAACCTGACTGACC-3′ and 5′-TCCTTCCACCAG CAAGAAGAA-3’; Osteocalcin, 5′-CTCACTCTGCTGGCCCTG-3′ and

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