EXPERIMENTAL CELL RESEARCH 319 (2013) 436–446

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Research Article

Apolipoprotein E inhibits osteoclast differentiation via regulation of c-Fos, NFATc1 and NF-κB

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ARTICLE INFORMATION

Article Chronology:
Received 18 July 2012
Received in revised form
15 November 2012
Accepted 5 December 2012
Available online 12 December 2012

Keywords:
Osteoclast differentiation
Apolipoprotein E
c-Fos
NFATc1
NF-κB

ABSTRACT

Apolipoprotein E (ApoE) plays a major role in the transport and metabolism of lipid. Other functions of ApoE include modulation of innate and adaptive immune responses. The expression of ApoE in osteoblasts and its relevance with bone formation have also been reported. However, the effect of ApoE on osteoclasts has not yet been examined. Here, we investigated the role of ApoE in osteoclast differentiation using bone marrow-derived macrophages (BMMs) and RAW264.7 cells. We found a down-regulation of ApoE gene expression during osteoclastic differentiation of those cells. Overexpression of ApoE in BMMs and RAW264.7 cells significantly blocked the induction of c-Fos and nuclear factor of activated T cell c1 (NFATc1), transcription factors critical for expression of osteoclast marker genes, by receptor activator of nuclear factor κB ligand (RANKL), the osteoclast differentiation factor. ApoE inhibited osteoclast differentiation, as measured by decreased number of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (MNCs). In addition, ApoE reduced the expression of dendritic cell-specific transmembrane protein (DC-STAMP) and ATPase, H^+ transporting, lysosomal 38 kDa, V0 subunit d2 (ATP6v0d2), genes involved in cell–cell fusion during osteoclastogenesis. Knock-down of ApoE using a specific siRNA promoted the RANKL-mediated induction of osteoclast differentiation. While ApoE did not affect the activation of ERK, JNK, and p38 MAPK signaling pathways by RANKL, the phosphorylation of p65 trans-activation domain on serine 536 and transcription activity of NF-κB were reduced by ApoE overexpression. These findings suggest that ApoE plays an inhibitory role in osteoclast differentiation via the suppression of RANKL-dependent activation of NF-κB and induction of c-Fos and NFATc1.

Abbreviations: ApoE, Apolipoprotein E; BMMs, Bone marrow-derived macrophages; RANKL, Receptor activator of NF-κB ligand; M-CSF, Macrophage colony-stimulating factor; NFATc1, Nuclear factor of activated T cell c1; TRAP, Tartrate-resistant acid phosphatase; OSCAR, Osteoclast-associated receptor; DC-STAMP, Dendritic cell-specific transmembrane protein; ATP6v0d2, ATPase, H^+ transporting, lysosomal 38 kDa, V0 subunit d2; MNCs, Multinucleated cells

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http://dx.doi.org/10.1016/j.yexcr.2012.12.004

Introduction

Apolipoprotein E (ApoE) is a structural component of lipoproteins. It mediates lipoprotein binding to endocytotic lipoprotein receptors and thereby plays a central role in lipoprotein metabolism, in particular in the hepatic clearance of postprandial triglyceride-rich lipoproteins from the circulation [1]. ApoE-deficient mice are characterized by severe hyperlipidemia and develop spontaneous atherosclerotic lesions [2,3]. ApoE can mediate cholesterol efflux from the macrophage and identified as an autocrine and paracrine factor [4–6]. In addition to an important role in cholesterol and lipid metabolism, ApoE has also been shown to alter both innate and adaptive immune responses [7].

Osteoclasts differentiate from hematopoietic stem cells upon stimulation with two essential cytokines, receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [8,9]. RANKL is the factor that drives the differentiation process while M-CSF supports precursor proliferation and cell survival during differentiation [10–12]. Gene expression for osteoclast differentiation is controlled by various transcription factors including c-Fos, NFATc1 and NF-κB [13–15]. During osteoclast differentiation, c-Fos gene expression is stimulated by RANKL and c-Fos binding to the promoter region of NFATc1 increases NFATc1 expression level [16,17]. c-Fos and NFATc1 act synergistically to induce the expression of key osteoclastogenesis-associated genes that include TRAP, cathepsin K, OSCAR, DC-STAMP, and ATP6v0d2 [18–23]. Especially, it was reported that DC-STAMP and ATP6v0d2 regulate cell-to-cell fusion of macrophage lineage cells [22,23]. Overstimulation of osteoclastogenesis is responsible for excessive bone resorption that leads to bone destruction in osteoporosis and rheumatoid arthritis. Therefore, it is important to identify novel molecular targets that regulate osteoclast differentiation or function for the development of new therapeutics.

The NF-κB pathway has been shown to be critical in osteoclast differentiation [15] and the majority of NF-κB is the p50/p65 heterodimer, which remains in an inactive state in the cytoplasm in association with the inhibitory protein IκB [24]. Upon stimulation, IκB is rapidly phosphorylated by IκB kinase (IKK) and degraded by proteasomes [25]. IKK also induces the phosphorylation of p65 trans-activation domain on serine 536 and subsequently allows translocation of p50/p65 to the nucleus [26,27].

ApoE knockout mice display hyperlipidemia [2,3]. It has been shown that lipid-lowering agents positively affect atherosclerotic calcification and osteoporosis [28,29]. Although, whether the lipid-lowering agents attenuate osteoporosis directly or indirectly remains controversial, lipid levels appear to be inversely related to bone volume. However, there have been only a few studies describing skeletal changes caused by ApoE deficiency. Some osteoblast associated studies reported that ApoE-deficient mice display a high bone mass phenotype by an increased bone formation rate [30,31]. On the other hand, in a high-fat diet condition, ApoE gene deficiency was shown to reduce bone formation due to the stimulation of p53-mediated apoptosis of osteoblastic cells [32]. It has also been reported that ApoE expression was induced upon mineralization of primary calvariae osteoblasts [33]. Collectively, the effect of ApoE on bone has not been well investigated and most of the studies have focused on osteoblasts.

Unlike the role of ApoE in osteoblasts, the possibility of involvement of ApoE in osteoclast regulation has not been well studied. Therefore, we investigated the potential role of ApoE in osteoclast differentiation in this study.

Materials and methods

Materials

Cell counting kit (CCK) was obtained from Dojindo (Kumamoto, Japan). Recombinant human soluble RANKL, M-CSF and ApoE4 were from Peprotech (Rocky Hill, NJ, USA). Anti-ApoE was purchased from Abcam (Cambridge, UK). Polyclonal antibodies against ERK, JNK, p38, IκBα, phospho-ERK (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), phospho-IκBα (Ser32), phospho-p65 (Ser536) and PARP were purchased from Cell Signaling Technology (Cambridge, MA, USA). Anti-c-Fos and anti-NFATc1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody against β-actin and HRP-conjugated secondary antibodies were from Sigma Aldrich (St Louis, MO, USA). Renilla-Firefly luciferase dual assay kit was purchased from Pierce (Rockford, IL USA). TRAP staining kit and other reagents were purchased from Sigma Aldrich.

Cell culture

RAW264.7 and PlatE cells were cultured in DMEM supplemented with 10% v/v heat-inactivated FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin and incubated at 37 °C in 5% CO_2. RAW264.7 cells were plated in 48-well culture plates at 1×10^4 cells per well and PlatE cells were plated in 60-mm culture dishes at 4×10^5 cells. Bone marrow-derived macrophages (BMMs) were cultured in α-MEM supplemented with 10% v/v heat-inactivated FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin and incubated at 37 °C in 5% CO_2. BMMs were plated in 48-well culture plates at 4×10^4 cells per well.

Gene cloning

To express mouse ApoE in BMMs, the 936 bp cDNA (GenBank accession number NM_009696) fragment of ApoE was obtained by RT-PCR of mRNA from BMMs. The following primers were used: full length ApoE, 5′-GGATCCGCCACCATGAAGGCTCTGTGG-3′ (sense) and 5′-GCGGCCGATCATTGATTCTCCTGGG-3′ (antisense). pMX-IG vector and the PCR product were cleaved with BamHI and NotI restriction enzymes and subjected to 1% agarose gel electrophoresis. Each DNA fragment was purified from the gel and ApoE fragment was cloned into identical sites of pMX-IG and the resulting plasmid was named as pMX-ApoE. To establish RAW264.7 cells stably overexpressing ApoE, ApoE was obtained by RT-PCR of mRNA from RAW264.7 cells. The following primers were used: full length ApoE, 5′-CGGGATCC-CACCATGAAGGCTCTGTGGGCC-3′ (sense) and 5′-TCCCCGCGGTT-GATTCTCCTGGGC-3′ (antisense). PCR product was cleaved with BamHI and SacII restriction enzymes and subjected to 1% agarose gel electrophoresis. DNA fragment was purified from the gel and cloned into identical sites of pLenti6 vector.