EXPERIMENTAL CELL RESEARCH 316 (2010) 3254–3262

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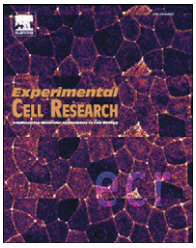

Research Article

Double stranded RNA-dependent protein kinase is involved in osteoclast differentiation of RAW264.7 cells in vitro

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ARTICLE INFORMATION

Article Chronology:
Received 8 April 2010
Revised version received 29 July 2010
Accepted 16 August 2010
Available online 20 August 2010

Keywords:
PKR
Osteoclast
RAW264.7
MFR
DC-STAMP
NF-κB

ABSTRACT

Double-stranded RNA-dependent protein kinase (PKR) plays a critical role in antiviral defence of the host cells. PKR is also involved in cell cycle progression, cell proliferation, cell differentiation, tumorigenesis, and apoptosis. We previously reported that PKR is required for differentiation and calcification of osteoblasts. However, it is unknown about the role of PKR in osteoclast differentiation. A dominant-negative PKR mutant cDNA, in which the amino acid lysine at 296 was replaced with arginine, was transfected into RAW264.7 cells. We have established the cell line that stably expresses the PKR mutant gene (PKR-K/R). Phosphorylation of PKR and α-subunit of eukaryotic initiation factor 2 was not stimulated by polyinosic–polycytidylic acid in the PKR-K/R cells. RANKL stimulated the formation of TRAP-positive multinuclear cells in RAW264.7 cells. However, TRAP-positive multinuclear cells were not formed in the PKR-K/R cells even when the cells were stimulated with higher doses of RANKL. A specific inhibitor of PKR, 2-aminopurine, also suppressed the RANKL-induced osteoclast differentiation in RAW264.7 cells. The expression of macrophage fusion receptor and dendritic cell-specific transmembrane protein significantly decreased in the PKR-K/R cells by real time PCR analysis. The results of RT-PCR revealed that the mRNA expression of osteoclast markers (cathepsin K and calcitonin receptor) was suppressed in the PKR-K/R cells and RAW264.7 cells treated with 2-aminopurine. Expression of NF-κB protein was suppressed in the PKR-K/R cells and 2-aminopurine-treated RAW264.7 cells. The level of STAT1 protein expression was elevated in the PKR-K/R cells compared with that of the wild-type cells. Immunohistochemical study showed that PKR was localized in osteoclasts of metatarsal bone of newborn mouse. The finding that the PKR-positive multinuclear cells should be osteoclasts was confirmed by TRAP-staining. Our present study indicates that PKR plays important roles in the differentiation of osteoclasts.

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doi:10.1016/j.yexcr.2010.08.006

Introduction

Double-stranded RNA-dependent protein kinase (PKR) is an abundantly expressed serine/threonine protein kinase which is activated by double-stranded RNA (dsRNA), interferons, cytokines, stress signals, and viral infection [1,2]. PKR is also involved in the several signal transduction pathways, such as Toll-like receptors (TLRs), mitogen-activated protein kinase (MAPK), nuclear factor of κB (NF-κB), and inhibitor of NF-κB (IκB) [3–5]. PKR becomes activated through autophosphorylation. Once activated, the enzyme phosphorylates certain substrates including the α-subunit of eukaryotic initiation factor 2 (eIF-2α) [6,7].

Osteoclasts are multinucleated cells derived from committed monocyte/macrophage lineage [8,9]. Formation of osteoclasts should be involved in the fusion of osteoclast precursor cells. Macrophage fusion receptor (MFR) [10] and dendritic cell-specific transmembrane protein (DC-STAMP) [11] were suggested to play roles in the cell fusion of osteoclast precursor macrophages and formation of active multinucleated osteoclasts. The receptor activator of NF-κB (RANK) and its ligand (RANKL) are central regulators of osteoclast formation and function. RANKL–RANK interaction activates a variety of downstream signaling pathways such as NF-κB [12,13]. As stimulation of RANK results in the strong NF-κB activation, NF-κB is essential for RANK-expressing osteoclast precursors to differentiate into tartrate resistant acid phosphatase (TRAP)-positive osteoclasts in response to RANKL and other osteoclastogenic cytokines [14,15]. RANKL was also reported to stimulate the signal transducers and activators of transcription 1 (STAT1) activation in bone marrow-derived macrophages [16]. These findings suggest that downstream signaling including NF-κB and STAT1 through RANK is essential for osteoclastogenesis.

We have previously reported that PKR is essential for osteoblast differentiation and mineralization in mouse osteoblastic MC3T3-E1 cells [17,18]. PKR-dependent differentiation of osteoblast was mediated by STAT1 signalling [17]. PKR was also involved in the ubiquitination of STAT1 and degradation of this protein [18]. However, the function of PKR in osteoclastogenesis has not been studied. In the present study, we clarified whether PKR could affect osteoclastogenesis. A dominant-negative PKR mutant cDNA, in which the amino acid lysine at 296 was replaced with arginine in catalytic domain of PKR and was transfected into the murine osteoclast lineage cell line RAW264.7. We have established cell lines that are stably expressing PKR-K/R mutant gene. We examined the osteoclast differentiation in the PKR-K/R mutant cells or 2-aminopurine-treated RAW264.7 cells.

Materials and methods

Reagents

Recombinant mouse RANKL was purchased from Peprotech EC (London, UK), 2-aminopurine (2-AP), G418 Geneticin, polyinosic–polycytidylic acid [poly (I:C)], Leukocyte acid phosphatase kit, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alfa-modification of Minimum Essential Medium (α-MEM) and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). FuGene HD was from Roche (Indianapolis, IN, USA). Fetal bovine serum was obtained from Equitech-Bio (Kerrville, TX, USA). Anti-phospho-eIF-2α (119A11) antibody was from Cell Signaling (Danvers, MA, USA). Anti-PKR (M-515) and anti-NF-κB p65 (C-20) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit monoclonal anti-PKR antibody (YE350) was obtained from abcam (Cambridge, MA, USA). Antibodies for STAT1 (610185) and STAT3 (610189) were obtained from BD Biosciences (San Jose, CA, USA). Plastic dishes were from IWAKI (Chiba, Japan). Other materials used were of the highest grade commercially available.

Establishment of the PKR-K/R mutant RAW264.7 cells

Human PKR cDNA and a PKR-K/R mutant cDNA (carrying a mutation of amino acid K to R at position 296) and their expression vector were kindly provided by Drs. A. Hovanessian (Institute Pasteur, France) [19] and T. Takizawa (Aichi Human Service Center, Aichi, Japan) [20], respectively. PKR-K/R cDNA was subcloned into pcDNA3.1-Flag (modified pcDNA3.1, Invitrogen). Transfection of pcDNA3.1-Falg-PKR-K/R into RAW264.7 cells was performed using FuGene HD Reagents. Two μg of pc-Flag and pc-Flag-PKR-K/R in 100 μl Opti-MEM were mixed with 8 μl FuGene HD Reagent for 15 min at room temperature. The DNA-FuGene HD complex was then added into 35-mm dishes containing 4×10^5 cells. The media were replaced at 24 h after transfection. The cells were cultured for 2 weeks with 500 μg/ml G418 Geneticin. Media were replenished every 3 days. The drag-resistant colonies were selected and cloned. We confirmed that dominant-negative PKR of human origin was expressed in RAW264.7 cells.

Differentiation of osteoclast in RAW264.7 cells

RAW264.7 cells were seeded into 96 well plates at a density of 750 cells per 150 μl of α-MEM containing 10% FBS with various concentrations of RANKL. After 2-day cultivation the media were completely replaced with fresh media with RANKL. At day 4, the cells were fixed for 50 s with acetone-citrate-formaldehyde (26:66:8), and stained for TRAP by using Leukocyte acid phosphatase kit according to the manufacturer's directions. TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclasts.

RNA preparation, real time PCR, and RT-PCR

RAW264.7 cells and the PKR-K/R cells were cultured in 35 mm dishes (1.0×10^4 cells/dish) in the medium containing 10 ng/ml of RANKL or 5 mM 2-AP for the desired days. Quantitative real time PCR analysis was performed using SYBER Premix Ex taq Perfect Real Time (Takara Bio, Kyoto, Japan). The sequences of the primers used are as follows: mouse MFR forward, ATGACACGCCCAAGAATCTC, mouse MFR reverse, CAACCGTGTGGAAGATGTTG, mouse DC-STAMP forward, ACTAGAGGAGAAGTCCTGGGAGTC, mouse DC-STAMP reverse, CACCCACATGTAGAGATAGGTCAG, mouse CD9 forward, TGTCTCAGTCGGTTGTCGAG, mouse CD9 reverse, GCTCGAA GATGCTCTTGGTC, mouse CD44 forward, GGCGACTAGATCCCT CCGTT, mouse CD44 reverse, ACCCAGAGGCATACCAGCTG, mouse GAPDH forward, TGTGTCCGTCGTGGATCT, mouse GAPDH reverse, TTGCTGTTGAAGTCGCAGGAG. DNA amplification and detection was performed in the ABI Prism 7500 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). PCR amplification (40 cycles) was performed