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Constitutive and functional expression of runt-related transcription factor-2 by microglial cells

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ABSTRACT

Runt-related transcription factor-2 (Runx2) is the master regulator of osteoblastogenesis with an ability to promote differentiation of mesenchymal stem cells into the osteoblastic lineage. We have previously shown constitutive and functional expression of Runx2 by astroglial cells. In this study, we investigated the possible expression of Runx2 by both murine microglia and microglial cell line BV-2 cells. *Runx2* expression was seen in cultured microglia and BV-2 cells, while sustained exposure to 1 mM ATP led to a significant but transient increase in mRNA and corresponding protein expression of Runx2 within 24 h. The increase in *Runx2* expression was invariably prevented by several chemicals with antagonistic properties for P2X7 purinergic receptor, calmodulin and calcineurin in BV-2 cells, with a P2X7 receptor agonist more than quadrupling *Runx2* expression. A significant increase in *Runx2* expression was seen in osteoclastic cells, but not in osteoblastic or chondrocytic cells, when exposed to a high concentration of ATP. In BV2-cells with control siRNA, a significant decrease was found in the number of cells with at least one process within 3 h after the exposure to 1 mM ATP, followed by an increase up to 24 h. However, Runx2 siRNA significantly deteriorated the property to induce delayed process extension during 6–24 h after exposure to ATP along with drastically decreased Runx2 protein levels. These results suggest that Runx2 is constitutively and functionally expressed by microglial cells with responsiveness to ATP for upregulation in the murine brain.

1. Introduction

Microglia are derived from parenchymal tissue macrophages at a population over 10% of cells found in the central nervous system (CNS). In the healthy adult brain, microglia are present with at least one branched process as often referred to as "ramified microglia", which are thought to be quiescent under the normal conditions, with actively surveying their environments using these branched processes (Wake et al., 2009). When they sense unusual signs and/or signals during infection, inflammation, trauma, ischemia and other neurodegenerative abnormalities with those motile processes, in contrast, they immediately transform their features from a surveying type into an active form called "activated microglia". Activated microglia show several typical profiles functionally different from those in ramified microglia, such as process retraction, migration, proliferation and phagocytosis, in addition to releasing pro-inflammatory cytokines (Hanisch and Kettenmann, 2007; Kreutzberg, 1996; Nakajima et al., 1996). In addition, ramified microglia are also shown to migrate with extremely motile processes to monitor brain parenchyma even in a presumed resting state *in vivo* (Nimmerjahn et al., 2005).

Abbreviations: αMEM, α-minimal essential medium; BzATP, 3′-O-(4-benzoyl)benzoyl ATP; Cbfβ, core binding factor β; CNS, central nervous system; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagle medium; DMEM/F12, Dulbecco's modified Eagle's and Ham's F-12 medium; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FK506, tacrolimus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hoechst33342, bis-benzimide trihydrochloride; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase extracellular signal-regulated kinase kinase; MG132, N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al; MMP13, matrix metalloproteinase-13; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; OSE2, osteoblast specific element; oxATP, oxidized ATP; PA, paraformaldehyde; PACAP, pituitary adenylyl cyclase activating peptide; PBS, phosphate-buffered saline; PPADS, 4-([4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo)-1,3-benzenedisulfonic acid; p38-MAPK, p38 mitogen-activated protein kinase; RT-PCR, reverse transcription polymerase chain reaction; Runx2, runt-related transcription factor-2; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole; siRNA, small interfering RNA; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; Wnt, W*ingless* and *INT-1*.

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A large amount of intracellular ATP is leaked from neighboring injured and dead cells to induce diverse functional alterations through activation of a variety of purinergic receptors expressed at the surface in ramified and activated microglia. Purinergic receptors are classified into P1 and P2 receptor subclasses according to the agonist specificity; adenosine for the P1 subclass and ATP for the P2 subclass, respectively (Burnstock and Kennedy, 1985; Koizumi et al., 2013). P1 receptors are all metabotropic receptor isoforms grouped into A1, A2A, A2B and A3 subtypes on the basis of their intracellular signals and molecular nucleotide sequential homology, whereas P2 receptors are further classified into ionotropic P2X and metabotropic P2Y subtypes. Recent studies indicate that purinergic receptor activation plays a central role in determining phenotypes of activated microglia (Koizumi et al., 2013).

Runt-related transcriptional factor-2 (Runx2; also known as Cbfa1, PEBP2α and AML3) is a cell-specific member of the Runt family of transcription factors (Runx1-3) with a critical role in cellular differentiation steps in osteoblasts and chondrocytes (Kagoshima et al., 1993). In osteoblasts, Runx2 forms a heterodimer with the partner protein core binding factor β (Cbfβ) to recognize the particular DNA element osteoblast specific element (OSE2) at the upstream of different target gene promoters, followed by induction of osteoblastic differentiation for subsequent regulation of expression of a variety of genes characteristic to the osteoblast phenotype. By contrast, recent studies including ours have demonstrated that Runx2 is also expressed in non-osseous tissues such as brain (Jeong et al., 2008; Takarada and Yoneda, 2009). We have shown that both *Runx*2 and *Cbf*β are expressed by rat neocortical astrocytes and C6 glioma cells to lead to transactivation of several downstream target genes such as matrix metalloproteinase-13 (MMP13) as seen in osteoblasts (Takarada and Yoneda, 2009). However, little attention has been paid to the expression of Runx2 by other cells residing in the CNS.

In the present study, therefore, we investigated expression of the master regulator of osteoblastic differentiation Runx2 by microglial cells to elucidate the possible novel physiological and/or pathological significance of the Runx2 signaling pathway outside bone in the maintenance of brain functions using primary cultured mouse brain microglia and mouse microglial cell line BV-2 cells.

2. Materials and methods

2.1. Materials

Pregnant ddY mice were supplied by SANKYO LABO SERVICE (Toyama, Japan). Mouse microglial BV-2 cells are a generous gift from Dr. Eui-Ju Choi (Korea University, Seoul, Korea) (Blasi et al., 1990). Pre-osteoblastic MC3T3-E1 cells, pre-chondrogenic ATDC5 cells and the macrophage cell line RAW264.7 cells were all purchased from RIKEN Cell Bank (Saitama, Japan). FK506 (tacrolimus, m.w. 822.03) was kindly given by Astellas Pharma, Inc. (Tsukuba, Japan). Dulbecco's modified Eagle medium (DMEM), penicillin–streptomycin and ethidium bromide solution were obtained from Gibco BRL (Gaithersburgm, MD, USA). Lipopolysaccharide (LPS) from Salmonella enteritidis, Poly-L-lysine, ATP, bis-benzimide trihydrochloride (Hoechst33342), oxidized ATP (oxATP), N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al (MG132), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole (SB203580), 3′-O-(4-benzoyl)benzoyl ATP (BzATP), propidium iodide (PI), Hoechst33342 and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was provided by JRH Biosciences, Inc. (Lenexa, KS, USA). Dulbecco's modified Eagle's and Ham's F-12 medium (DMEM/F12), α-minimal essential medium (αMEM), 5xFirst strand buffer, M-MLV Reverse transcriptase, Ultrapure agarose, Plus reagent, LipofectamineRNAiMAX, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG were supplied by Invitrogen (San Diego, CA, USA). ISOGEN, trypsin, paraformaldehyde (PA), EDTA, dimethyl sulfoxide (DMSO), tunicamycin, rapamycin, cyclosporin A (CsA) and anti-Iba1 antibody were purchased from Wako (Osaka, Japan). Fluoro Save™ was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). Tissue-Tek OCT compound 4583 was purchased from SAKURA (Tokyo, Japan). dNTP mix and 10xbuffer were purchased from Takara Bio Inc. (Otsu, Japan). 4-([4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy) methyl]-2-pyridinyl]azo)-1,3-benzene-disulfonic acid (PPADS) and isoPPADS were purchased from Tocris (Ballwin, MO, USA). Bio-Rad Protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Horseradish peroxidase conjugated secondary antibody and ECL™ detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). 1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) was purchased from CST Japan (Tokyo, Japan). Anti-Runx2 antibody was purchased from MBL (Nagoya, Japan). EGTA was purchased from Dojindo (Kumamoto, Japan). Other chemicals used were all of the highest purity commercially available.

2.2. Cell lines

Mouse microglial cell line BV-2 cells were plated at a density of 2×10^4 cells/cm^2 in DMEM containing 10% FBS in appropriate dishes, and then cultured at 37 °C for 1 day under 5% CO_2. Pre-osteoblastic MC3T3-E1 cells were plated at a density of 1×10^4 cells/cm^2 in αMEM containing 10% FBS, followed by culture for 7 days with 50 μg/ml ascorbic acid and 5 mM β-glycerophosphate for induction of cellular differentiation and subsequent replacement of culture medium with DMEM containing 10% FBS. Pre-chondrocytic ATDC5 cells were plated at a density of 1×10^4 cells/cm^2 in a 1:1 mixture of DMEM/F12 containing 5% FBS, followed by culture for 7 days with 10 μg/ml transferrin, 3×10^{-8} M sodium selenite and 10 μg/ml bovine insulin for induction of differentiation and subsequent replacement of culture medium with DMEM containing 10% FBS. RAW264.7 cells were plated at a density of 2×10^4 cells/cm^2 in DMEM containing 10% FBS in appropriate dishes and then cultured at 37 °C for 1 day under 5% CO_2. These cell lines were then exposed to 1 mM ATP for 1–24 h, followed by extraction of total RNA and subsequent determination of mRNA expression on quantified reverse transcription polymerase chain reaction (RT-PCR).

2.3. Primary microglia and astroglia

Animal experiments conducted here were based on the Act on Welfare and Management of Animals (1973 Law Number 105), the Standards Relating to the Care and Management of Experiment Animals (2006 Ministry of the Environment Announcement Number 88), the Policy on Animal Euthanasia Methods (1995 General Administrative Agency of the Cabinet Announcement Number 40), the MEXT Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (2006 Ministry of Education, Culture, Sports, Science and Technology Announcement Number 71). In addition, this study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Japanese Society for Pharmacology. The protocol was approved the Committee on the Ethics of Animal Experiments of the University of Kanazawa (permit number; AP-122394) with an effort to minimize the number of animals used and their suffering. Microglia were isolated from neonatal mouse brains by the method