Free Radical Biology & Medicine 51 (2011) 424–431

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Increase in CpG DNA-induced inflammatory responses by DNA oxidation in macrophages and mice

Hiroyuki Yoshida *, Makiya Nishikawa, Tsuyoshi Kiyota, Hiroyasu Toyota, Yoshinobu Takakura

Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Yoshidashimoadachi-cho 46–29, Sakyo-ku, Kyoto 606–8501, Japan

ARTICLE INFO

Article history:
Received 5 February 2011
Revised 4 April 2011
Accepted 19 April 2011
Available online 22 April 2011

Keywords:
8-Hydroxydeoxyguanosine containing DNA
CpG DNA
TLR9
Inflammatory response

ABSTRACT

Unmethylated CpG dinucleotide (CpG motif) is involved in the exacerbation of DNA-associated autoimmune diseases. We investigated the effect of DNA containing 8-hydroxydeoxyguanosine (oxo-dG), a representative DNA biomarker for oxidative stress in the diseases, on CpG motif-dependent inflammatory responses. ODN1668 and ODN1720 were selected as CpG-DNA and non-CpG DNA, respectively. Deoxyguanosine in the CpG motif (G9) or outside the motif (G15) of ODN1668 was substituted with oxo-dG to obtain oxo(G9)-1668 and oxo(G15)-1668, respectively. Oxo(G15)-1668 induced a significantly higher amount of tumor necrosis factor (TNF)-α from RAW264.7 macrophage-like cells than ODN1668, whereas oxo(G9)-1668, oxo(G8)-1720, or oxo(G15)-1720 hardly did. CpG DNA-induced TNF-α production was significantly increased by addition of oxo(G8)-1720 or oxo(G15)-1720, but not of ODN1720. This oxo-dG-containing DNA-induced increase in TNF-α production was also observed in primary cultured macrophages isolated from wild-type mice, but not observed in those from Toll-like receptor (TLR)-9 knockout mice. In addition, TNF-α production by ligands for TLR3, TLR4, or TLR7 was not affected by oxo-dG-containing DNA. Then, the footpad swelling induced by subcutaneous injection of ODN1668 into mice was increased by coinjection with oxo(G8)-1720, but not with ODN1720. These results indicate that oxo-dG-containing DNA increases the CpG motif-dependent inflammatory responses, which would exacerbate DNA-related autoimmune diseases.

Introduction

Progressive joint destruction with chronic inflammation is one of the chief complaints of patients with rheumatoid arthritis (RA), a disease that affects 1% of the population worldwide. Its complete pathogenic mechanism still remains to be elucidated, but the involvement of DNA in the pathogenesis has been suggested. In the inflamed joints, nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) are continuously released from dead cells and the concentration of mtDNA in the joint cavity and circulation of RA patients is higher than that of healthy subjects [1]. In addition to mtDNA, various viral or bacterial DNA were also detected in the joints of arthritis patients [2–4].

mtDNA and viral/bacterial DNA contain many unmethylated CpG dinucleotides (CpG motifs), which are a known activator of dendritic cells, B cells, and macrophages. Upon recognition by cells through Toll-like receptor-9 (TLR9), the specific receptor for CpG motif, such DNA induces the production of TNF-α, interleukin-6 (IL-6), and interferon-γ (IFN-γ) [5]. An intraarticular injection of CpG DNA induced synovial cytokine production and aggravated RA symptoms [6,7]. Accumulation of macrophages into inflamed sites was associated with these pathological changes [6,8,9].

Loss of homeostatic balance between reactive oxygen species (ROS) and antioxidants in the cellular milieu results in oxidative stress that causes oxidative DNA damages, including chemical modification of both pyrimidine and purine bases [10]. The most general marker for oxidative DNA is 8-hydroxydeoxyguanine (oxo-dG) and the level of which in plasma is known to increase in various diseases, such as Alzheimer's disease [11], Parkinson's disease [12], cystic fibrosis [13], systemic lupus erythematosus [14], and RA [15]. mtDNA is continuously exposed to more oxidative stress than nDNA because of its location close to the respiratory chain and lack of protective histones [16], which makes mtDNA, but not nDNA, a major component for oxidation [17]. Moreover, it has been reported that a rheumatoid joint is under high oxidative stress [18,19]. These pieces of evidence will explain the finding that the synovial fluid of RA patients contains large amounts of DNA, especially oxidized DNA [1].

Although there are multiple reports of oxo-dG as a biomarker of various diseases, few studies have evaluated the immunological aspects of oxo-dG on RA. Collins et al. investigated the involvement of

Abbreviations: CpG motif, unmethylated CpG dinucleotide; TLR, Toll-like receptor; LPS, lipopolysaccharide; ROS, reactive oxygen species; RA, rheumatoid arthritis; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; ODN, oligodeoxynucleotide; PO, phosphodiester; PS, phosphorothioate; bp, base pair; oxo-dGTP, 8-hydroxydeoxyguanosine triphosphate; oxo-dG, 8-hydroxydeoxyguanosine; oxo-G, 8-hydroxyguanosine; oxo-Gua, 8-hydroxyguanine; oxo-DNA, oxo-dG-containing DNA; ds, double-stranded; ss, single-stranded; LA2000, Lipofectamine2000; lipoplex, plasmid DNA/LA2000 complex; MFI, mean fluorescence intensity; PAGE, polyacrylamide gel electrophoresis; MyD88, myeloid differentiation factor 88.

* Corresponding author. Fax: +81 75 753 4614.
E-mail address: h.yoshida@nihs.go.jp (H. Yoshida).

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.04.035

oxo-dG in the aggravation of RA and demonstrated that intraarticular injection of oligodeoxynucleotide (ODN) containing oxo-dG into mouse knee joint provoked histopathological arthritis at a high frequency despite lacking a CpG motif in the ODN [20]. Accordingly, it has been postulated that oxo-dG or oxo-dG-containing DNA (oxo-DNA) is immunologically active, but its detail has not been clarified yet. Therefore, in this study, we evaluated the immunological activity of oxo-DNA by measuring the production of proinflammatory cytokines in murine macrophages. Assuming the conditions in inflamed joints, oxo-DNA was added to cells in combination with CpG DNA. Finally, the induction of experimental arthritis was examined by subcutaneous injection of a mixture of CpG DNA and oxo-DNA into the mouse footpad.

Materials and methods

Chemicals

RPMI 1640 medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). Lipofectamine2000 (LA2000) and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). DNase I and 20-base pair (bp) DNA ladder were purchased from Takara Bio, Inc. (Otsu, Japan). DNase II, lipopolysaccharide (LPS), poly(I:C), and polymyxin B sulfate salt were purchased from Sigma (St. Louis, MO, USA). Recombinant murine IFN-γ was purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). Triton X-114 was purchased from Nacalai Tesque (Kyoto, Japan). Imiquimod was purchased from Imgenex (San Diego, CA, USA).

DNA and oxo-DNA

ODNs were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The sequences of ODNs were as follows: CpG ODN1668, 5′-TCCATGACGTTCCTGATGCT-3′; non-CpG ODN1720, 5′-TCCATGAGCTTCCTGATGCT-3′; and A-type ODN2216, 5′-ggggACGATCGTCgggggG-3′. Capital letters represent phosphodiester (PO) linkages 3′ of the base and lowercases represent phosphorothioate (PS) ones. B-type ODN1668, all internucleotide linkages of which were PS, was also prepared. Double-stranded (ds-) ODN was prepared by annealing each ODN with its fully complementary ODN, and double-strand formation was confirmed by 21% polyacrylamide gel electrophoresis (PAGE). Oxo(G9)-1668 and oxo(G15)-1668, whose deoxyguanosine in the CpG motif (G9) or outside the motif (G15) of ODN1668 was substituted with 8-hydroxydeoxyguanosine (oxo-dG), were purchased from Nihon Gene Research Laboratories (Sendai, Japan). Similarly, oxo(G8)-1720 and oxo(G15)-1720 were obtained from Nihon Gene Research Laboratories. ODN1668 fluorescently labeled with Alexa488 was purchased from Nihon Bioservice (Saitama, Japan). ODN1668 fluorescently labeled with ATTO488 was purchased from Nihon Gene Research Laboratories. dGTP was purchased from Sigma. 8-Hydroxy-dGTP (oxo-dGTP) was purchased from Jena Bioscience (Jena, Germany). Oxo-dG was purchased from Wako Pure Chemical (Osaka, Japan). 8-Hydroxyguanosine (oxo-G) and 8-hydroxyguanine (oxo-Gua) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Plasmid vector pCMV-Luc, a CpG motif replete circular ds-DNA, was constructed as previously reported [21]. pCMV-Luc has 33 Pur-Pur-CpG-Pyr-Pyr sequences including two GACGTT, a most potent CpG motif for mice [22]. pCpG-ΔLuc, another plasmid with no CpG motifs, was constructed as previously reported [23]. Plasmid DNA/LA2000 complex (lipoplex) was prepared at a ratio of 2 μl LA2000 and 1 μg plasmid DNA according to the manufacturer's instructions.

Sample preparation

To minimize the activation of cells by contaminated LPS, plasmid DNA samples were extensively purified with Triton X-114, a nonionic detergent, before use according to a previously published method [24]. The level of contaminated LPS was checked by a Limulus amebocyte lysate assay using the Limulus F Single Test kit (Wako Pure Chemical). The level of contaminated LPS was reduced to below the detection limit of 0.01 EU/μg DNA. For poly(I:C) and imiquimod, polymyxin B, which binds to LPS, was added to cells at a final concentration of 5 μg/ml. ODNs, nucleotides, and nucleosides were used as obtained without further purification or addition of polymyxin B.

Animals

TLR9$^{-/-}$ mice were purchased from the Oriental Yeast Company (Tokyo, Japan). C57BL/6 wild-type mice and Institute for Cancer Research (ICR) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained on a standard food and water diet under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Institutional Animal Experimentation Committee of the Graduate School of Pharmaceutical Sciences, Kyoto University.

Cell cultures

Splenic macrophages were collected as previously described [25] and cultured on 96-well culture plates at a density of 3×10^5 cells/well in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (292 μg/ml), and 2-mercaptoethanol (10^{-5} M). These cells were used for the cytokine release experiment soon after isolation. The murine macrophage-like cell line, RAW264.7 cells, was cultured on 96-well culture plates at a density of 5×10^4 cells/well in RPMI 1640 supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (292 μg/ml). These cells were used after 24-h incubation.

Cytokine release from macrophages

RAW264.7 cells or splenic macrophages were incubated with ODNs, oxo-ODNs, oxo-dGTP, oxo-dG, oxo-G, oxo-Gua, dGTP, lipoplex, poly(I:C), LPS, or imiquimod for 8 h (TNF-α assay) or for 24 h (IL-6 assay), then the supernatants were collected for ELISA and kept at −80 °C until use. LPS was used as a positive control in most experiments to check the viability and reactivity of cell preparations. In the case of splenic macrophages, 10 units/ml of IFN-γ was added to the culture medium to prime cells. In addition, 5 μg/ml polymyxin B was also added to avoid cell activation by LPS in the IFN-γ sample. Separately, RAW264.7 cells were incubated with lipoplex for 2 h, the cells were washed with RPMI 1640 and incubated with fresh growth medium for an additional 6 h, and then the supernatants were collected for ELISA and kept at −80 °C until use. The level of TNF-α and IL-6 in the media was determined by ELISA using the OptEIA set (BD Biosciences, San Diego, CA, USA).

Cellular uptake of ODN in RAW264.7 cells

RAW264.7 cells were incubated with ATTO488-labeled ODN1668 or ATTO488-labeled oxo(G15)-1668 for 30, 90, or 240 min and washed three times with phosphate-buffered saline (PBS). Separately, cells were incubated with Alexa488-labeled ODN1668 with or without ODN1720 or oxo(G8)-1720 for 30, 90, or 240 min and washed three times with PBS. Then, the intensity of cell fluorescence was analyzed by flow cytometry (FACScan; BD Biosciences) using CellQuest software (version 3.1; BD Biosciences). Cellular uptake was estimated by subtracting the mean fluorescence intensity (MFI) at 4 °C from that at 37 °C (ΔMFI) and plotted against the incubation time.