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Inhibition of Rac and Rac-linked functions by 8-oxo-2′-deoxyguanosine in murine macrophages

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Abstract
Rac is a protein involved in the various functions of macrophages (Mϕ), including the production of reactive oxygen species (ROS), phagocytosis, chemotaxis and the secretion of cytokines (such as γ-INF). This study tested the effects of nucleosides containing 8-oxoguanine(8-hydroxyguanine) such as 8-oxo-2′-guanosine (8-oxoG) or 8-oxo-2′-deoxyguanosine (8-oxodG), on Rac and the above-listed Rac-associated functions of Mϕ using mouse peritoneal Mϕ (MpMϕ). It is reported that 8-oxodG was able to effectively inhibit Rac and the Rac-associated functions of MpMϕ. Compared to 8-oxodG, 8-oxoG showed negligible effects. Furthermore, normal nucleosides such as deoxyguanosine (dG), guanosine (G) and adenosine (A) did not exert any effects. These results suggested that 8-oxodG could be used as a potential tool to modulate the functions of Mϕ that are intimately related to various pathological processes.

Keywords: *chemotaxis, cytokines, macrophage, 8-oxo-deoxyguanosine, phagocytosis, Rac, ROS*

Abbreviations: *A, adenosine; dG, deoxyguanosine; G, guanosine; LPS, lipopolysaccharides; Mϕ, macrophage; MpMϕ, mouse peritoneal macrophages; 8-oxoGua, 8-oxoguanine(8-hydroxyguanine); 8-oxodG, 8-oxo-2′-deoxyguanosine(8-hydroxy-2′-deoxyguanosine); 8-oxoG, 8-oxoguanosine(8-hydroxyguanosine); PMA, phorbol myristate acetate*

Introduction

Rac, a small GTP (guanosine triphosphate) binding protein is known to be involved in various functions of leukocytes, particularly those of macrophages (Mϕ), which play important roles in inflammation, immune responses and atherosclerosis. These functions include phagocytosis [1], generation of reactive oxygen species (ROS) [2], chemotaxis [3] and production of cytokines such as INF-γ [4,5]. Thus, the modulation of these Mϕ functions is intimately related to the control of various immunological [6] and pathological processes [7].

As with DNA, the guanine base of 2′-deoxyguanosine triphosphate(dGTP) and guanosine triphosphate (GTP) is oxidized into 8-oxo-guanine(8-oxoGua; 8-hydroxyguanine) by ROS [8,9]. Thus far, the resulting 8-oxodGTP and 8-oxoGTP have been regarded simply as mutagenic waste, since they are incorporated into DNA and RNA, making 8-oxoGua-containing DNA and RNA, respectively.

Interestingly, we recently reported that 8-oxoGTP inactivates Rac [10], which is activated by GTP. Furthermore, we also observed that 8-oxoGTP inhibited Rac-associated function. For example, 8-oxoGTP inhibited phorbol myristate acetate (PMA)-induced activation of NADPH oxidase in human neutrophil lysates and, thus, PMA-induced production of ROS [11]. NADPH oxidase is a typical Rac-linked effector, requiring Rac-GTP complex for its activation to produce ROS [12]. Further study

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revealed the possibility that 8-oxoGTP may act as a terminator of ROS production during respiratory burst of neutrophils [11]. This was based on the understanding that 8-oxoGTP, formed by ROS, may sufficiently accumulate in the cytosol of neutrophils such that this enzyme is inhibited as the respiratory burst proceeds.

These observations suggested that 8-oxoGTP might affect other Rac-linked functions of Mϕ. Because of its impermeability to cell membranes, however, the Rac-inhibiting action of 8-oxoGTP was demonstrated only in cell lysates [10,11] and not in intact cells. Thus, its inhibiting action on other Rac-linked functions of Mϕ could not be demonstrated. In an attempt to identify modulators of Mϕ functions, we tested whether 8-oxoguanosine (8-oxoG) and 8-oxo-2′-deoxyguanosine (8-oxodG), 8-oxoGua-containing membrane-permeable nucleosides inhibited Rac and the Rac-associated functions of Mϕ in intact mouse peritoneal Mϕ (MpMϕ). Here, we report that 8-oxodG effectively inhibited all Rac-associated functions tested, proposing that 8-oxodG could act as a modulator of Mϕ functions.

Materials and methods

Animals

C57BL/6 male mice, 5-weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free conditions and used at $\sim$6 weeks of age weighing 25–28 g. Animal experiments were performed in accordance with the *Guidelines for Animal Experimentation* issued by the Ethics Committee of Seoul National University.

Isolation of mouse peritoneal macrophage (MpMϕ)

MpMϕ were obtained as previously described [13]. Briefly, mice were injected intraperitonially with 500 μl of thiolglycollate medium (Renel, Lenexa, KS) containing 3% thiolglycollate. Three days later, 10 ml of Hanks' balanced salt solution (HBSS) was injected into the peritoneal cavity. Then, HBSS was collected and centrifuged at 1300 g for 5 min. The cell pellet obtained contained MpMϕ of $\sim 1 \times 10^7$ cells/mouse and these were pooled and suspended in RPMI1640(RPMI)-supplemented with 10% foetal bovine serum (RPMI/10% FBS). The cell viability was found to be greater than 80% by flow cytometric analysis. MpMϕ, which was in the stimulated state due to the presence of thioglycollate, were starved to bring them to the resting state. Starvation of MpMϕ was achieved by 30-times dilution of the MpMϕ suspension in RPMI/10% FBS with FBS-free RPMI to 1×10^6 cells/ml and then incubation in a CO_2 incubator for 24 h at 37°C. The starved MpMϕ were used directly for the same day experiments.

Rac assay

Rac activity was assayed as previously described [10] using Rac activation kits (Upstate Biotechnology, Lake Placid, NY) containing a mouse monoclonal anti-Rac1. MpMϕ (1×10^6 cells) in 3 ml of RPMI was transferred into a culture dish (60 mm) and allowed to adhere to the bottom of the dish for 3 h. After replacing the media with fresh RPMI (3 ml) containing 8-oxodG, 8-oxoG, dG, G or A (0–100 μg/ml; 0–353 μM), cells were incubated for 1 h and then treated with PMA (30 μg in 30 μl DMSO) for 15 min. After discarding the media, the cells were washed with PBS twice, suspended in 1 ml of lysis buffer [10] on ice for 30 min and centrifuged at 1300 g for 15 min. The supernatants were collected as cell lysates, from which active Rac1 was affinity-precipitated and detected by Western blotting. The band densities were quantified using the BAS 2500 imaging analyser (Fuji Photo Film, Japan).

Measurement of ROS

MpMϕ (1×10^5 cells) in 100 μl of RPMI was transferred into a 96-well plate and allowed to adhere to the bottom of the wells for 3 h. After replacing the media with fresh RPMI (200 μl) containing 8-oxodG, 8-oxoG, dG, G or A (0–100 μg/ml), the cells were incubated for 1 h. This was followed by a further incubation with PMA (2 μg in 2 μl DMSO) for 15 min. In order to detect ROS, 2 μg OxyBurst Green H_2HFF BSA in 2 μl PBS and 2 mM sodium azide were added into each well. Two minutes later, the fluorescence was detected continuously using excitation and emission wavelengths of 488 nm and 530 nm, respectively, over a period of 2 min in a spectrofluorometer (Uvicon 933, Kontron instrument, Italy).

Measurement of NADPH oxidation

MpMϕ (1×10^6 cells) in 3 ml RPMI was transferred into culture dishes (60 mm) and allowed to adhere to the bottom of the dish for 3 h. The cells were treated with 8-oxodG, 8-oxoG, dG, G or A (0–100 μg/ml) or diphenyleneiodonium (DPI) (10 μM) and then stimulated with PMA as was previously done in the Rac assay. NADPH in 300 μl PBS was added to give a final concentration of 0.07 mM. Aliquots (0.5 ml) were taken every 5 min and NADPH oxidation was monitored at A_{330} using a spectrophotometer (Uvikon 933, Kontron instrument, Italy).

Phagocytosis assay

MpMϕ (1×10^6 cells) in 3 ml RPMI were treated with 8-oxodG (0–100 μg/ml) and stimulated with PMA as previously done in the Rac assay. In order to monitor phagocytosis, a suspension (5 μl) of

fluorescently labelled beads (1×10^{10} beads, 1 μm/ml: FluoSpheres® polystyrene microspheres, Molecular Probes, Eugene, OR) was added to the media and incubated for 6 h. The media was then discarded and the cells were washed three times with cold PBS. The cells were collected in 500 μl PBS and subjected to flow cytometric analysis using FACScalibur flow cytometry (Becton Dickinson, Mountain View, CA).

Chemotaxis assay

MpMϕ (1×10^3 cells) in 25 μl of RPMI were incubated with 8-oxodG (0–100 μg/ml) for 1 h and then transferred to the upper compartments of each well of a Boyden-modified, 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD). Each well of this chamber was separated into two compartments by a polycarbonate filter with a pore of 5 μm diameter. The lower compartments were filled with RPMI (25 μl) with or without formyl-Met-Leu-Phe (fMLP) (10^{-8} M). The chamber was incubated in a CO_2 incubator for 6 h at 37°C. Chemotaxis was evaluated by determining the number of the cells that adhered to the filter, according to the manufacturer' instructions.

IFN-γ assay

MpMϕ (1×10^5 cells) in 0.3 ml RPMI were transferred into a 48-well plate and allowed to adhere to the bottom of the wells for 3 h. The media was replaced with fresh RPMI containing 8-oxodG of 0–100 μg/ml, incubated for 1 h and then further incubated for 24 h after mixing lypopolysaccharides (LPS) (100 ng/ml). The media was then assayed for IFN-γ using an ELISA assay kit (R&D Systems, Minneapolis, MN) containing a monoclonal antibody specific to IFN-γ, according to the manufacturer's instructions.

Results

Rac activity

In the present study, 8-oxodG and other nucleosides of 8-oxoGua were tested for Rac-inhibiting activity in intact cells using MpMϕ. Rac1 was examined since it is the predominant form of Rac in mice [14]. As shown in Figure 1, the activity of Rac1 in the resting state was abruptly increased upon stimulation by PMA. This PMA-induced increase in Rac1 activity was inhibited by pre-treatment of MpMϕ with 8-oxodG in a dose-dependent manner. In contrast, 8-oxoG showed almost no effect on the activity of Rac1. No inhibition was also observed with normal nucleosides such as dG, G and A.

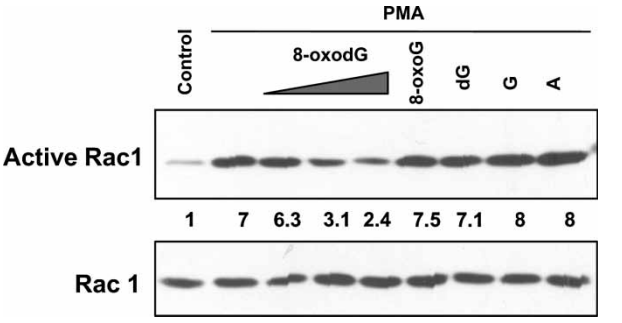

Figure 1. Effects of 8-oxoGua-containing or normal nucleotides on Rac in intact MpMϕ. MpMϕ were incubated with 8-oxodG (0, 25, 50 or 100 μg/ml; 0, 88, 177 or 353 μM), 8-oxoG, dG, G or A (100 μg/ml; 353 μM, each) and then treated with PMA. The cells were subjected to lysis. The active Rac1 was determined from these lysates. Details are described in Materials and methods. The results are shown in the upper row indicated by *Active Rac 1*. The numbers shown below each band are relative densities. The lower row indicated by *Rac1* shows total amounts of Rac protein. The data are the representative of four experiments.

NADPH oxidase

In the following experiments, the effects of 8-oxodG and other nucleosides on Rac-associated functions of Mϕ were investigated. First, we examined the effect of these agents on ROS production by NADPH oxidase. As shown in Figure 2A, ROS production in the resting state of the cells was increased 2-fold after PMA stimulation. This PMA-induced increase in ROS production was effectively inhibited by pre-treatment with 8-oxodG in a dose-dependent manner (Figure 2A). In contrast to 8-oxodG, 8-oxoGTPγS, which was previously reported to inhibit Rac and NADPH oxidase in cell lysates [11], could not inhibit Rac (data not shown) and ROS production (Figure 2A) in intact cells. 8-oxoG, dG and G showed no inhibition (Figure 2B). Some inhibition was observed with A, but this was not statistically significant (Figure 2B). As shown in Figure 2C, NADPH oxidase activity was assessed in terms of NADPH oxidation. As expected, the results obtained were similar to that shown in Figure 2A and B. Diphenyleneiodonium (DPI) also inhibited the oxidation of NADPH since it is known to inhibit this enzyme by interacting with its flavin moiety [15]. However, DPI did not inhibit Rac1 (data not shown).

Phagocytosis

In Figures 1 and 2, substantial inhibition of Rac1 and ROS production was observed only with 8-oxodG. Therefore, other Rac-associated functions were studied with 8-oxodG alone. In Figure 3A, 8-oxodG was tested for phagocytosis. Panel (a) in Figure 3A shows the curves plotted from data obtained from the flow cytometric analysis of

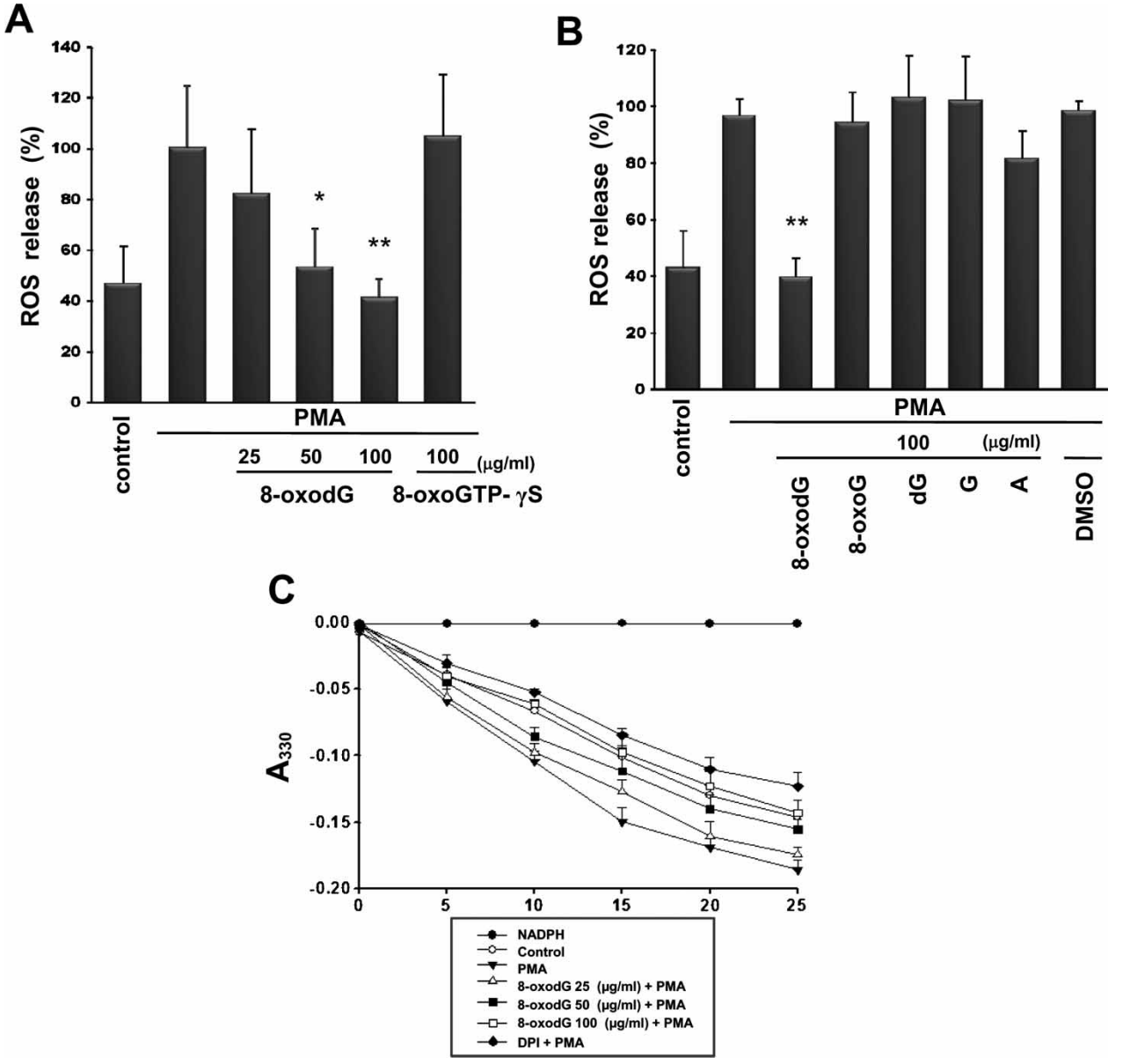

Figure 2. Effects of 8-oxoGua-containing and normal nucleotides on ROS production and NADPH oxidation in intact MpMϕ. MpMϕ were incubated with 8-oxodG, 8-oxoG, dG, G, A, 8-oxoGTPγS or DMSO (2 μl) and then treated with PMA. OxyBurst Green H_2HFF BSA (A and B) or NADPH (C) was added and the fluorescence or A_{330} was monitored, respectively. Details are described in Materials and methods. (C) NADPH; NADPH only, control; MpMϕ+NADPH, PMA; MpMϕ+NADPH+PMA. Data are mean±SD ($n=5$). * and **; $p<0.05$ and <0.01, respectively, vs the value obtained with no 8-oxodG by Student's t-test.

MpMϕ incubated with the fluorescent polystylene beads for 6 h. This showed that the majority of the cell population (left large curve) did not phagocytose the beads while only 15.8% of MpMϕ (right small curve) phagocytosed them. However, in panel (b) in Figure 3A, the population of MpMϕ that engulfed the beads was increased to 39.4% when MpMϕ were treated with PMA. When the same experiment was conducted with MpMϕ pre-treated with 8-oxodG at concentrations of 25, 50 and 100 μg/ml (panel c, d and e, respectively), the sizes of the right curve diminished (43. 7, 25.3 and 14.4%, respectively) while those of the left curve increased.

Chemotaxis

In Figure 3B, 8-oxodG was tested for chemotaxis. Chemotaxis was measured by determining the number of cells captured by the filter during movement of MpMϕ from one compartment (a) toward the other compartment (b) containing the chemo-attractant (fMLP). In the control experiments where fMLP was not added to (b), cells in (a) moved slowly toward

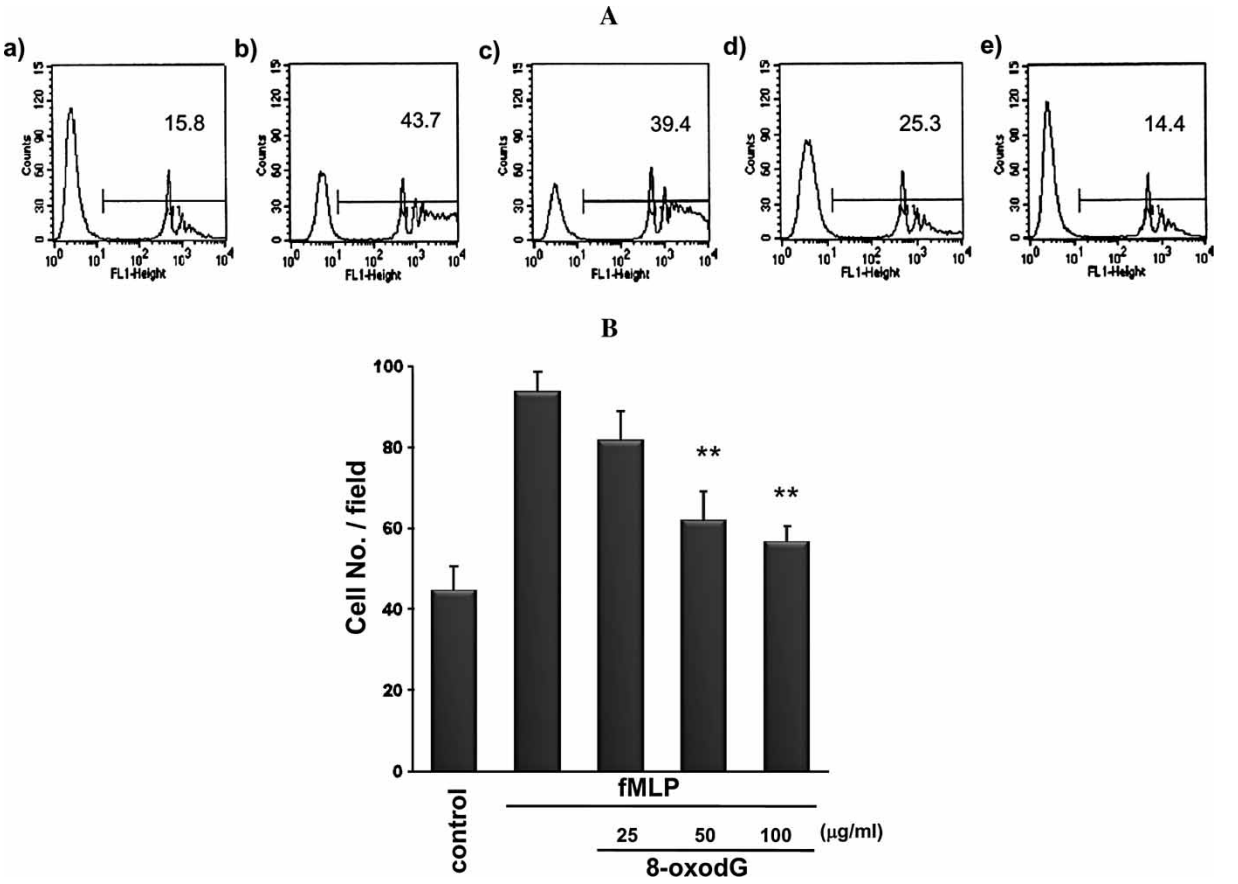

Figure 3. Effect of 8-oxodG on phagocytosis and chemotaxis of MpMϕ. (A) MpMϕ were treated with 8-oxodG, incubated with fluorescent beads and stimulated with PMA. The cells were then subjected to flow cytometric analysis. Panel a; neither 8-oxodG nor PMA and Panels b–e; treated with 0, 25, 50 and 100 μg/ml (0, 88, 177 or 353 μM), respectively, and then PMA stimulation. The number in each panel is percentage of cell population in the right curve. (B) Chemotaxis was assayed by determining the number of the cells captured to the filter during their movement toward the compartment containing fMLP. Details are described in Materials and methods. Data are mean ± SD ($n = 4$). * and **; $p < 0.05$ and < 0.01, respectively, vs the value obtained with no 8-oxodG by Student's *t*-test.

(b) (control in Figure 3B). Upon the addition of fMLP to (b), MpMϕ in (a) moved twice as fast as that of the control. However, when the same experiment was conducted with MpMϕ that were pre-treated with 8-oxodG (25, 50 or 100 μg/ml), the movement of MpMϕ became slower as the concentration of 8-oxodG increased.

Secretion of IFNγ

INF-γ activates the Mϕ to kill the phagocytosed microbes [4]. As shown in Figure 4, the secretion of IFN-γ was abruptly increased by LPS (~6-times greater than that of the control), but this LPS-stimulated secretion of IFN-γ was inhibited dose-dependently when MpMϕ were pre-treated with 8-oxodG.

Toxicity of 8-oxodG

MTT test [16] of 8-oxodG on MpMϕ showed that the viability of MpMϕ was not affected by up to 200 μg/ml of 8-oxodG (data not shown), indicating the observed inhibitory effects of 8-oxodG were not due to toxicity of this compound.

Discussion

Previously, 8-oxoGTP was reported to inhibit Rac only in cell lysates [10,11] but not in intact cells (Figure 2A). In the present study, we report that 8-oxodG, an 8-oxoGua-containing but phosphate-less nucleoside was capable of inhibiting Rac (Figure 1), ROS production (Figure 2), phagocytosis (Figure 3A), chemotaxis (Figure 3B) and INF-γ secretion (Figure 4) in intact cells, namely MpMϕ. This action was unique to 8-oxodG since 8-oxoG showed negligible effects (Figure 1 and 2) while normal nucleosides such as dG, G and A showed no effects on Rac activity (Figure 1 and 2). These results suggest that 8-oxodG could be a potential tool to modulate the functions of Mϕ.

8-oxodG is formed from 8-oxoGua in DNA by nucleotide excision [17,18] or from 8-oxodGTP by

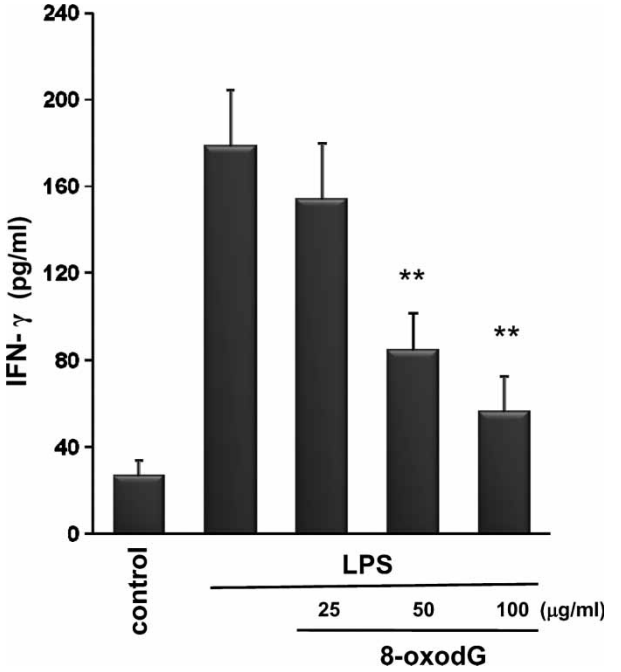

Figure 4. Effect of 8-oxodG on secretion of IFN-γ from MpMϕ. MpMϕ were incubated with 8-oxodG and then treated with LPS. IFN-γ was determined from the cell lysates by ELISA using monoclonal antibody specific to IFN-γ. Data are mean $\pm$ SD ($n = 5$). * and **; $p < 0.05$ and < 0.01, respectively, vs the value obtained with no 8-oxodG by Student's t-test.

hydrolytic activity of hMT [19,20] and nucleotidase [21] and exists in nucleus or cytosol where it is formed. In rats, the amount of 8-oxodG formation was estimated to be between 165–481 pmol/kg body weight/day [22], which is 2.80–8.16 μg/kg body weight/day. In humans, the amount of 8-oxodG formation will be much less, since the number of oxidative hits to DNA per cell per day in humans was reported to be one tenth that in rats [23]. Thus far, the endogenously formed 8-oxodG has been used as a marker of oxidative stress [24] and is not expected to exert Mϕ-modulating action since its amount is not high enough. Therefore, only the exogenously administered 8-oxodG can have Mϕ-modulating action.

The Rac-associated functions of Mϕ are involved in defense against invading microorganisms, which is the major role of Mϕ. The invading microorganisms are removed by the concerted actions of these functions but at the expense of tissue damage caused by various tissue reactions, collectively referred to as inflammation. Since Rac is involved in all these functions of Mϕ, suppression of the functions of Mϕ via Rac inhibition could be used as a means to control pathological processes associated with inflammation. Based upon this notion, we tested the anti-inflammatory action of 8-oxodG in LPS-treated mice [25]. We observed that 8-oxodG suppressed the LPS-induced increases of pro-inflammatory cytokines levels in serum and the infiltration of inflammatory cells into lung tissue. In this study [25], we also observed that Rac was activated by LPS in lung tissues but 8-oxodG inhibited LPS-induced Rac activation, suggesting that the anti-inflammatory properties of 8-oxodG occurred via Rac-inactivation.

Phagocytosis of Mϕ is an essential step in the presentation of an antigen to T-cells [26], which is the first step in immune response. In addition to phagocytosis of antigens by Mϕ or dendritic cells to present antigens to T-cells, Rac is also known to be involved in T-cell activation induced by the antigen presenting cells as well as B-cell activations by antigen-holding T-cells, where Rac activates MAP kinases and leads to activation of AP-1 [27]. Future studies, therefore, involve the testing of this compound for immune reaction-associated diseases such as asthma, atopic dermatitis and autoimmune arthritis. Phagocytosis also assists in the formation of foam cells, which are Mϕ that engulf oxidized lipids [28]. The accumulation of foam cells beneath the vascular endothelia is an initiating step for atherosclerosis [28]. So we are also trying to test 8-oxodG for anti-atherosclerotic effect since it is expected that the formation of foam cells will be inhibited by inactivation of Rac.

An X-ray crystallophic study [29] showed that the GTPase catalytic site of Rac is formed by Lys 16, Thr 35 and Gln 61. The γ-phosphate oxygen of GTP is retained to the Lys 16 side chain and Thr 35. Gln 61 is involved in the hydrolysis of GTP to GDP. In this Rac-GTP complex, the C_8 of the guanine base is only a C-C bond length away from the Lys 16 side chain. In solution, the 8-enol tautomeric form of 8-hydroxydG exists in dynamic equilibrium with the 6,8-diketo tautomer of 8-oxodG, which is the major form. Therefore, the C=O group on C_8 of 8-oxo-dG is highly expected to hinder the formation of the Rac-GTP complex by affecting the coordination of the Lys 16 side chain to the γ-phosphate oxygen of GTP. This may explain how the phosphate-less 8-oxodG can inactivate Rac as nucleotide, 8-oxoGTP can. However, at this stage, we are unable to explain why 8-oxodG, but not 8-oxoG, inactivates Rac. The structural difference between both compounds is that the latter has an oxygen at position 2′.

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