Mutat Res Gen Tox En 828 (2018) 10–14

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

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

Impact of DNA polymerase ζ mutations on genotoxic thresholds of oxidative mutagens

Tetsuya Suzuki[a,1], Kyomu Matsumoto[b], Masamitsu Honma[a], Takehiko Nohmi[a,*]

[a] Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Toxicology Division, The Institute of Environmental Toxicology, 4321 Uchimoriya, Joso-shi, Ibaraki, 303-0043, Japan

ARTICLE INFO

Keywords:
DNA polymerase zeta
Translesion DNA synthesis
Practical threshold
Genotoxicity
Oxidative stress

ABSTRACT

In regulatory genetic toxicology, it is an axiom that there is no threshold for genotoxicity of chemicals, such that genotoxic chemicals may impose carcinogenic risk on humans even at very low doses. This paradigm is counterintuitive, however, because humans possess a number of self-defense mechanisms that may suppress the genotoxicity at these low doses and therefore manifest a practical threshold. DNA polymerase zeta (Pol ζ) is a specialized Pol that plays an important role in DNA synthesis across DNA damage, thereby modulating cell survival and genotoxicity. In this study, we compared the sensitivity of three types of human cells: D2781N, L2618M, and their wild-type (WT) cells, to the low dose effects of genotoxicity of the oxidizing agents, potassium bromate ($KBrO_3$) and sodium dichromate ($Na_2Cr_2O_7$). D2781N cells express a variant form of Pol ζ, whose activity is weaker than that of the WT enzyme. L2618M cells express another variant form of Pol ζ, whose fidelity of DNA replication is lower than that of the WT enzyme. D2781N exhibited the highest sensitivity for *TK* gene mutation and micronucleus (MN) formation and displayed the lowest practical threshold for MN induction by $KBrO_3$. In contrast, L2618M exhibited the lowest practical threshold for sister-chromatid exchange (SCE) induction by both chemicals. These results suggest that Pol ζ mutations have significant impacts on practical thresholds of genotoxicity; the factors affecting the practical threshold can differ depending on the endpoint of genotoxicity. Roles of the variant forms of Pol ζ in genotoxicity by the oxidizing agents are discussed.

1. Introduction

In modern society, people are inevitably exposed to a variety of chemical agents. These man-made chemicals primarily exist to sustain and improve the quality of life; however, some of the chemicals have unexpected adverse effects on humans. In particular, carcinogenicity of chemicals is a major public concern because cancer is a leading fatal disease in many countries [1]. To protect human health and regulate the use of chemical carcinogens, international organizations have established guidelines [2–4] that classify them into two groups: genotoxic carcinogens, which induce cancer via genotoxic mechanisms such as mutation induction; and non-genotoxic carcinogens, which induce cancer via non-genotoxic mechanisms, such as hormonal effects, cytotoxicity, cell proliferation, or inflammation [5]. In general, genotoxic carcinogens are assumed to not have thresholds or safety doses and are thus severely regulated [6–8], such that they are not considered acceptable for use as pesticides, food additives, or veterinary drugs [9]. In contrast, non-genotoxic carcinogens are believed to have a defined threshold or dose below which the response cannot be distinguished from the ever present background and can thus be used in various applications, when the exposure level is below the threshold [10].

Recently, the paradigm that genotoxic carcinogens have no threshold has been challenged by a number of studies performed with low doses of chemical mutagens and carcinogens [11–19]. In fact, humans and other organisms possess a number of self-defense mechanisms, such as antioxidants, detoxification mechanisms, DNA repair, error-free translesion DNA synthesis (TLS), cell-cycle checkpoint, and others [20]. Several possible mechanisms underlying a non-linear threshold-type dose response are reported [21]. Therefore, these mechanisms may suppress genotoxicity of chemicals at low doses and

Abbreviations: TLS, translesion DNA synthesis; Pol, DNA polymerase; $KBrO_3$, potassium bromate; $Na_2Cr_2O_7$, sodium dichromate; 8-oxo-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; D2781N, a human cell line that expresses a variant form of Pol ζ having asparagine instead of aspartate at 2781; L2618M, a human cell line that expresses a variant form of Pol ζ having methionine instead of leucine at 2618; TK, thymidine kinase; MN, micronucleus; SCE, sister-chromatid exchange; BrdU, 5-bromo-2′-deoxyuridine; WT, wild-type; %MN, percentage of cells with micronucleus; PBS, phosphate-buffered saline; DSBs, double-strand breaks

* Corresponding author. Present address: Biological Safety Research Center, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki-shi, Kanagawa, 210-9501, Japan.
E-mail address: nohmi@nihs.go.jp (T. Nohmi).
[1] Present address: Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan.

https://doi.org/10.1016/j.mrgentox.2018.02.001
Received 17 September 2017; Received in revised form 31 January 2018; Accepted 2 February 2018
Available online 07 February 2018
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constitute a practical threshold, below which no genotoxicity over the spontaneous level is detectable [22]. The term "practical threshold" used in this study is interchangeable to no-observed-genotoxic-effect-level (NOGEL) [23,24]. However, the exact mechanisms underlying the practical threshold or NOGEL are still unclear.

Humans possess multiple specialized Pols, in addition to the replicative Pols, *i.e.*, Pol alpha, delta and epsilon [25–27]. If specialized Pols are absent or inactivated, DNA synthesis ceases at or before DNA lesions, which may result in DNA strand-breaks. Pol zeta (Pol ζ) is one of these specialized Pols that is involved in TLS [28,29], in which specialized Pols continue to synthesize DNA across DNA lesions that otherwise strongly block replicative Pols. Unlike DNA repair, TLS does not remove DNA lesions but simply aids the completion of DNA replication, thereby preventing DNA strand-breaks [30,31] and enhancing cell survival [22,32]. In fact, human cells lacking the catalytic activity of Pol ζ exhibit high sensitivity to the cytotoxic effects of a variety of genotoxic carcinogens [33]. However, the fidelity of DNA synthesis by specialized Pols is much lower than that of DNA synthesis by replicative Pols [34,35]. Therefore, mutations are sometimes induced by TLS in favor of cell survival [30].

Previously, we established human cell lines that express variant forms of Pol ζ [36]. One cell line expresses a variant form of Pol ζ that has weak catalytic activity, due to a mutation of the aspartate at 2781 of Pol ζ to an asparagine (D2781N). The other cell line expresses another variant form of Pol ζ with a leucine to methionine mutation (L2618M), which has low fidelity of DNA synthesis. Fidelity is an index of error frequency of Pols and L2618M is supposed to increase the error-rate during DNA synthesis. Using these cell lines, we examined thymidine kinase (*TK)* gene mutation, micronucleus (MN) formation, and sister-chromatid exchange (SCE) induction by low-dose exposure to potassium bromate ($KBrO_3$) and sodium dichromate ($Na_2Cr_2O_7$) with these cell lines to analyze how Pol ζ modulates the genotoxic threshold. These chemicals induce 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) in DNA [37,38], which is representative of oxidative DNA damage that can lead to deletions and point mutations *in vivo* [39–41].

2. Materials and methods

2.1. Chemicals

$KBrO_3$, $Na_2Cr_2O_7$, and colcemid were purchased from Wako Pure Chemical Industries, Ltd. and dissolved in sterilized distilled water. 5-Bromo-2′-deoxyuridine (BrdU) was purchased from Sigma-Aldrich.

2.2. Cell culture

The human cell line Nalm-6-MSH+ [42] and the derivatives, *i.e.*, D2781N and L2618M [36], were cultured in RPM1640 supplemented with 10% calf serum, 50 μg/mL kanamycin, and 50 μM 2-mercaptoethanol at 37 °C under a 5% CO_2 atmosphere. The derivatives have been established by gene targeting with oligonucleotides having DNA sequences directing amino acid changes of D2781N or L2618M, as previously described [36]. The original Nalm-6-MSH+ cells are referred to as the wild-type (WT) cells in this study.

2.3. Genotoxicity assays

Genotoxicity assays *in vitro* were conducted as described previously [43]. A brief outline of the assays is described below.

2.3.1. TK mutation assay

Cell suspensions of 12 mL (4×10^5 cells/mL) were treated with $KBrO_3$ or $Na_2Cr_2O_7$ at various doses for 4 h. After the treatment, the cells were washed once and re-suspended in a fresh medium (2.5×10^5 cells/mL). After a 96-h expression period, the cell suspension was seeded in 96-well plates (2.5×10^4 cells/well) in the presence of trifluorothymidine at a concentration of 3 μg/mL. Plating efficiency was determined by seeding cells (2.5 cells/well) in 96-well plates. All 96-well plates were incubated for more than 20 days at 37 °C. Mutation frequency was calculated according to the previously described method [44].

2.3.2. Cytokinesis-block MN assay

The assay was conducted according to OECD test guideline 487 [45]. The cells (4×10^5 cells/mL) were treated with $KBrO_3$ or $Na_2Cr_2O_7$ at various doses for 4 h. After washing, the cells were suspended in fresh medium and incubated for 28 h in the presence of cytochalasin B (3 μg/mL). The cells were fixed and stained with acridine orange (40 μg/mL). At least 1000 intact binucleated cells were examined for each treatment, and the cells with MN were scored microscopically. The results are presented as percentage of cells with MN (%MN) [46].

2.3.3. SCE assay

The cells were inoculated on 60-mm plates (2.5×10^5 cells/plate) and incubated for 2 days at 37 °C. The cells were then treated with $KBrO_3$ or $Na_2Cr_2O_7$ at various doses for 4 h. After washing twice with phosphate-buffered saline (PBS), the cells were suspended in fresh media containing 2 μg/mL of BrdU and incubated at 37 °C for 40 h for WT and D2781N cells and 49 h for L2618M cells. After a 2-h colcemid treatment (0.2 μg/mL), the cells were swollen with 0.075 M KCl for 10 min at room temperature, fixed with Carnoy's solution (methanol:acetic acid = 3:1), and then air-dried on glass slides. The slides were stained with 3% (v/v) Giemsa solution containing 2% (w/v) EDTA-4Na for 4–6 min at 40 °C [47]. By microscopy, 50 well-spread metaphase cells were analyzed for SCE.

2.4. Statistical analyses

Statistically significant differences in frequencies of *TK* mutation, MN, or SCE in treated *versus* untreated solvent groups were examined by the Dunnett's test. The Tukey's test was performed to examine statistical significance of the differences in frequencies among cell lines at the same doses.

3. Results

In this study, we defined the practical threshold of genotoxicity as the highest dose at which the frequencies of *TK* mutation, MN formation, or SCE induction did not increase significantly over the untreated levels. The cell line D2781N exhibited higher sensitivity to the mutagenicity of $KBrO_3$ than L2618M or WT cells both at higher doses (0–4 mM) and lower doses (0–1 mM) (Fig. 1A and B and Table S1). Although the practical threshold for L2618M was less than 1 mM, while those for D2781N and WT cells were 3 mM in the higher-dose experiments (Fig. 1A), the practical thresholds at the lower doses were not clear because of the relatively large standard deviations and negative responses of all three cell lines (Fig. 1B). D2781N cells also exhibited higher sensitivity to the mutagenicity of $Na_2Cr_2O_7$ at doses between 0 and 10 μM, although none of the cell lines exhibited significantly higher mutation frequencies over the untreated levels (Fig. 1C and Table S1). Therefore, we could not determine reliable practical thresholds for *TK* mutation frequencies under the conditions tested.

Next, we conducted the *in vitro* MN assay to examine the practical threshold for MN induction of the oxidative agents. The cell line D2781N exhibited higher sensitivity to MN induction by $KBrO_3$ than either L2618M or WT cells at doses below 1 mM (Fig. 2A and Table S2). The practical threshold for D2781N was 0.25 mM, which was lower than that for WT (0.5 mM) and L2618M cells (0.75 mM). MN induction (%MN) in D2781N was significantly higher than that in L2618M at doses of 0.75 mM and 1.0 mM. There was no difference in MN induction between L2618M and WT at the doses. D2781N also exhibited higher sensitivity to MN induction by $Na_2Cr_2O_7$ at doses below 10 μM,

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