ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2018, Vol. 73, No. 3, pp. 124–129. © Allerton Press, Inc., 2018.
Original Russian Text © G.V. Morgunova, A.A. Klebanov, 2018, published in Vestnik Moskovskogo Universiteta, Seriya 16: Biologiya, 2018, Vol. 73, No. 3, pp. 153–159.

GERONTOLOGY

Impairment of the Viability of Transformed Chinese Hamster Cells in a Nonsubcultured Culture under the Influence of Exogenous Oxidized Guanoside is Manifested Only in the Stationary Phase of Growth

G. V. Morgunova[a],* and A. A. Klebanov[a]

[a] *Evolutionary Cytogerontology Sector, School of Biology, Lomonosov Moscow State University, Moscow, 119234 Russia*
**e-mail: morgunova@mail.bio.msu.ru*

Received January 29, 2018; in final form, May 15, 2018

Abstract—Despite the fact that oxidation products of nucleotides and nucleosides are markers of oxidative stress, reports of the paradoxical ability of these compounds to protect cells from the harmful effects of reactive oxygen species began to appear more often. Among all nitrogenous bases, guanine is most susceptible to the influence of oxidative stress; therefore, guanosine is oxidized more often than other bases. In the present work, the effect of exogenous 8-oxo-2'-deoxyguanosine on the growth and "stationary phase aging" (accumulation of "age-related" changes in cultured cells during cell proliferation slowing down within a single passage and subsequent "aging" in the stationary growth phase) of nonsubcultured transformed Chinese hamster cells was studied. We showed that the nucleoside is rapidly absorbed by the cells from the medium, but it does not affect the growth of the culture, and impairs the viability of the cells in the late stationary growth phase. Thus, no mitogenic or geroprotective effect of 8-oxo-2'-deoxyguanosine was found.

Keywords: cell aging, survival curve, 8-oxo-2'-deoxyguanosine, oxidative stress, DNA damage, geroprotectors.

DOI: 10.3103/S0096392518030136

DNA in cells experiences a constant impact of damaging endogenous and exogenous factors. Errors are eliminated by the DNA repair system, but the capabilities of the latter are not limitless. Cells with unrepaired DNA damage usually die or lose the ability to divide. For an actively proliferating culture, the appearance of such cells is not very hazardous because they can be "diluted" with the descendants of other cells [1, 2]. However, when the cells that, for certain causes, are unable to divide are damaged, the only hope is the DNA repair system [3, 4]. Situations when the damage that occurred in DNA leads to the emergence of mutations that do not prevent the cell from proliferation and copying the error are also dangerous. Such failures may lead to tumor development [5–7].

The effects of oxidative stress on DNA have been studied most comprehensively. The intracellular damaging factors are the reactive oxygen species (hydrogen peroxide, superoxide radical, and hydroxyl radical) emerging as a result of accepting the leakage electrons by oxygen molecules [8–10] and the so-called reactive nitrogen species (peroxynitrite and nitrous anhydride) [6]. Among the purine and pyrimidine bases in nucleic acids, guanine is most susceptible to the impact of the reactive oxygen species because it has the lowest redox potential [7, 11, 12]. For this reason, the main oxidative stress markers are the guanine oxidation products—8-oxo-7,8-dihydrodeoxyguanosine triphosphate, 7,8-dihydro-8-oxo-2'-deoxyguanosine, etc. In total, there are more than 100 oxidation products of nitrogen bases [13]. According to the calculations by Park et al. [14], approximately 10^5 oxidized guanines emerge in DNA and are eliminated by the DNA repair system in rat cells per day [14]. RNA nucleosides [15–17], as well as free nucleotides [6, 18], are also susceptible to oxidation. 8-Oxoguanosine is one of the most hazardous forms of oxidized nucleosides, since it can form a Hoogsteen pair with adenosine [7, 11, 13]. If this error is not eliminated, the G:C pair may be replaced with the T:A pair. In addition, the incorporation of the oxidized guanosine into DNA during replication may cause a double-strand DNA break [5].

The oxidized nucleotides are eliminated and their occurrence is prevented by the base excision repair system. Base excision repair is performed primarily by DNA glycosylases [19]. In bacteria, these are mutM (formamidopyrimidine-DNA glycosylase), mutT (8-oxo-dGTP diphosphatase), and mutY (adenine DNA glycosylase) [20, 21]. Their mammalian homologs are MTH1 (mutT homolog 1), OGG1 (8-oxoguanine-

DNA glycosylase 1), and MUTYH (mutY homolog), respectively [7]. Yeast lack mutY and MutT homologs [3] but have several variants of oxoguanine-DNA glycosylase [11]. MutM/OGG1 eliminates oxidized guanosine (OG) from the OG:C pair in DNA. MutY/MUTYH eliminates the inappropriate adenine base from the incorrect OG:A pair. MutT/MTH1 hydrolyzes 8-oxo-7,8-dihydrodeoxyguanosine triphosphate, eliminating the compound from the free nucleotide pool, and prevents its incorporation into DNA. It was shown that mutations in the genes encoding these enzymes are the cause of many tumor diseases [7, 10, 22]. Since only several of the 10^6 guanosines in DNA are oxidized [7], their search is highly complicated. Some authors believe that the presence of a certain amount of oxidized nucleosides is essential for the cell, because the oxidized guanosine may stimulate transcription via the activation of the base excision repair system, thus performing epigenetic regulation [12]. There is also a standpoint that a certain amount of oxidized guanosines is not removed from DNA due to the hormesis effect [16]. Temporary retaining of a baseline amount of oxyguanosines may be more beneficial than their reduction. The incorporation of oxidized nucleosides from the free nucleotide pool into DNA is of considerable interest [6, 8, 9, 18, 23]. Unfortunately, some enzymes may contribute to the accumulation of errors (e.g., bacterial DNA polymerase, which sometimes inserts an oxidized nucleoside from the free nucleotide pool into the *de novo* synthesized DNA [18]).

The chronological aging of yeast and the "stationary phase aging" of cultured mammalian cells are accompanied by proliferation restriction due to contact inhibition. As a result, cells are not renewed, and various changes (similar to the changes developing in aging multicellular organisms) occur in them. Among these, DNA lesions are most important, because any other macromolecules and organelles can be replaced [24–26]. It was previously shown that DNA–protein crosslinks [27] and single-strand breaks [28], DNA demethylation [29], inhibition of poly(ADP-ribosyl)ation of chromatin proteins [30], and changes in the spontaneous sister chromatid exchange level [31, 32] occur in this model system. The content of 8-oxo-2'-deoxyguanosine (8-oxo-dG) in the DNA of cells "aging" in the stationary growth phase increases 4–5 times [33]. Interestingly, 8-oxo-dG accumulates in the cells "aging" according to Hayflick [34].

Recently, it was reported that exogenous oxidized nucleosides may have a positive effect on the viability of cells and tissues. For example, 8-oxo-dG can paradoxically function as an antioxidant and regulate the production of proinflammatory cytokines [35]. It is assumed that it can be used in the treatment and prevention of diseases and disorders involving oxidative stress and inflammation [36–38]. In this study, we investigated the effect of exogenous 8-oxo-dG on the viability, growth, and death of nonsubcultured transformed Chinese hamster cells.

MATERIALS AND METHODS

Experiments were performed on transformed Chinese hamster cells of the subcultured line B11-dii-FAF28 (clone 237), which was obtained from the Medical Genetics Research Center (Moscow). The cells were cultured at 37°C in Carrel glass flasks in Dulbecco's Modified Eagle's Medium (Ivanovsky Institute of Virology, Ministry of Healthcare of the Russian Federation, Moscow) supplemented with 5–10% bovine serum (PAA, Austria), penicillin (100 U/mL), and streptomycin (100 μg/mL). The culture was maintained by subculturing the cells at a ratio of 1:10 to 1:3 every 3–4 days. The cells were removed from the growth surface using a mixture (1:1) of 0.02% Versene and 0.25% trypsin (Ivanovsky Institute of Virology). 8-Oxo-dG was synthesized at the Department of Bioorganic Chemistry, School of Biology, Lomonosov Moscow State University from 2'-deoxyguanosine (Sigma, United States). The purity of the synthesized compound was confirmed by nuclear magnetic resonance as well as liquid chromatography and tandem mass spectrometry.

In preliminary studies aimed at determining the cytotoxic and mitogenic properties of 8-oxo-dG, 3–4-day-"old" cells (i.e., grown without subculturing for 3–4 days) were seeded in hermetically sealed penicillin flasks at a density of approximately 40 000 cells/cm^2. After 24 hours of incubation, medium containing 8-oxo-dG at final concentrations of 10^{-6} to 10^{-3} M was added to the experimental flasks, and medium containing respective amounts of distilled water of Milli-Q quality was added to the control flasks. The flasks were then placed in an incubator (37°C) for 4 days, after which the cells were removed from the growth surface with a mixture of trypsin and Versene solutions, and their number was estimated using the standard hemocytometer. In addition, we determined the effect of 8-oxo-dG in the lowest and highest concentrations in a long-term experiment wherein cells were not subcultured for 19 days (the nucleoside also was added 24 hours after seeding).

To assess the effect of 8-oxo-dG on the growth kinetics of the cells and their subsequent death in the stationary growth phase, 3-day-"old" cells were seeded into hermetically sealed penicillin flasks at a density of 40 000 cells/cm^2. The next day, the number of adherent cells was determined, after which the experimental flasks were supplemented with medium containing 8-oxo-dG at a final concentration of 10^{-3} M, and the control flasks were supplemented with medium containing a respective volume of water. After certain time intervals, three flasks of each group were withdrawn from the incubator. Cells were removed from the growth surface using a mixture of Versene

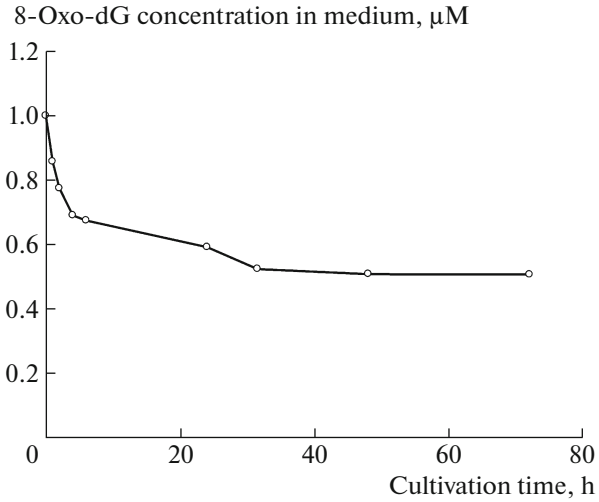

Fig. 1. Dynamics of changes in the concentration of 8-oxo-2'-deoxyguanosine in the culture medium during the first 70 h after its addition to the medium with 5-day-"old" (cultured for 5 days without subculturing) transformed Chinese hamster cells in the stationary growth phase. The content of 8-oxo-2'-deoxyguanosine in the medium, measured by reverse-phase high performance liquid chromatography, is shown.

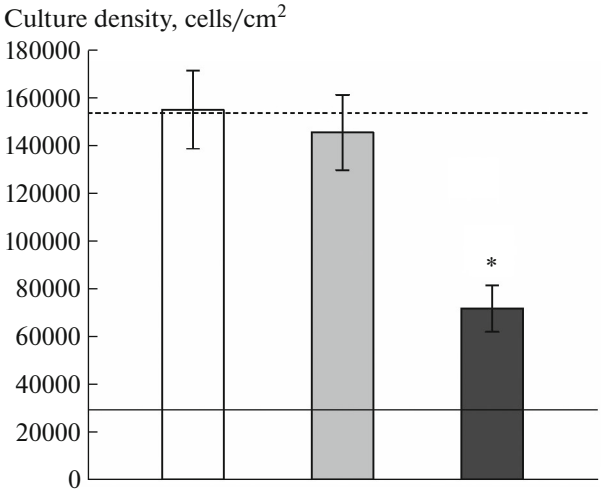

Fig. 2. Culture density of transformed Chinese hamster cells at 19 days after seeding under the influence of 8-oxo-2'-deoxyguanosine. 8-Oxo-2'-deoxyguanosine was added to the growth medium 24 hours after seeding to a final concentration of 10^{-6} M (gray column) and 10^{-3} M (black column). The solid horizontal line is cell culture density 24 hours after seeding, the dotted horizontal line is the culture density in the control group at 10 days after seeding. Data are represented as the mean value ± standard error of the mean, the asterisk (*) marks the significant difference from the control group (white column).

and trypsin solutions, and then their number was evaluated using the standard hemocytometer (three flasks per point, four chambers per flask). The experiment was repeated twice.

To determine the absorption dynamics of 8-oxo-dG, the cells were cultured in hermetically sealed flasks for 5 days until the formation of a confluent monolayer, after which the flasks were supplemented with solution of oxidized nucleoside at a final concentration of 10^{-3} M. Then, at regular time intervals, small (20–30 μL) aliquots of the medium were taken (the last aliquot was taken on day 20). In addition, the stability of 8-oxo-dG in a cell-free medium containing 10% serum during incubation at 37°C for 21 days was determined in a similar manner. The content of 8-oxo-dG in the medium was kindly determined by the staff of the Department of Bioorganic Chemistry, School of Biology, by HPLC with an LC-20AD chromatograph (Shimadzu Scientific Instruments, Japan) equipped with an SPD-M20A spectrophotometric detector (Shimadzu Corporation, Japan) and an SIl-20A autosampler (Shimadzu Corporation). HPLC was performed in an Equivalent to Phenomenex® Luna® C18(2) column (Phenomenex Inc., United States). The chromatograms were recorded using a Shimadzu LCSolution program (Shimadzu Corporation).

The data were compared using the Student's t-test or the Mann–Whitney U test (depending on the results of checking the normality assumption). All statistical data analysis were performed using the SigmaPlot 12.0 and R software (version 3.4.4) with the additional "nortest" package.

RESULTS

8-Oxo-dG remains stable when incubated in a serum-containing medium. Its concentration, determined by HPLC, does not change within 21 days. The estimation of the absorption by cells of 8-oxo-dG from the medium showed that its concentration rapidly decreased during the first few hours (Fig. 1), dropped twice relative to the initial value on the second day, and then remained at this level (the last measurement was performed on day 20).

When assessing the cytotoxic and mitogenic properties of 8-oxo-dG, we determined its effect at concentrations of 10^{-6} to 10^{-3} M. Even at the highest concentration (10^{-3} M), the oxidized nucleoside had no effect on the growth and viability of the cells, and no differences between the groups were detected 4 days after addition of 8-oxo-dG. In the case of a long-term (18-day) exposure of the cells to 8-oxo-dG, the culture density was significantly lower in the group with the highest content of the nucleoside in the medium (Fig. 2) but comparable to the control value in the group with its low content. In view of this, the concentration of 10^{-3} M was selected for the next experiment.

Control and experimental data obtained in the evaluation of the effect of 8-oxo-dG on the dynamics

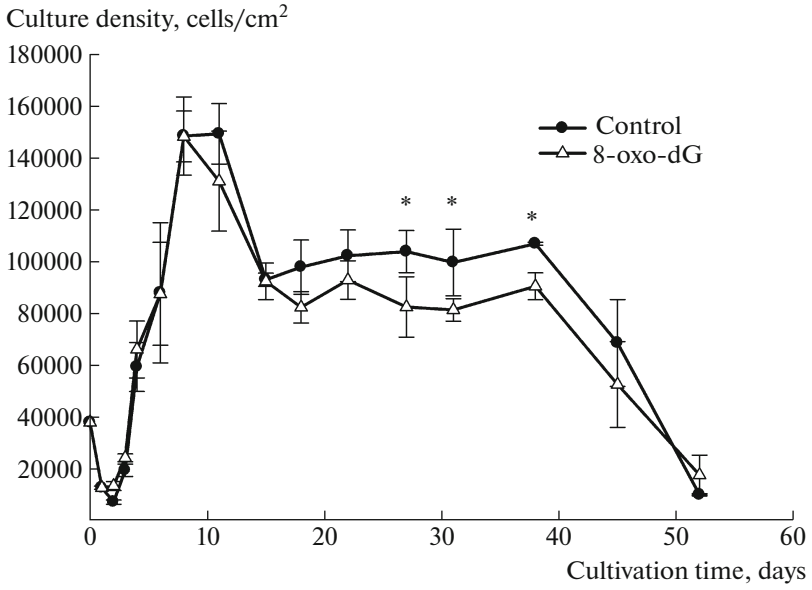

Fig. 3. Effect of the addition of 8-oxo-2'-deoxyguanosine at a concentration of 10^{-3} M on the growth kinetics and the stationary phase aging of the culture of transformed Chinese hamster cells (for methodological details, see the text). Data are represented as the mean value ± standard error of the mean. The black curve shows the control, and the gray curve shows the experiment (the addition of 8-oxo-2'-deoxyguanosine). Data are represented as the mean value ± standard error of the mean, the asterisk (*) marks the significant difference from the control group.

of growth and "stationary phase aging" of the cell culture were compared at each point of counting the number of living cells. We performed two experiments. The first experiment (data not shown), which was completed on day 22 (when the cells only entered the extinction phase), showed no significant differences between the groups. The second experiment was completed on day 52 (Fig. 3), when almost all cells in both groups died. It can be seen in Fig. 3 that the curve describing the kinetics of growth and death of cells in the experimental group, starting from the plateau phase, lies somewhat lower than the control curve; significant differences were found on the 27th, 31st, and 38th days. Thus, 8-oxo-dG at a concentration of 10^{-3} M does not change the kinetics of growth and impairs viability of "aged" cells.

DISCUSSION

8-Oxo-dG even at a high concentration has no effect on the growth and viability of "young" cells that actively proliferate or that just entered the stationary growth phase. Wherein the nucleoside is actively absorbed from the medium during the first day (especially in the first few hours). It can be assumed that, since cancer cells are better adapted to the impact of oxidative stress and the level of MTH1 activity in them is high [10], they can convert the absorbed potentially harmful oxidized products into harmless ones. On the other hand, although 8-oxo-dG is absorbed by cells in the first few hours, its effect is observed only at the late stage of the stationary growth phase.

The available published data indicate that the positive effect of 8-oxo-dG is manifested primarily in the model systems where objects are exposed to adverse or damaging factors that cause the development of oxidative stress [36–38]. In our case, a certain impact of 8-oxo-dG on the viability was also detected only when the cells remained in the stationary growth phase for a long time. However, this effect was not positive; conversely, it was manifested as decline of the cell viability (Fig. 2, 3). It remains obscure how a compound that is absorbed by cells almost immediately exerts its effect only on day 19 or 40. A question arises as to whether 8-oxo-dG is incorporated in the cellular structures or it affects certain metabolic reactions.

A certain difference between the control and experimental curves describing the growth and death of the cells can be noticed in the late stationary growth phase (Fig. 3), although significant differences were found only on 28th, 31th and 38th days. It should be noted that the complexity of the experiments on assessing the kinetics of growth and death of cells does not allow for using a sufficiently large number of flasks in each group. In the cytotoxicity experiments, in which the differences were detected on day 19 after seeding, the sample was larger (seven flasks per point). Therefore, it cannot be ruled out that increasing the number of flasks in each group from three to five or seven would allow us to detect significant differences in other

points of counting the number of living cells which are in the stationary phase of growth.

The negative effect was more expected then positive effect, because the accumulation of the oxidized nucleosides, which indicates the oxidative stress, exerts a significant load on the cell, causing it to waste resources on the functioning of the glycosylases involved in the base excision repair. A certain activation of this repair system possibly improves transcription; however, a significant increase in the amount of oxidation products of nucleic acids cannot have a positive effect on the viability of cells and only complicates their existence, especially in the late stationary growth phase.

The oxidized nucleotides from the free pool significantly contribute to DNA stability disturbance. Russo et al. [8] showed that the DNA mismatch repair system is involved in the protection of DNA from the insertion of oxidized nucleosides. The disturbance of this system functioning causes a significant compensatory activation of MTH1. The addition of normal purine nucleotides (dGTP and dATP) to a bacterial suspension caused no increase in the mutation rate; however, after the addition of oxidized form of the same nucleotides , the mutation rate increased 12 and 9 times, respectively [23].

Thus, there is reason to assume that, when 8-oxo-dG gets into the cell, it accumulates in cellular structures. The vital functions of "young" actively proliferating cells are not disturbed. However, when the cell culture enters the stationary growth phase, when replicative DNA synthesis does not take place, the accumulated oxidized nucleotides become ballast. This leads to an increase in the number of errors, whose frequency, in any case, rises with an increase in the duration of incubation of cells during stationary phase aging, when the functioning of the enzymes that maintain the correct DNA structure can not track all occurring lesions. The response of cells to exogenous and endogenous lesions is largely determined by the functioning of the enzymes involved in the base excision repair. Therefore, the studies aimed at investigating their functioning in the cells aging in the stationary phase of growth are most promising.

ACKNOWLEDGMENTS

We are grateful to D.S. Esipov and N.V. Marmiy for providing 8-oxo-dG and assistance with the determination of the substance concentration in the medium. This work was supported by the Russian Foundation for Basic Research (project no. 18-34-00813 mol_a).

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Translated by M. Batrukova