Free Rad. Res., Vol. 20, No. 2, pp. 113–117
Reprints available directly from the publisher
Photocopying permitted by license only

8-HYDROXYDEOXYGUANOSINE IN DNA FROM TPA-STIMULATED HUMAN GRANULOCYTES

H.C. BIRNBOIM*, LINDSAY MAIDT**, TINA RAYNOR* and R.A. FLOYD**

**Ottawa Regional Cancer Centre and University of Ottawa, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6 and **Free Radical Biology & Aging Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, USA*

(Received August 16th, 1993; in revised form September 29th, 1993)

8-Hydroxydeoxyguanosine (8-OHdG) is now widely used as a sensitive marker of oxidative damage to DNA. When human granulocytes are stimulated with TPA, they release a large quantity of reactive oxygen species (superoxide, hydrogen peroxide) which might be expected to generate hydroxyl radicals (OH·) which in turn could produce 8-OHdG in the DNA. There had been considerable debate as to whether OH· is detectable in stimulated granulocytes; most workers now agree that none can be detected, unless exogenous iron is added. An earlier report had described that 8-OHdG (a marker of OH·) was increased in the DNA of TPA-stimulated, compared to control, granulocytes. We have repeated this experiment and have been unable to reproduce this finding. We conclude that the amount of 8-OHdG produced in the DNA of TPA-stimulated human ganulocytes is indistinguishable from that seen in control (unstimulated) cells (less than one 8-OHdG/10^5 dG).

KEY WORDS: Granulocytes, hydroxyl radical, 8-hydroxydeoxyguanosine, DNA strand breaks.

INTRODUCTION

The presence of 8-hydroxydeoxyguanosine (8-OHdG) (detected by HPLC-EC[1]), is now widely used as an indicator of oxidative damage to DNA. 8-OHdG can be produced by hydroxyl radical (OH·) but also by other oxidizing species such as lipid peroxides, singlet oxygen and UV irradiation and certain carcinogenic agents.[2-5] An important biological source of oxyradicals is the respiratory burst of granulocytes which produce large quantities of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), effectively exposing these cells to relatively high concentrations of these oxidizing species. In the presence of transition metals, these species would be expected to produce OH· via the Haber-Weiss reaction. However, evidence that OH· is *actually* produced in or around granulocytes as a result of the respiratory burst had been conflicting. Most workers now agree that OH· is not found in activated granulocytes unless exogenous iron is added, likely because of the iron-binding properties of lactoferrin.[6-12] In this context, an earlier report identifying a large increase in 8-OHdG in the DNA of granulocytes following stimulation with TPA was of particular interest since it seemed to indicate that OH· was being produced intracellularly at a location where it could reach the DNA[13]. Granulocytes have been

Corresponding author: H.C. Birnboim, Ottawa Regional Cancer Centre, 501 Smyth Road, Ottawa, Ontario Canada K1H 8L6. ph. 613-737 6719. FAX 613-737 6745. Internet: HCBME@acadvm1.uottawa.ca

TABLE 1
8-OHdG content of human granulocyte DNA, expressed as moles per 10^5 moles dG[a]. Cells were untreated or treated with TPA as described in Materials and Methods

Donor no.	(a) Incubation at 0°C (control)	(b) Incubation at 37° (control)	(c) Incubation at 37° (with TPA)
I	<1.39 ($n = 3$)	7.8 ± 2.1 ($n = 2$)	5.5 ± 0.0 ($n = 3$)
II	7.0 ± 1.4 ($n = 3$)	7.5 ± 2.1 ($n = 3$)	7.5 ± 4.5 ($n = 3$)

[a]Means ± SEM are shown except for $n = 2$ where mean ± ranges are shown.

studied extensively with respect to DNA strand breaks associated with the respiratory burst.[14–19] The identification of large amounts of 8-OHdG would lend support to the notion that strand breaks observed in activated granulocytes might be due to oxidative attack on the DNA backbone by OH·. Because of the importance of this observation to the study of the mechanism of DNA strand breakage, we reinvestigated the question of whether 8-OHdG could be detected in increased amounts in DNA from activated granulocytes. We now report that preparations of purified DNA from highly purified granulocytes show no significant increase in 8-OHdG following stimulation with TPA.

MATERIALS AND METHODS

Methods used for preparing purified human granulocytes and for measuring DNA strand breaks have been described[19]. Preparations contained greater than 97% granulocytes and less than one red blood cell per white cell. Purification of DNA followed our published procedure[20] with the following modifications. No phenol extraction step was carried out and ribonuclease was not used since granulocytes have only very small amounts of RNA compared to DNA[21]. Precipitated DNA was sent under ethanol from Ottawa to Oklahoma City for 8-OHdG analysis by HPLC-EC as described[22]. DNA strand break analysis was carried out by the FADU procedure[19]. Measurements of O_2^- production was carried out by a 10-min pulse method.[15] Granulocytes were suspended at a concentration of 1×10^6 cells per mL in a balanced salt solution for all experiments[19]

RESULTS

Freshly isolated, highly purified human granulocytes were suspended in a balanced salt solution containing 5 mM glucose and incubated at 0°C or at 37°C in the absence or presence of 50 nM TPA (12-*O*-tetradecanoylphorbol 13-acetate). Using granulocytes from 2 different normal donors (I and 11), 3 separate incubations and DNA isolations were carried out for 8-OHdG analysis. Results are shown in Table 1. There was no statistically significant difference ($p > 0.1$, one way ANOVA) in 8-OHdG content amongst samples of DNA from cells from either donor incubated at 37°C in the absence or presence of TPA. That is, within the limits of detection of the system, there was no indication that stimulation of granulocytes with TPA, a treatment known to cause generation of oxyradicals and DNA strand breaks, caused an increase in 8-OHdG. The value observed in both control and TPA-stimulated cells

TABLE 2
Superoxide production and DNA strand breaks in TPA-stimulated human granulocytes.

Donor no.	O_2^- Production (nmoles/10^6 cells/40 min)[a]	DNA strand breaks (Qd units)[b]
I	149.7 ± 8.2	
III–VIII		70.8 ± 17.2 (n = 5)

[a] O_2^- production was measured on the same granulocyte preparation as used in Table 1. Result shown is the mean ± SEM of one analysis carried out in triplicate. This value is within the range of O_2^- measurements carried out on samples prepared from 14 different donors under similar conditions (148.3 ± 10.1) (average ± SEM).

[b] DNA strand break measurements were carried out under the same conditions on samples from 5 different donors. 1 Qd unit is estimated to indicate 120 SSB per cell[23].

is well below the value reported earlier (43 8-OHdG/10^5 dG residues or 1.3 pmole/μg DNA)[13].

The only significantly different result from all the others in Table 1 was obtained using 0°C incubated cells from donor 1. The 8-OHdG content was below detection limit, which was estimated to be $\approx$1.4 moles of 8-OHdG per 1×10^5 moles of dG for the analyses reported. 8-OHdG was below the detection limit in at least 12 other control samples from other donors (data not shown).

Superoxide production by TPA-stimulated granulocytes was measured at the same time that 8-OHdG was measured in cells from donor 1. The result is shown in Table 2. O_2^- levels were in the expected range for normal human granulocytes, indicating that the cells were metabolically active at the time of TPA stimulation prior to DNA isolation for 8-OHdG measurement. DNA strand breaks were also measured by the FADU method. 70 Qd units corresponds to about 8400 SSB (single strand breaks) per cell[23] induced by TPA treatment, or 0.6 SSB/10^5 dG residues, assuming 6×10^9 nucleotides per cell.

DISCUSSION

Our results differ from those reported earlier as to the content of 8-OHdG in DNA from TPA-stimulated human granulocytes[13]. The reason for the discrepancy is uncertain; it is possible that an impurity such as copper in the earlier preparations of granulocytes may have been the cause of the discrepancy between the present and the earlier report.[17,24] The present results do not rule out the possibility that small amounts of 8-OHdG are induced in granulocytes. Rather, we conclude that no 8-OHdG was detectable in TPA-stimulated cells above the background observed in control cells. Other workers have detected 8-OHdG in the DNA of "target" cells co-incubated with TPA-stimulated granulocytes[25]. The relatively large amount of H_2O_2 scavenging enzymes such as myeloperoxidase and catalase present in granulocytes may explain why these cells suffered little or no oxidative base damage to their DNA.[26]

The cause of the observed "background" levels of 8-OHdG is of considerable interest. 8-OHdG may arise after cells have been lysed, perhaps during DNA purification and/or enzymatic digestion[27]. Typically, most workers have described levels of 1 to 2 8-OHdG residues/10^5 dG in control DNA.[26,28-30] An increase in 8-OHdG content (0.3 residues/10^5 dG/hr digestion of DNA with alkaline phosphatase) has been reported by Kasai and coworkers[31]. They also observed that 8-OHdG content could

be increased by the use of phenol during DNA isolation and during "prolonged" homogenization of mouse liver prior to extraction of DNA. The effect of exposure of DNA to phenol and other organic solvents on 8-OHdG yield has been shown to be significant[32]. Others have observed a variety of oxidized bases, including 8-OHdG, in DNA from "untreated" normal and tumour tissues,[28,29] but whether these are present *in situ* or whether they arise during DNA extraction and processing has been questioned[27].

Kasai *et al.*[31] reported that 8-OHdG was induced by ionizing irradiation of HeLa cells and whole mice at a rate of 0.008–0.018 residue/10^5 dG/10 Gy. Ionizing radiation is expected to induce 0.8 SSB per 10^5 dG in DNA per 10 Gy (calculated from 1,200 SSB/6 × 10^9 bases/Gy[33]). Our estimate (see above) is that TPA induces 0.6 SSB/10^5 dG in DNA. *If an ionizing radiation-like mechanism were operative* to produce both SSB and 8-OHdG, then one might expect 0.006–0.014 8-OHdG/10^5 dG to be formed. Clearly, such values are too low to be detected in granulocytes until conditions which reliably produce ultra-low backgound levels of 8-OHdG in DNA are established.

Acknowledgements

HCB is a career scientist of the Ontario Cancer Treatment and Research Foundation. This work was supported by a grant from the Medical Research Council of Canada to HCB and by grant CA 42854 from the NIH to RAF.

References

1. R.A. Floyd, J.J. Watson, P.K. Wong, D.H. Altmiller and R.C. Richard (1986) Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. *Free Radical Research Communications*, **1**, 163–172.
2. R.A. Floyd (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB Journal*, **4**, 2587–2597.
3. J.-W. Park and R.A. Floyd (1992) Lipid peroxidation products mediate the formation of 8-hydroxydeoxyguanosine in DNA. *Free Radical Biology & Medicine*, **12**, 245–250.
4. R.A. Floyd, M.S. West, K.L. Eneff, W.E. Hogsett and D.T. Tingey (1988) Hydroxyl free radical mediated formation of 8-hydroxyguanine in isolated DNA. *Archives of Biochemistry and Biophysics*, **262**, 266–272.
5. T.P.A. Devasagayam, S. Steenken, M.S.W. Obendorf, W.A. Schulz and H. Sies (1991) Formation of 8-hydroxy(deoxy)guanosine and generation of strand breaks at guanine residues in DNA be singlet oxygen. *Biochemistry*, **30**, 6283–6289.
6. H. Kaur, I. Fagerheim, M. Grootveld, A. Puppo and B. Halliwell (1988) Aromatic hydroxylation of phenylalanine as an assay for hydroxyl radicals: application to activated human neutrophils and to the heme protein leghemoglobin. *Analytical Biochemistry*, **172**, 360–367.
7. A. Samuni, C.D.V. Black, C.M. Krishna, H.L. Malech, E.F. Berstein and A. Russo (1988) Hydroxyl radical production by stimulated neutrophils reappraised. *Journal of Biological Chemistry*, **263**, 13797–13801.
8. B.E. Britigan, D.J. Hassett, G.M. Rosen, D.R. Hamill and M.S. Cohen (1989) Neutrophil degranulation inhibits potential hydroxyl-radical formation. Relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques. *Biochemical Journal*, **264**, 447–455.
9. S. Pou, G.M. Rosen, B.E. Britigan and M.S. Cohen (1989) Intracellular spin-trapping of oxygen-centered radicals generated by human neutrophils. *Biochimica Biophysica Acta*, **991**, 459–464.
10. R.A. Greenwald, S.W. Rush, S.A. Moak and Z. Weitz (1989) Conversion of superoxide generated by polymorphonuclear leukocytes to hydroxyl radical: A direct spectrophotometric detection system based on degradation of deoxyribose. *Free Radical Biology & Medicine*, **6**, 385–392.
11. B.E. Britigan, T.J. Coffman and G.R. Buettner (1990) Spin trapping evidence for the lack of significant hydroxyl radical production during the respiration burst of human phagocytes using a spin

adduct resistant to superoxide-mediated destruction. *Journal of Biological Chemistry*, **265**, 2650–2656.
12. B.E. Britigan and B.L. Edeker (1991) *Pseudomonas* and neutrophil products modify transferrin and lactoferrin to create conditions that favor hydroxyl radical formation. *Journal of Clinical Investigation*, **88**, 1092–1102.
13. R.A. Floyd, J.J. Watson, J. Harris, M. West and P.K. Wong (1986) Formation of 8-hydroxydeoxyguanosine, hydroxyl free radical adduct of DNA in granulocytes exposed to the tumor promoter, tetradecanoylphorbolacetate. *Biochemical and Biophysical Research Communications*, **137**, 841–846.
14. H.C. Birnboim (1982) DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate. *Science*, **215**, 1247–1249.
15. H.C. Birnboim and M. Kanabus-Kaminska (1985) The production of DNA strand breaks in human leukocytes by superoxide anion may involve a metabolic process. *Proceedings of the National Academy of Science U.S.A.*, **82**, 6820–6824.
16. H.C. Birnboim (1988) Superoxide anion may trigger DNA strand breaks in human granulocytes by acting at a membrane target *In Annals of the New York Academy of Sciences. vol.* 551. *Membrane in Cancer Cells.* (eds. T. Galeotti, A. Cittadini, G. Neri and A. Scarpa)), The New York Academy of Sciences, New York, New York. pp. 83–94.
17. H.C. Birnboim (1992) Effect of lipophilic chelators on oxyradical-induced DNA strand breaks in human granulocytes: Paradoxical effect of 1,10-phenanthroline. *Archives of Biochemistry and Biophysics*, **294**, 17–21.
18. H.C. Birnboim (1988) A superoxide anion-induced DNA strand break metabolic pathway in human leukocytes: Effects of vanadate. *Biochemistry and Cell Biology*, **66**, 374–381.
19. H.C. Birnboim (1990) Fluorometric analysis of DNA unwinding to study strand breaks and repair in mammalian cells. *Methods in Enzymology*, **186**, 550–555.
20. H.C. Birnboim (1993) Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Methods in Enzymology*, **216**, 154–160.
21. H.C. Birnboim (1988) Rapid extraction of high molecular weight RNA from cultured cells for Northern analysis. *Nucleic Acids Research*, **16**, 1487–1497.
22. R.A. Floyd, M.S. West, K.L. Eneff *et al.* (1990) Conditions influencing yield and analysis of 8-hydroxy-2′-deoxyguanosine in oxidatively damaged DNA. *Analytical Biochemistry*, **188**, 155–158.
23. R.S. McWilliams, W.G. Cross, J.G. Kaplan and H.C. Birnboim (1983) Rapid rejoining of DNA strand breaks in resting human lymphocytes after irradiation by low doses of ^{60}CO τ rays or 14.6-MeV neutrons. *Radiation Research*, **94**, 499–507.
24. O.I. Aruoma, B. Halliwell, E. Gajewski and M. Dizdaroglu (1991) Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochemical Journal*, **273**, 601–604.
25. M. Dizdaroglu, R. Olinski, J.H. Doroshow and S.A. Akman (1993) Modification of DNA bases in chromatin of intact target human cells by activated human polymorphonuclear leukocytes. *Cancer Research*, **53**, 1269–1272.
26. I. Schraufstatter, P.A. Hyslop, J.H. Jackson and C.G. Cochrane (1988) Oxidant-induced DNA damage of target cells. *Journal of Clinical Investigation*, **82**, 1040–1050.
27. T. Lindahl (1993) Instability and decay of the primary structure of DNA. *Nature*, **362**, 709–715.
28. R. Olinski, T. Zastawny, J. Budzbon, J. Skokowski, W. Zegarski and M. Dizdaroglu (1992) DNA base modifications in chromatin of human cancerous tissues. *FEBS Letters*, **309**, 193–198.
29. C.G. Fraga, M.K. Shigenaga, J.W. Park, P. Degan and B.N. Ames (1990) Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. *Proceedings of the National Academy of Science U.S.A.*, **87**, 4533–4537.
30. E.M. Fiala, C.C. Conaway and J.E. Mathis (1989) Oxidative DNA and RNA damage in the livers of Sprague-Dawley rats treated with the hepatocarcinogen, 2-nitropropane. *Cancer Research*, **49**, 5518–5522.
31. H. Kasai, P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama and H. Tanooka (1986) Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenests*, **7**, 1849–1851.
32. H.G. Claycamp (1992) Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanine assays. *Carcinogenesis*, **13**, 1289–1292.
33. D.K. Myers (1977) *Low level radiation: a review of current estimates of hazards to human populations. Publication No. 5715.* Atomic Energy of Canada Limited, Chalk River, Ontario.