Free Rad. Res. Comms., Vol. 6, No. 5, pp. 323-328
Reprints available directly from the publisher
Photocopying permitted by license only

FREE RADICAL MEDIATED CYTOTOXICITY OF DESFERRIOXAMINE

MARTIN POOT, PETER S. RABINOVITCH*, and HOLGER HOEHN

Department of Human Genetics, University of Würzburg, 8700 Würzburg, West Germany and Department of Pathology, University of Washington, Seattle, WA 98195, USA*

(Received December 7, 1988)

Toxic effects of desferrioxamine (DFO) upon cell growth were assayed with continuous bromodeoxyuridine (BrdU) labeling and bivariate ethidium bromide/Hoechst 33258 flow cytometry. At 5% oxygen DFO caused a dose-dependent inhibition of cell growth, which was potentiated at 20% oxygen, and by cumene hydroperoxide but not by paraquat. An irreversible arrest in the G2 phase of the cell cycle was the cell-kinetic mechanism underlying this growth inhibition. The G2 arrest was not dependent upon the BrdU concentration in the medium, thus ruling out a direct attack of a free radical on thymidine residues. The observed cytotoxicity of DFO cautions against its use in the treatment of conditions of elevated oxidative stress.

KEY WORDS: Desferrioxamine (desferal), cytotoxicity, BrdU/Hoechst 33258 flow cytometry, cumene hydroperoxide.

INTRODUCTION

Because of its excellent sequestration of ferric iron[1-4] desferrioxamine (DFO 1,desferal) is the agent of choice for clinical iron chelation therapy in conditions like haemochromatosis, haemosiderosis, chronic inflammation, arthritis and reperfusion injury.[5-7] In several trials, however, DFO failed to alleviate the toxicity of paraquat.[8,9] Some reports even mention adverse side-effects including visual and auditory neurotoxicity, coma and retinal pigmentation during prolonged clinical use of DFO.[10-15] One chronic haemodialysis patient with osteomalacia presented upon DFO administration with acute thrombocytopenia.[16] Thus, not only caution should be applied in the interpretation of beneficiary effects from DFO treatment, but also a toxic action of DFO should be considered. Here, we report inhibition of cell growth in human fibroblast cultures, which was potentiated by elevated oxygen concentrations and by cumene hydroperoxide, but alleviated by paraquat.

MATERIALS AND METHODS

Desferrioxamine

Desferrioxamine mesylate was obtained from Sigma (St Louis, MI) and stored at −20°C until use. Immediately before use, DFO was dissolved in phosphate-buffered saline (PBS), and further diluted to the appropriate concentration with culture

Corresponding author: Martin Poot, Department of Human Genetics, University of Würzburg, Koellikerstrasse 2, 8700 Würzburg, FRG. Tel: +49-931-31298

medium (see below). DFO was either added to cells during plating or incubated during 48 hours with confluent cultures experiencing serum starvation. The latter type of incubation will be referred to as incubation during quiescence.

Cells and Culture

Human diploid skin derived fibroblast-like (HDFL) cells[17] were cultured in Minimal Essential Medium (MEM) (Gibco, NY) supplemented with 10% fetal bovine serum (FBS). Confluent cultures rendered quiescent by serum starvation (48 hrs in MEM supplemented with 0.1% FBS). Subsequently, cells were trypsinized and resuspended in MEM containing 10% FBS and 65 μM of bromodeoxyuridine (BrdU) and deoxycytidine (dC) each. Cells were plated in 80 cm^2 culture flasks (Nunc, Denmark) at a density of 2,000 to 2,500 cells per cm^2. Experiments were carried out at 37°C in incubators with sensors regulating CO_2 and oxygen supply (Heraeus, Hanau, FRG). CO_2 was maintained at 5% (vol/vol), and in some experiments the oxygen concentration was adjusted to 5% (vol/vol) by mixing nitrogen and air. All culture flasks were carefully wrapped in aluminium foil, and care was taken to avoid exposure to light of short wavelengths during all stages of handling. Cells were harvested 72 hours after plating by trypsinization and stored at -20°C in MEM supplemented with 10% FBS and 10% dimethylsulfoxide.

Cell Staining and Flow Cytometry

After thawing, cell pellets were resuspended in staining buffer containing 1.2 μg Hoechst 33258 and 2.0 μg ethidium bromide per ml of staining buffer.[18] Flow cytometric analysis was carried out with an epiillumination system of conventional design (ICP 22, Ortho Diagnostic Systems, Raritan, NJ). Bivariate cytograms of Hoechst 33258 (blue) and ethidum bromide (red) fluorescence were collected with a PDP 11/23 microcomputer (Digital Equipment Corporation, Maynard, MA). Simultaneous analysis of BrdU-quenched Hoechst 33258 fluorescence and non-quenched ethidium bromide fluorescence allows identification of three consecutive cell cycles.[19] By electronic framing, rotation and deconvolution each of the component cell cycles was obtained from the bivariate cytogram. Each histogram was analysed by automated curve fitting.[18] Cell numbers in each cell cycle compartment were normalised to the percentage of original cells by dividing the number of cells that underwent one division by two, and those that underwent two divisions by four.

RESULTS AND DISCUSSION

Impairment of cell growth is a very sensitive indicator of toxic insult. BrdU/Hoechst 33258 flow cytometry not only reveals the overall growth rate of a cell culture, but also allows to assess the extent to which cells are arrested in the various compartments of the cell cycle.[18,19] Cell growth was assayed in cultures after 72 hours of exposure to a concentration series of DFO at 5 or 20% (vol/vol) oxygen, at 5% oxygen with 10 μM cumene hydroperoxide (Chp), or at 5% oxygen with 10 μM paraquat (Par). A dose-dependent decrease in cell growth rates was obtained with DFO at 5% oxygen and a stronger decrease was found at 20% oxygen (Figure 1), which indicates a synergism between DFO and oxygen. At 5% oxygen, with 10 μM of the lipophilic

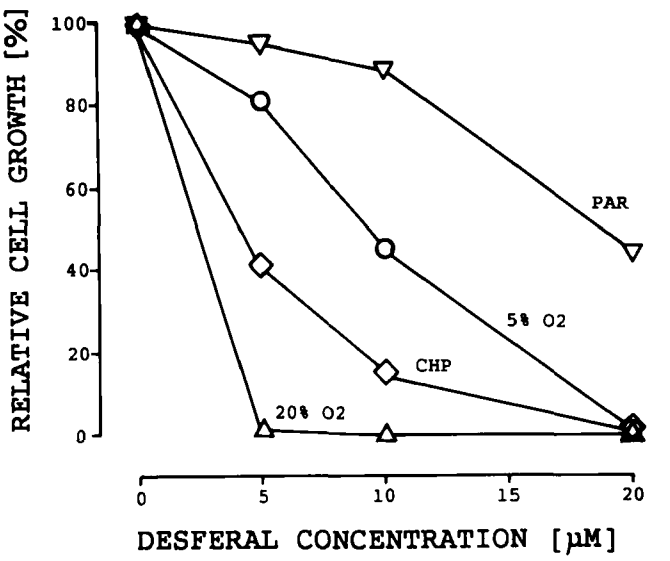

FIGURE 1 Relative cell growth determined with BrdU/Hoechst flow cytometry after 72 hours of culture with a concentration series of DFO at 5 and 20% oxygen, at 5% oxygen with 10 μM paraquat, and at 5% oxygen with 10 μM Chp. The mean and standard deviations of 4 independent determinations are shown.

hydroperoxide Chp, cells showed a diminished growth rate akin to what was found with 20% oxygen. This result suggests that the oxygen-dependent potentiation of the DFO effect can in part be accounted for by lipophilic hydroperoxides. On the other hand, paraquat, an intracellular generator of superoxide, prevented part of the growth inhibitory effect of DFO. Presumably, superoxide radicals are less effective than Chp in promoting the formation of a toxic species from DFO in this cell culture system.

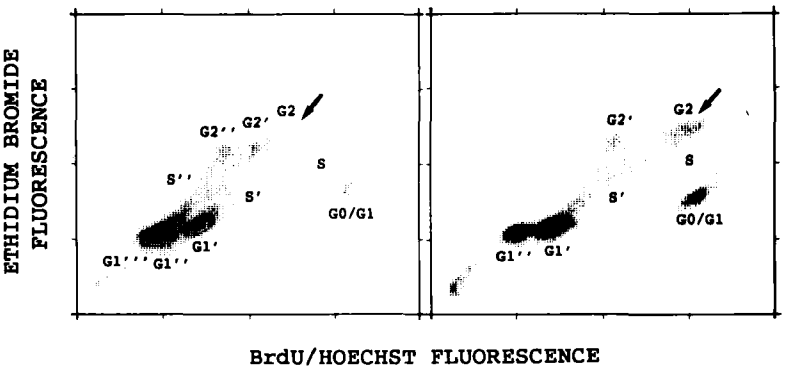

FIGURE 2 Bivariate cytogram of a cell culture after 72 hours at 5% oxygen (left panel) and at 5% oxygen with 5 μM DFO. Note the prominent accumulation of cells in the first G2 phase after release from quiescence (arrows).

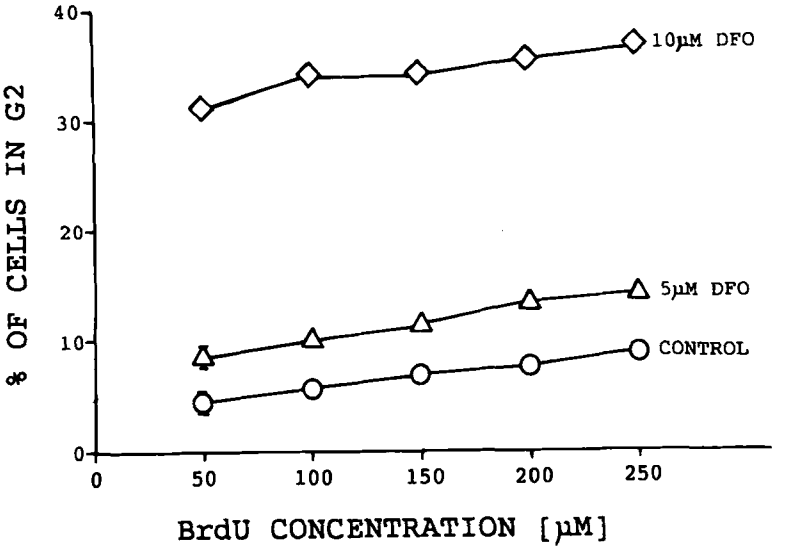

FIGURE 3 Fraction of cells in G2 after 72 hours of culture with a concentration series of Brdu at 5% oxygen (○), at 5% oxygen with 5 (△) and 10 (◇) μM DFO. Data are mean and standard deviation from four independent determinations.

To detect the cell kinetic mechanism by which DFO perturbs cell growth, the bivariate cytgrams obtained with BrdU/Hoechst 33258 flow cytometry were analysed. An accumulation of cells in the G2 phase of the first cell cycle is conspicuous in the DFO-treated cells (Figure 2). Such an accumulation in G2 was also seen in cells cultured at 20 or 35% oxygen,[20] and in cells incubated with Chp or 4-hydroxynonenal, a breakdown product of lipid hydroperoxides.[21] The accumulation of cells in G2 elicited by 35% oxygen is related to the formation of a thymidinyl-like radical, which is enhanced BrdU substitution of the DNA.[22] As a result the fraction of cells of G2 increases with the increasing BrdU concentration in the culture medium. To test whether DFO acts via a similar mechanism, cells were incubated with 5 or 10 μM of DFO and 50 to 200 μM of BrdU. Figure 3 shows that the fraction of cells in G2 in cultures exposed to DFO does not increase more than in the control cultures with increasing BrdU concentrations in the culture medium. Hence, the formation of a thymidinyl-like radical in the DNA is not likely to be the mechanism by which DFO affects cell growth.

In vivo, most cells are in a quiescent state, i.e. they do not proliferate. In evaluating toxicity of prospective clinical drugs, tests with quiescent cells should therefore be included. Figure 4 displays the growth rate of cultures exposed to DFO during quiescence and during proliferation and maintained at 5 or 20% oxygen. At 5% oxygen, cells exposed to DFO during quiescence show no reduction in growth rate, while proliferating cells are sensitive to DFO. In cultures kept at 20% oxygen, DFO elicits a diminished growth rate in cells exposed during quiescence or during proliferation, albeit to a lesser extent in cells exposed during quiescence only. Proliferative activity clearly increases the sensitivity of cells towards DFO.

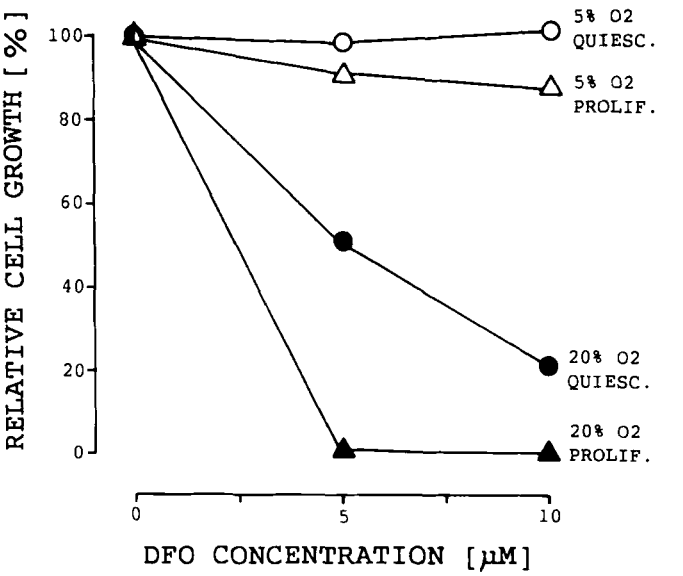

FIGURE 4 Relative growth of cells cultured during 72 hours at 5 and 20% oxygen with 10 μM DFO during logarithmic growth or after exposure to 10 μM DFO during quiescence and without DFO during serum stimulation.

The potentiation of DFO-induced growth inhibition by oxygen and Chp (Fig. 1) suggest a free radical mechanism of DFO toxicity. A one-electron reduced species with a redox potential of -420 to -450 mV has been detected upon reduction with paraquat.[23] Also a nitroxide free radical has been obtained upon oxidation of DFO.[24,25] Our data suggest that free radical mediated oxidation, rather than reduction, promote the formation of a toxic product from DFO. Clearly, further experimentation is required to determine the precise free radical mechanism of DFO toxicity. Nevertheless, our data demonstrate a clear cytotoxicity of DFO, which prompts to caution against indiscriminate use of DFO, particularly in conditions associated with generation of lipid hydroperoxides and tissue renewal (eg. rheumatoid arthritis, inflammation).

Acknowledgements

Helpful discussions with Dr. D.C. Borg and Dr. R.P. Mason on free radical activation of DFO are kindly acknowledged. This work is supported by Deutsche Forschungsgemeinschaft grant no. DFG 849/2-1 and by NIH grant no. AG-01751.

References

1. Keberle, H., *Ann. N.Y. Acad. Sci.*, **119**, 758–768 (1964).
2. Hershko, C., *Blood*, **51**, 415–423 (1978).
3. Graf, E., Mahoney, J.R., Bryant, R.G. and Eaton, J.W., *J. Biol. Chem.*, **259**, 3620–3624 (1984).
4. Cumming, R.L.D., Miller, J.A., Smith, J.A. and Goldberg, A., *Br. J. Haematol.*, **17**, 257–265, (1969).
5. Gutteridge, J.M.C., Richmond, R. and Halliwell, B., *Biochem. J.*, **184**, 469–472 (1979).
6. Bernier, M., Hearse, D.J. and Manning, A.S., *Circ. Res.*, **58**, 331–340 (1986).

7. Jeremy, J.Y., Kontoghiorghes, G.J., Hoffbrand, A.V. and Dandona, P., *Biochem. J.*, **254**, 239–244, (1988).
8. Osheroff, M.B., Schaich, K.M., Drew, R.T. and Borg, D.C., *J. Free Rad. Biol. Med.*, **1**, 71–82, (1985).
9. Bagley, A.C., Krall, J. and Lynch, R.E., *Proc. Natl. Acad. Sci. USA*, **83**, 3189–3193, (1986).
10. Davies, S.C., Marcus, R.E., Hungerford, J.L., Miller, M.H., Arden, G.B. and Huehns, E.R., *Lancet*, **ii**, 181–184, (1983).
11. Simon, P., Ang, K.S., Meyrier, A., Alalin, P. and Mauras, Y. *Lancet*, **ii**, 512–513, (1983).
12. Lakhanpal, V. Shocket, S.S. and Jiji, R., *Opthalmol.*, **91**, 443–451, (1984).
13. Guerin, A., London, G., Marchais, S., Metivier, F. and Pelisse, J.M., *Lancet*, **ii**, 39–40, (1985).
14. Orton, R.B., De Veber, L.L. and Sulh, H.M., *Can. J. Ophtalmol.*, **20**, 153–156, (1985).
15. Olivieri, N.F., Buncic, J.R., Chew, E., Gallant, T., Harrison, R.V., Keenan, N., Logan, W., Mitchell, D., Ricci, G., Skarf, B., Taylor, M. and Freedman, M.H., *New Engl. J. Med.*, **314**, 869–873, (1986).
16. Walker, J.A., Sherman, R.A. and Eisinger, R.P., *Am. J. Kidney Dis.*, **6**, 254–256, (1985).
17. Harper, R.H. and Grove, G., *Science*, **204**, 526–527, (1979).
18. Rabinovitch, P.S., *Proc. Natl. Acad. Sci. USA*, **70**, 2951–2955, (1983).
19. Rabinovitch, P.S., Kubbies, M., Chen, Y.C., Schindler, D., Hoehn, H., *Exp. Cell Res.*, **174**, 309–318, (1988).
20. Poot, M., Schindler, D., Kubbies, M., Hoehn, H., Rabinovitch, P.S., *Cytometry*, **9**, 332–338, (1988).
21. Poot, M., Esterbauer, H., Rabinovitch, P.S., Hoehn, H., *J. Cell. Physiol.*, **137**, 421–429 (1988).
22. Lett, J.T., Parkins, G., Alexander, P., Ormerod, M.G., *Nature*, **203**, 593–596, (1964).
23. Borg, D.C. and Schaich, K.M., *J. Free Radicals Biol. Med.*, **2**, 237–243, (1986).
24. Davies, M.J., Donkor, R., Dunster, C.A., Gee, C.A., Jonas, S., Willson, R.L. *Biochem. J.*, **246**, 725–729, (1987).
25. Morehouse, K.M., Flitter, W.D., Mason, R.P., *FEBS Lett.*, **222**, 246–250, (1987).

Accepted by Prof. H. Sies