RESEARCH COMMUNICATION

Copper-1,10-phenanthroline induces internucleosomal DNA fragmentation in HepG2 cells, resulting from direct oxidation by the hydroxyl radical

Shui Ying TSANG*†, Shuk Ching TAM†, Ian BREMNER* and Mark J. BURKITT*‡

*Boyd Orr Research Centre§ at the Rowett Research Institute, Division of Biochemical Sciences, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K., and †Boyd Orr Research Centre§ at the University of Aberdeen, Department of Chemistry, Meston Walk, Old Aberdeen AB9 2UE, Scotland, U.K.

In view of the current speculation regarding the possible role of reactive oxygen species (ROS) in apoptosis, both under physiological conditions and in response to chemicals that promote their intracellular formation, the present investigation was undertaken to examine whether DNA fragmentation during oxidative stress results from endonuclease activity (apoptosis) or from direct attack by ROS. We report that the incubation of HepG2 cells (a human-derived hepatoma cell line) with the copper(II) complex of 1,10-phenanthroline, $\text{Cu}^{\text{II}}(\text{OP})_2$, results in internucleosomal DNA fragmentation, which is widely recognized as being a hallmark of apoptosis. DNA fragmentation did not occur at low temperature, but activity was restored by the addition of ascorbic acid. It is proposed that DNA fragmentation

results from the direct attack of hydroxyl radicals upon DNA. Hydroxyl radicals are produced from oxygen by the redox-cycling of $\text{Cu}^{\text{II}}(\text{OP})_2$, which is supported by metabolic processes at normal temperature. At low temperature ascorbic acid provides an artificial cellular reducing environment, thereby restoring hydroxyl radical formation. These findings were confirmed by the detection of internucleosomal DNA fragmentation following the exposure of isolated chromatin to a biomimetic $\text{Cu}^{\text{II}}(\text{OP})_2$ redox-cycling system. We conclude that DNA laddering, the widely employed hallmark of apoptosis, is not unique to endonuclease activity and may also result from direct attack upon DNA by the hydroxyl radical.

INTRODUCTION

As well as being an important mechanism of cell deletion in response to physiological stimuli, apoptosis [or programmed cell death (PCD)] may also be responsible for cell killing during the metabolism of chemical agents that stimulate the intracellular generation of reactive oxygen species (ROS) [1–3]. Indeed, evidence suggests that ROS, which are generated in trace amounts by all aerobic cells, may serve a physiological role in the mediation of cell killing by apoptosis [4–6]. Antioxidants, for example, have been reported to block apoptosis [7,8], and the prevention of cell killing by expression of the *Bcl-2* proto-oncogene has been attributed to the suppression of cellular ROS [9,10]. The suggested role of ROS in apoptosis, however, and the mechanism by which *Bcl-2* is suggested to operate, have been challenged [11–13]. For example, Shimizu et al. have reported that the expression of *Bcl-2* and a related protein, *Bcl-xL*, prevents apoptotic cell death induced by hypoxia [12]. These authors demonstrated that ROS are not involved in hypoxia-induced cell killing, and concluded, therefore, that *Bcl-2* and *Bcl-xL* do not prevent this form of cell death via their regulation of cellular ROS levels. It was suggested that the proteins may regulate proteases directly, or might function, for example, through protein kinase cascades [12]. Similar conclusions were reached by Jacobson and Raff [13]. They found that although near-anaerobic conditions and antioxidants prevent PCD induced by ROS-generating agents, such conditions do not inhibit PCD induced by other means. *Bcl-2* was found to prevent cell death under both aerobic

and anaerobic conditions, leading to the suggestion that although ROS can activate the cell-death programme, they do not seem to be required to execute it, and that *Bcl-2* protects cells from PCD in ways that do not depend on its ability to regulate ROS [13].

Internucleosomal DNA fragmentation is often observed during apoptosis and has become widely accepted as one of the most reliable biochemical markers for the process [1–3,14]. In view of the fact that ROS, particularly the hydroxyl free radical ($\cdot\text{OH}$), can themselves bring about DNA fragmentation [15,16], relatively little attention appears to have been given to the possibility that the internucleosomal DNA fragmentation that is often observed in cells following exposure to ROS is caused, not by endonucleases (apoptosis), but by the hydroxyl radical [17,18]. With this in mind, we embarked upon a study of DNA fragmentation in HepG2 cells exposed to the copper(II) complex of 1,10-phenanthroline (*o*-phenanthroline, OP), $\text{Cu}^{\text{II}}(\text{OP})_2$. In the presence of a reducing agent, $\text{Cu}^{\text{II}}(\text{OP})_2$ is known to promote hydroxyl radical formation from molecular oxygen by redox-cycling, and was therefore considered to be a suitable agent for the stimulation of ROS formation [19–22]. We demonstrate that internucleosomal DNA fragmentation is induced in exposed cells, and that although this is suppressed if incubations are carried out at 4 °C (indicating a requirement for enzyme activity), this is due entirely to hydroxyl radical attack upon the DNA, demonstrating that internucleosomal DNA fragmentation cannot be used alone to distinguish between apoptosis and other forms of cell killing.

Abbreviations used: ROS, reactive oxygen species; PCD, programmed cell death; LDH, lactate dehydrogenase; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; OP, *o*-phenanthroline (1,10-phenanthroline); $\text{Cu}^{\text{II}}(\text{OP})_2$, copper(II) complex of 1,10-phenanthroline.

‡ To whom correspondence should be addressed.

§ The Boyd Orr Research Centre is a member of Aberdeen Research Consortium.

MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Company Ltd. (Poole, Dorset, U.K.) and were used without further purification. HepG2 cells (a gift from Dr J. E. Hesketh, Rowett Research Institute) were seeded at a density of $3.7 \times 10^5 \text{ ml}^{-1}$ in 9.6 cm² culture dishes in Williams' E medium (ICN Biomedicals Ltd., Thame, Oxfordshire, U.K.) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) Fungizone, 60 mg·ml⁻¹ penicillin G and 100 µg·ml⁻¹ streptomycin sulphate, and grown in a 5% CO₂, 37 °C humidified atmosphere. After five days, the medium was removed and the confluent cells were washed twice with PBS before exposure to OP and CuCl₂ in RPMI 1640 medium, as indicated in the Figure legends. CuCl₂ and OP were added from aqueous stock solutions. Toxicity was determined as the percentage of total cellular lactate dehydrogenase (LDH) released into the medium using a standard spectrophotometric assay in the presence of NADH and pyruvic acid [23]. Total LDH activity was obtained from Triton X-100 lysed cells.

For the analysis of DNA fragmentation using agarose-gel electrophoresis, exposed cells were lysed in 0.5 ml of lysis buffer (0.5% Triton X-100 in 5 mM Tris/20 mM EDTA, pH 8.0), microcentrifuged (11600 g for 15 min), and the supernatants, containing non-sedimentable DNA fragments, were treated with RNases A and T1, followed by proteinase K, and then extracted for DNA using the standard phenol/chloroform procedure [3,24]. Extracted DNA was resolved in 1.2% agarose gels using a constant current of 60 mA, and visualized with ethidium bromide [3,24]. For the determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG), total DNA was extracted from cells, as described above, following treatment in lysis buffer. The DNA from each incubation was then dissolved in 20 mM sodium acetate buffer, pH 4.8, and heated to 95 °C for 2 min, followed by immediate cooling in an ice/ethanol/water mixture to prevent renaturation. After adjustment of the sample volumes to give absorbance values at 260 nm of 2.1, 350 µl aliquots were hydrolysed enzymically [25] and 8-OHdG and dG were measured using HPLC, as described [24].

In order to prepare chromatin, 3.5×10^7 cells were suspended in 6 ml of homogenization buffer (0.25 M sucrose/50 mM Tris/HCl/25 mM KCl/5 mM MgCl₂·6H₂O, pH 7.5) containing 1 mM PMSF plus 0.5% (v/v) Nonidet P-40, and placed on ice for 15 min. The cells were then subjected to 12 passes of a hand-operated Dounce homogenizer. Following the addition (with mixing) of 2 ml of glycerol, the cells were centrifuged for 10 min at 255 g using a swing-out rotor (Jouan MR1822). The supernatant was discarded and the pellet (nuclei) was washed by resuspension in homogenization buffer (without Nonidet P-40) followed by centrifugation. Chromatin was then isolated from the nuclei using the method of Gajewski et al. [26], but with omission of the final homogenization step. Following incubation, total DNA was extracted and examined by agarose-gel electrophoresis, as described above for the extraction and examination of non-sedimentable DNA fragments from cells. Chromatin was also incubated briefly with micrococcal nuclease to provide a positive control for the generation of nucleosomal DNA fragments [27].

RESULTS AND DISCUSSION

When the supernatants from detergent-lysed HepG2 cells, which had been incubated with Cu^{II}(OP)₂ for 2 h, were extracted for DNA and examined by agarose-gel electrophoresis, DNA fragments were detected, indicating that double-strand-breakage had occurred (Figure 1). No fragments were detected in cells incu-

Figure 1 Dose-dependent internucleosomal DNA fragmentation in HepG2 cells following incubation with CuCl₂ and OP detected using agarose-gel electrophoresis

Following incubation of cells in the presence of various concentrations of CuCl₂ and OP for 2 h, non-sedimentable DNA fragments that had been released from the chromatin upon treatment with lysis buffer were extracted and resolved in a 1.2% agarose gel and visualized using ethidium bromide, as described in the Materials and methods section. Lane 1, no addition; lane 2, 0.1 mM CuCl₂/0.5 mM OP; lane 3, 0.2 mM CuCl₂/1.0 mM OP; lane 4, 0.5 mM CuCl₂/2.5 mM OP; lane 5, 1.0 mM CuCl₂/5 mM OP; lane 6, 2.0 mM CuCl₂/10.0 mM OP. DNA size markers are given in base pairs (lambda DNA bstII digest).

Table 1 Toxicity of CuCl₂ and OP towards HepG2 cells as determined by LDH release

HepG2 cells were exposed to CuCl₂ and OP at the concentrations indicated for 2 h. Toxicity was determined as the percentage of total cellular LDH activity in the medium, as described in the Materials and methods section. Total LDH activity was obtained from Triton X-100-lysed cells. Values are means $\pm$ 1 S.D. ($n = 3$).

Incubation conditions	LDH released into medium (% of total)
Control (no addition)	1.4 $\pm$ 0.6
10 µM CuCl ₂ /50 µM OP	2.5 $\pm$ 0.1
20 µM CuCl ₂ /100 µM OP	5.3 $\pm$ 0.9
50 µM CuCl ₂ /250 µM OP	36.5 $\pm$ 3.7
100 µM CuCl ₂ /500 µM OP	56.2 $\pm$ 3.3
500 µM CuCl ₂ /2.5 mM OP	58.8 $\pm$ 2.6
500 µM CuCl ₂ alone	0.4 $\pm$ 0.0
2.5 mM OP alone	2.3 $\pm$ 0.7

bated in the absence of the copper complex. The fragments formed a typical apoptotic ladder in the gel, with most of the DNA accumulated in the lower bands at the higher Cu^{II}(OP)₂ concentrations, indicating that fragmentation was internucleosomal and dose-dependent. Extensive fragmentation was observed at 100 µM Cu(II) (Figure 1), and was also detectable down to 20 µM (results not shown).

Although the ability of Cu^{II}(OP)₂ to act as a chemical nuclease via the promotion of $\cdot$ OH formation has been reported extensively [19–21], the laddering of the DNA fragments in agarose gels reported here is more commonly associated with the activity of endonucleases [1–3,7,11,14]. Incubation of HepG2 cells with Cu^{II}(OP)₂ caused cell killing (Table 1) and the detection of 8-OHdG in the DNA confirmed attack by $\cdot$ OH (Table 2) [17,25]. When the reducing agent ascorbic acid was included in incubations containing Cu^{II}(OP)₂, fragmentation was not affected, suggesting that it is not limited by the rate of

Table 2 Levels of 8-OHdG detected in DNA from HepG2 cells incubated with CuCl₂ and OP

HepG2 cells were exposed to CuCl₂ and OP at the concentrations indicated for 2 h. Total cellular DNA was then extracted and analysed for 8-OHdG and dG content using HPLC, as described in the Materials and methods section. Values are means $\pm$ 1 S.D. ($n = 4$); n.d. = not detectable.

Incubation conditions	8-OHdG/ 10^5 dG
No addition	n.d.
10 μ M CuCl ₂ /50 μ M OP	5.2 $\pm$ 6.0
20 μ M CuCl ₂ /100 μ M OP	6.1 $\pm$ 3.4
50 μ M CuCl ₂ /250 μ M OP	20.3 $\pm$ 3.5
100 μ M CuCl ₂ /500 μ M OP	96.4 $\pm$ 7.2
500 μ M CuCl ₂ /2.5 mM OP	233.3 $\pm$ 22.4
500 μ M CuCl ₂ alone	n.d.
2.5 mM OP alone	n.d.

Figure 2 Effects of ascorbic acid, H₂O₂ and temperature on DNA fragmentation in HepG2 cells induced by CuCl₂ and OP

Cells were incubated for 2 h in the presence of 50 μ M CuCl₂ and 250 μ M OP. Incubations at 4 °C were carried out on ice in a 5% CO₂ humidified atmosphere. Following exposure, non-sedimentable DNA fragments were extracted and analysed by electrophoresis as described in the Materials and methods section. Samples in lanes 1–4 are from incubations carried out at 37 °C; those in lanes 5–8 are from incubations at 4 °C. Lanes 1 and 5, Cu^{II}(OP)₂ alone; lanes 2 and 6, Cu^{II}(OP)₂ plus 1 mM ascorbic acid; lanes 3 and 7, Cu^{II}(OP)₂ plus 100 μ M H₂O₂; lanes 4 and 8, Cu^{II}(OP)₂ plus 1 mM ascorbic acid and 100 μ M H₂O₂. DNA size markers are given in base pairs (lambda DNA bstIIIE digest).

endogenous reduction of the metal complex (which is necessary for $\cdot$ OH formation [22]) (Figure 2). Similarly, H₂O₂, which is the immediate precursor to $\cdot$ OH [22], had little effect on fragmentation. However, when cells were incubated with Cu^{II}(OP)₂ on ice, no DNA fragmentation was observed (Figure 2). Although this would appear to be good evidence for the involvement of an endonuclease, fragmentation was restored if ascorbic acid was present (Figure 2). Ascorbic acid alone did not induce DNA fragmentation at either temperature (results not shown). H₂O₂ did not restore fragmentation on ice, but fragmentation was observed when H₂O₂ was included in incubations conducted on ice and containing ascorbic acid (Figure 2). These observations reflect those of Jonas et al., who showed that the toxicity of H₂O₂ to epithelial cells is greatly diminished at 4 °C, but can be restored by adding ascorbic acid [28]. Jonas et al. suggested the involvement of transition-metal ions in the toxicity of H₂O₂ and proposed a mechanism of cell damage that depends on $\cdot$ OH

Figure 3 Electrophoretic resolution of DNA fragments generated following the incubation of isolated chromatin with either micrococcal nuclease or CuCl₂, OP, H₂O₂ and ascorbic acid

Chromatin from approx. 4.4×10^6 cells was incubated at room temperature in 1 mM phosphate buffer (pH 7) as indicated. Total DNA was then extracted and examined by agarose-gel electrophoresis as described in the Materials and methods section. Lane 1, 30 min control incubation; lane 2, 3 min incubation with 2 units of micrococcal nuclease plus 1 mM CaCl₂; lane 3, 30 min incubation with 5 μ M CuCl₂, 25 μ M OP, 20 μ M H₂O₂ and 200 μ M ascorbic acid. DNA size markers are given in base pairs (lambda DNA bstIIIE digest).

formation from the reaction of a reduced metal-ion with the peroxide. The restoration of H₂O₂ cytotoxicity by ascorbic acid at low temperature was suggested to reflect the artificial production of an intracellular reducing environment that at normal temperature is sustained by cellular metabolism [28]. Similarly, the findings reported here are consistent with a mechanism of DNA fragmentation involving $\cdot$ OH formation via the redox-cycling of Cu^{II}(OP)₂. Again, it would appear that at low temperature, ascorbic acid is able to substitute for the metabolism-dependent cellular reducing systems that serve to reduce Cu^{II}(OP)₂ at normal temperature. Metabolism-dependent reducing systems for Cu^{II}(OP)₂ might include the mitochondrial respiratory chain and NADPH-cytochrome *P*-450 reductase.

The fact that the DNA fragmentation observed at 4 °C in the presence of ascorbic acid is as extensive as that observed at 37 °C in the absence of the reductant, and the detection of 8-OHdG, suggest that fragmentation results from chemical processes, involving direct attack upon DNA by $\cdot$ OH [17]. A mechanism involving nuclease activation as a consequence of $\cdot$ OH formation, as suggested by Cantoni et al. [29], would be unlikely to proceed with such efficiency at low temperature. Internucleosomal DNA fragmentation, however, is generally not associated with DNA oxidation by $\cdot$ OH. In order to investigate DNA fragmentation by $\cdot$ OH in the absence of any nucleases, isolated chromatin was incubated with a hydroxyl-radical-generating system [18] consisting of Cu^{II}(OP)₂, H₂O₂ and ascorbic acid. Under these conditions DNA laddering was observed (Figure 3), demonstrating that $\cdot$ OH radicals can indeed bring about internucleosomal DNA fragmentation. A similar pattern of DNA fragmentation was observed following the brief incubation of chromatin with micrococcal nuclease (Figure 3), which provides a positive control for internucleosomal DNA fragmentation [27]. These observations are in agreement with those of Cartwright and Elgin, who observed internucleosomal DNA fragmentation when Cu^I(OP)₂ was used as a chemical nuclease to investigate chromatin secondary structure in *Drosophila* nuclei [30], and with our own recent findings on OP-induced DNA fragmentation in isolated rat-liver nuclei [24]. Therefore whilst we

acknowledge a possible role for ROS in apoptosis, it must be concluded that internucleosomal DNA fragmentation is not unique to endonuclease activity (apoptosis) and can also occur as a result of direct DNA oxidation by the hydroxyl radical, thereby underpinning the significance of oxidation by the hydroxyl radical as a mechanism of DNA damage and cell killing during oxidative stress.

This work was supported by the Scottish Office Agriculture and Fisheries Department. S.Y.T. acknowledges receipt of an Aberdeen Research Consortium Postgraduate Studentship, funded by the University of Aberdeen and the Rowett Research Institute. The authors thank Mrs Sharon Wood for assistance with the HPLC measurement of 8-OHdG.

REFERENCES

- Arends, M. J., Morris, R. G. and Wyllie, A. H. (1990) *Am J. Pathol.* **136**, 593–608
- McConkey, D. J., Hartzell, P., Nicotera, P., Wyllie, A. H. and Orrenius, S. (1988) *Toxicol. Lett.* **42**, 123–130
- Dypbukt, J. M., Ankarcrona, M., Burkitt, M., Sjöholm, Å., Ström, K., Orrenius, S. and Nicotera, P. (1994) *J. Biol. Chem.* **269**, 30553–30560
- Sarafian, T. A. and Bredesen, D. E. (1994) *Free Radical Res.* **21**, 1–8
- Buttke, T. M. and Sandstrom, P. A. (1994) *Immunol. Today* **15**, 7–10
- Rothstein, J. D., Bristol, L. A., Hosler, B., Brown, Jr., R. H. and Kuncel, R. W. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4155–4159
- Abello, P. A., Fidler, S. A., Bulkley, G. B. and Buchman, T. G. (1994) *Arch. Surg.* **129**, 134–141
- Sandstrom, P. A., Mannie, M. D. and Buttke, T. M. (1994) *J. Leukocyte Biol.* **55**, 221–226
- Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Butler Gralla, E., Selverstone Valentine, J., Örd, T. and Bredesen, D. E. (1993) *Science* **262**, 1274–1277
- Steinman, H. M. (1995) *J. Biol. Chem.* **270**, 3487–3490
- Muschel, R. J., Bernhard, E. J., Garza, L., McKenna W. G. and Koch, C. J. (1995) *Cancer Res.* **55**, 995–998
- Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H. and Tsujimoto, Y. (1995) *Nature (London)* **374**, 811–813
- Jacobson, M. D. and Raff, M. C. (1995) *Nature (London)* **374**, 814–816
- Zhivotovsky, B., Wade, D., Gahm, A., Orrenius, S. and Nicotera, P. (1994) *FEBS Lett.* **351**, 150–154
- Schraufstatter, I., Hyslop, P. A., Jackson, J. H. and Cochrane, C. G. (1988) *J. Clin. Invest.* **82**, 1040–1050
- Burkitt, M. J., Fitchett, M. and Gilbert, B. C. (1989) in *Medical, Biochemical and Chemical Aspects of Free Radicals* (Hayaishi, O., Niki, E., Kondo, M. and Yoshikawa, T., eds.), pp. 63–70, Elsevier, Amsterdam
- Halliwell, B. and Aruoma, O. I. (1991) *FEBS Lett.* **281**, 9–19
- Burkitt, M. J., Milne, L., Tsang, S. Y. and Tam, S. C. (1994) *Arch. Biochem. Biophys.* **311**, 321–328
- Que, B. G., Downey, K. M. and So, A. G. (1980) *Biochemistry* **19**, 5987–5991
- Dizdaroglu, M., Aruoma, O. I. and Halliwell, B. (1990) *Biochemistry* **29**, 8447–8451
- Milne, L., Nicotera, P., Orrenius, S. and Burkitt, M. J. (1993) *Arch. Biochem. Biophys.* **304**, 102–109
- Burkitt, M. J., Tsang, S. Y., Tam, S. C. and Bremner, I. (1995) *Arch. Biochem. Biophys.* **323**, 63–70
- Anuforo, D. C., Acosta, D. and Smith, R. V. (1978) *In Vitro* **14**, 981–988
- Burkitt, M. J., Milne, L., Nicotera, P. and Orrenius, S. (1996) *Biochem. J.* **313**, 163–169
- Richter, C., Park, J.-W. and Ames, B. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6465–6467
- Gajewski, E., Rao, G., Nackerdien, Z. and Dizdaroglu, M. (1990) *Biochemistry* **29**, 7876–7882
- Wolfe, A. (1992) *Chromatin Structure and Function*, Academic Press, London
- Jonas, S. K., Riley, P. A. and Willson, R. L. (1989) *Biochem. J.* **264**, 651–655
- Cantoni, O., Sestili, P., Cattabeni, F., Bellomo, G., Pou, S., Cohen, M. and Cerutti, P. (1989) *Eur. J. Biochem.* **182**, 209–212
- Cartwright, I. L. and Elgin, S. C. R. (1982) *Nucleic Acids Res.* **10**, 5835–5852