BioMetals 1997, **10**, 119–122

Hydroxyl free radicals generated by vanadyl[IV] induce cell blebbing in mitotic human Chang liver cells

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Received 27 May 1996; accepted for publication 5 September 1996

Vanadium has recently been reported to induce interphase and M-phase (mitotic) programmed cell death via the generation of hydroxyl free radicals (OH*). In this paper, the effects of antioxidants on: (a) vanadyl[IV]-generated OH* free radical levels; and (b) cellular glutathione in vanadyl [IV]-treated Chang liver cells were evaluated. The surface morphology of vanadyl-treated mitotic cells was studied by confocal and scanning microscopy. The free radical scavengers zinc chloride, glucose and thiourea reduced the levels of vanadyl-induced OH* free radicals and partially prevented the depletion of cellular glutathione. Concurrent with OH* free radical production, vanadyl-treated telophase cells exhibited excessive cell blebbing and cell shrinkage. The morphological features demonstrated in vanadyl-induced mitotic programmed cell death as a consequence of oxidative stress is novel.

Keywords: confocal microscopy, glutathione, hydroxyl free radicals, scanning electron microscopy, vanadium

Introduction

Vanadium is a trace element which is present in the bioreduced vanadyl[IV] oxidative state in mammalian cells (Chasteen 1983). Being a transition metal, vanadium may also exhibit the ability to produce reactive oxygen species such as hydroxyl free radicals (OH*), which have the potential to attack a variety of cellular targets (Farber *et al*. 1990). Amongst other effects, OH* free radicals have been reported to induce: (a) DNA damage (Rieth *et al*. 1992); (b) changes in nucleic acids such as base hydroxylation, nicking and scission of DNA strands (Southern & Powis 1988); and (c) lipid peroxidation and DNA fragmentation (Stohs & Bagchi 1995). In addition, the OH* free radical has been cited as the mediator of apoptosis/programmed cell death (PCD) in endothelial and FL5 cell lines via H_2O_2 (de Bono & Yang 1995, Hockenberry 1995); PC 12 neural cells via serum deprivation (Ferrari *et al*. 1995); HL 60 leukaemic cells via UV radiation (Verhaegen *et al*. 1995); and GT1-7 renal cells via buthionine sulfoxamine (Kane *et al*. 1993). PCD, a physiological mode of cell death, is implicated in developmental morphogenesis and a host of pathologic disorders (Thompson 1995). We have recently shown that vanadyl[IV] induced programmed cell death in both interphase and M-phase (mitotic) Chang liver cells via oxidative stress (Sit *et al*. 1996a,b). We now extend the study to show: (a) the effect of antioxidants on vanadyl-induced OH* free radicals and glutathione depletion; and (b) the surface morphology of vanadyl-treated M-phase cells.

Materials and methods

Chemicals

Vanadyl oxide sulphate pentahydrate was obtained from Fluka (Buchs, Switzerland) and monochlorobimane from Molecular Probes (Oregon, USA). All the other chemicals were analar grade from BDH (Poole, UK).

Cell culture

Human Chang liver cells (ATCC CCL 13) were purchased from the American Type Culture Collection (Maryland,

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USA). Parallel cultures were seeded at 3×10^5 cells per 25 cm^2 Costar culture flask, and grown in Dulbecco's modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, USA) containing 10% foetal calf serum (Biological Industries, Haemak, Israel) (DMEM10). For confocal microscopy, cells were grown in Nunc coverglass chambers. For scanning electron microscopy, cells were grown on 0.5 cm by 0.5 cm slides in sterile containers.

Protocol for Chang liver cells incubated with vanadyl[IV]

Vanadyl oxide sulphate pentahydrate ($V_2O_5SO_4.5H_2O$) was dissolved in water at 4 mM concentration. The pH was adjusted to 5.5 with sodium hydroxide, resulting in a dark grey precipitate. After one hour, the precipitate dissolved to form a black solution. Chang liver cells were incubated in ammonium chloride buffer (50 mM NH_4Cl, 50 mM glucose) containing 4 mM vanadyl[IV] oxide sulphate for 30 min. Controls were incubated in ammonium chloride buffer only. Cells were allowed to recover for 2 h in DMEM10 before estimation of cellular glutathione and examination under confocal and scanning microscopy.

Spectrofluorimetric evaluation of OH free radical*

Vanadyl-generated OH* free radicals were evaluated in a Spex spectrofluorimeter on line with the computer-based DM-3000 programme, at excitation/emission wavelengths of 304/413 nm as previously described (Sit *et al.* 1996a). The effect of 200 mM glucose, 10 mM zinc chloride and 10 mM thiourea were also ascertained.

Estimation of cellular glutathione (GSH) content

Monochlorobimane (mcb, mol. wt. 226.66) was added to the recovery medium (DMEM10) to a final concentration of 40 μM during the last 15 min of incubation in the recovery medium. Cellular content of glutathione (GSH) was evaluated by GSH–monochlorobimane adduct fluorescence in the Spex spectrofluorometer at excitation/emission wavelengths of 395/470 nm.

Laser scanning confocal microscopy

Cells were fixed in 5% glutaraldehyde and stained with 2% bromothymol blue. Examination was done under fluorescence and transmitted light with the Carl Zeiss LSM 410 Inverted Laser Confocal Microscope (equipped with a 10 mW argon laser and a Windows based LSM software version 3.5).

Scanning electron microscopy

Cultures were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 30 min. After osmication, cells were dehydrated in increasing concentration of methanol, and critical point dried in the Balzer's CPD 030 using liquefied carbon dioxide (Sit *et al.* 1992). Samples were sputter coated with 20 nm gold in a Balzers Sputter Coater SCD 004 before examination in a JEOL JSM 5300LV.

Results and discussion

Vanadyl [IV] produced a dramatic rise in OH* free radicals (Figure 1). Vanadium, being a transition variable metal, is capable of generating free radicals via the Fenton and Haber–Weiss reactions as shown below (Liochev & Fridovich 1991, Hamada 1994):

$V[IV] + H_2O_2 \rightarrow V[V] + OH^* + OH^-$
(Fenton reaction)

$O_2^- + H_2O_2 \rightarrow O_2 + OH^* + OH^-$
(catalysed by V[IV] salt)
(Haber–Weiss reaction)

Hence, vanadyl[IV] has the potential to induce programmed cell death via oxidative stress (Sit *et al.* 1996a,b). DNA fragmentation (the hall mark of PCD) due to free radical production has been attributed to the activation of endogenous endonuclease (Fernandez *et al.* 1995).

Glutathione, which is the main reducing agent in mammalian systems, is depleted in the presence of vanadyl-generated OH* free radicals. This is evidenced by the precipitous decline in the cellular

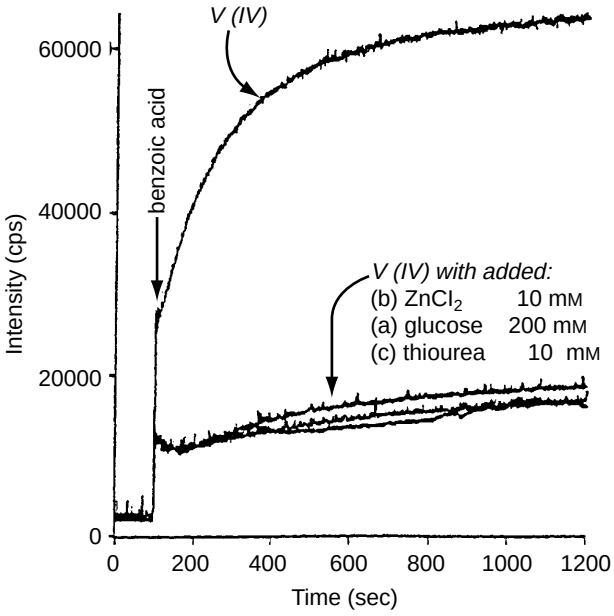

Figure 1. Spectrofluorimetric evaluation of vanadyl [IV]-generated hydroxl free radicals in the presence and absence of the antioxidants zinc chloride (10 mM), glucose (200 mM) and thiourea (10 mM). Addition of benzoic acid in excess increases the fluorescence due to OH*–benzoic acid adduct formation.

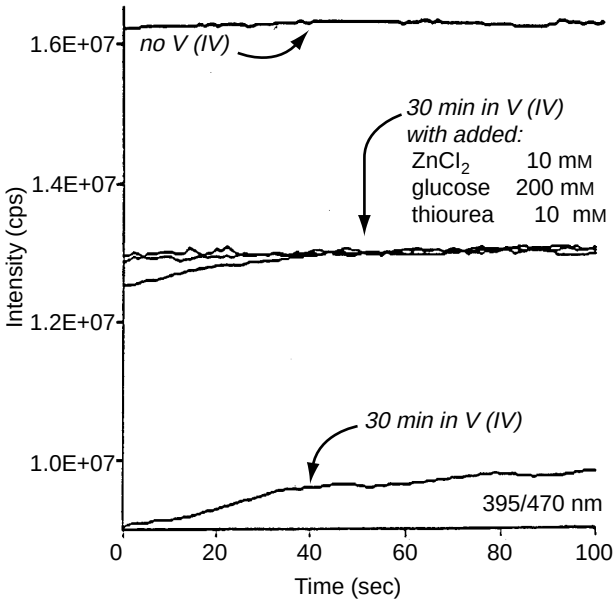

Figure 2. Spectrofluorimetric evaluation of GSH levels in human Chang liver cells in the absence and presence of vanadyl[IV] and the antioxidants zinc chloride (10 mM), glucose (200 mM) and thiourea (10 mM).

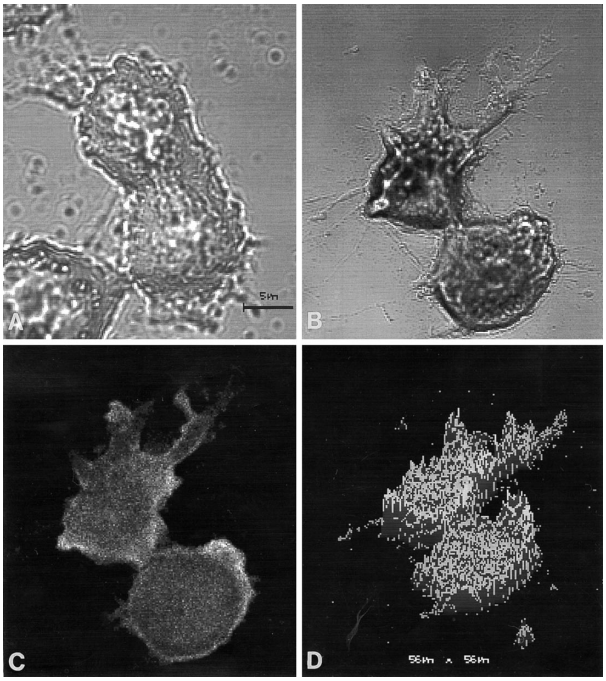

Figure 3. Confocal microscopic images of telophase Chang liver cells. **(A)** Control cells under transmitted light. **(B)** Vanadyl-treated cells under transmitted light. **(C)** Fluorescence image of vanadyl-treated cells. **(D)** Bitmap of fluorescence intensity of image in (C). Bar = 5 μm.

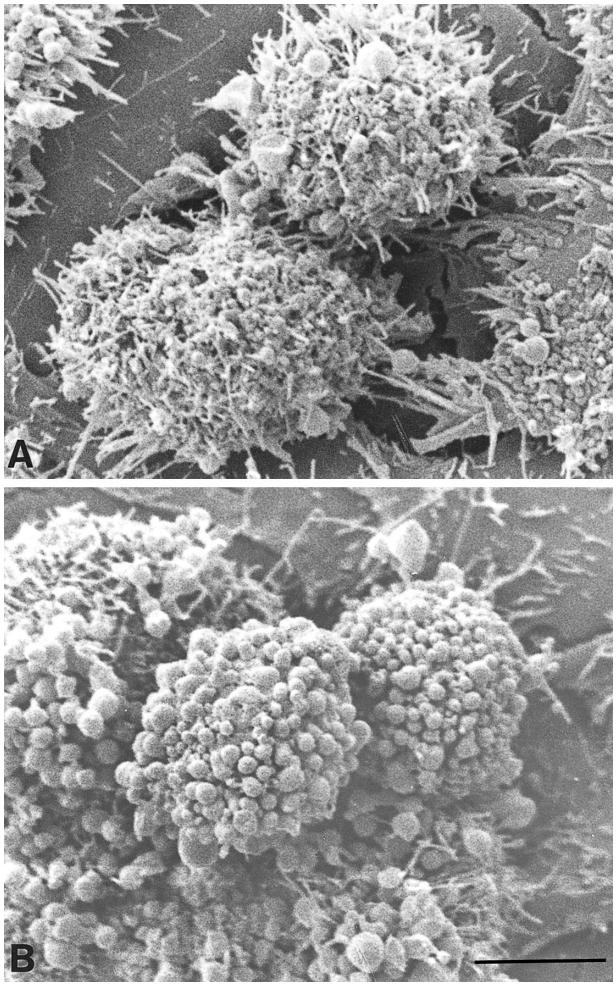

Figure 4. Scanning electron micrographs of telophase Chang liver cells. **(A)** Control cells. **(B)** Vanadyl-treated cells. Numerous blebs are present on the cell surface of treated cells. Bar = 5 μm.

GSH levels of the vanadyl[IV]-treated cells (Figure 2). The result is consistent with the observations that depletion of the cellular glutathione content could lead to increased susceptibility of the cells to programmed cell death induced by reactive oxygen species (Kane *et al.* 1993). It would appear that modification of glutathione metabolism could give rise to resistance to anticancer drugs, since the mode of action of various chemotherapeutic drugs has been reported to be mediated via the production of OH* free radicals (Powis 1987, Moscow & Gowen 1988).

In the presence of the free radical scavengers zinc chloride, glucose and thiourea, the OH* free radicals generated by vanadyl [IV] were reduced (Figure 1). These antioxidants also had the effect of

partially preventing the decline in GSH levels of vanadyl-treated cells (Figure 2). These observations are in accordance with those of Verhaegen *et al*. (1995) who reported that antioxidants protect against oxidative stress induced PCD in response to a wide variety of agents.

Telophase Chang liver cells treated with 4 mM vanadyl[IV] (the concentration which is known to induce mitotic PCD) exhibited distortion of the cell outline with abnormal protrusions underconfocal microscopy (Figure 3). When examined under scanning electron microscopy, telophase cells had excessive cell surface blebbing compared with the controls (Figure 4). The vanadyl-treated cells also appeared smaller than the controls. Morphological features typical of apoptosis in interphase cells are well cited in the literature. They include compaction of chromatin into sharply defined masses close to the cell membrane, condensation of cytoplasm with preservation of the membrane integrity, and formation of rounded apoptotic bodies (Kerr *et al*. 1972, Wyllie *et al*. 1980, Arends & Wyllie 1991, Sit *et al.* 1994). However, there has been no description of the surface features of apoptotic M-phase cells. Hence, oxidative stress mediated cell blebbing observed in vanadyl-treated telophase cells is novel.

Acknowledgements

The authors thank the National University of Singapore for financial support for this research project. R.P. is a recipient of the National University of Singapore Research Scholarship. We are grateful to Mr L. S. Liau for technical assistance.

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