Transient G2M arrest and subsequent release of apoptotic and mitotic cells in vanadyl(4)-prepulsed human Chang liver cells

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Abstract

The relationship between cell cycling and apoptosis/programmed cell death has been perceived as either checkpoint arrests or mitotic aberration where common pathways between mitosis and apoptosis seem suggested. We show here evidence implicating both perceptions of cell cycle involvement. The process was initiated by hydroxyl free radicals (OH^*) generated intracellularly from internalized vanadyl(4). Intranuclear sequestration of vanadyl(4) was verified by nuclear microscopy. Resultant high oxidative reactivity in the nucleus was shown by the redox indicator methylene blue, suggesting direct oxidative damage to genomic DNA. Oxidative stress was further enhanced by depletion of glutathione which is the main cellular reducing agent. Genomic degradation and fragmentation was confirmed by flow cytometric evaluation of terminal deoxynucleotidyl transferase (TdT)-mediated 3'OH end-labelling (TUNEL) of DNA nicks, and cell cycle DNA profiling demonstrating sub-G1 (sub-2N) accumulation. With DNA degradation, there was a G2M transient with hyperdiploid right-shifting, consistent with G2 arrest. G2 arrest was subsequently 'released' with abolition of G2M and all other cell cycle phases except for a solitary sub-G1 (apoptotic) peak. The cytological profile of this 'release' phenomenon was initially marked by the appearance of clusters of mitotic and apoptotic cells. At later stages, the cell population was composed exclusively of nuclear ghosts, apoptotic cells, mitotic cells, and mitotic cells with both chromosomes and apoptotic condensations. Concurrent and conjoint expression of cell death and cell division as the exclusive process of an entire cell population refuted the notion of mutual exclusivity between life and death. Zn^{2+} , an endonuclease inhibitor, abolished all observed cytological and DNA profile changes.

Keywords: G2M apoptosis and mitosis, mitotic apoptosis, vanadyl(4), oxidative stress, population suicide

Abbreviations: OH^* , hydroxyl free radical; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-

labelling; V(4), vanadyl(4), vanadium ion with oxidative valence 4; V(5), dioxovanadium ion with oxidative valence 5; PIXE, particle-induced X-ray emission; RBS, Rutherford backscattering; STIM, scanning transmission ion microscopy; FBS, foetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; pH_i , intracellular pH; BCECF, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; GSH, glutathione, reduced form; MCB, monochlorobimane; GST, glutathione-S-transferase; PMT4, flow cytometer photomultiplier channel number 4; PI, propidium iodide

Introduction

Mitosis seems linked by morphological and molecular similarities with apoptosis (King and Cidlowski, 1995) the suicidal or programmed cell death implicated in tissue homeostasis and a host of hypo- and hyper-proliferative disorders including cancer (Thompson, 1995). Recent findings further suggest a functional linkage between cell proliferation and cell death. Inositol 1,4,5-trisphosphate (IP_3)-receptor binding which triggers intracellular calcium accumulation and proliferation also triggers apoptosis (Khan *et al*, 1996). Oncogenic lesions that generate uncontrolled cell proliferation have been shown to be also potent triggers of apoptosis (Evan *et al*, 1995; Harrington *et al*, 1994; Gazitt and Erdos, 1994). Epidermal growth factor (EGF)-induced signal transduction pathways that promote cell proliferation seem to mediate apoptosis as well (Reddy, 1996). Reactive oxygen species, either directly applied or generated via glucocorticoid hormones, are mediators of apoptosis and second messengers in cell proliferation (Sundaresan *et al*, 1996; Fernandez *et al*, 1995; Briehl and Baker, 1996; Slater *et al*, 1996). However, while the signaling pathways for apoptosis and proliferation seem to be in common, concurrent cytological expressions in cell cycling have not been demonstrated as yet. Interphase checkpoint surveillance at G1/S and G2/M transitions are known to prevent cells with incomplete replication or unrepaired DNA damage from progressing into cytokinesis. The result of cell cycle transition arrest is phase-specific accumulations and mitotic depletion (Piacentini *et al*, 1993; Hartwell and Kastan, 1994; Earnshaw, 1995). Nevertheless, some cells have been noted to continue dividing despite cell cycle arrest in apoptotic systems (Traganos *et al*, 1992), and there have been arguments for the possibility that the interphase checkpoint surveillance could be bypassed allowing apoptosis to be committed with mitosis in common pathways (King and Cidlowski, 1995; Evan *et al*, 1995).

We have used vanadyl(4)-generated hydroxyl free radicals (OH^*) to initiate apoptosis/programmed cell death in human Chang liver cells (Sit *et al*, 1996a,b). The advantage of this system is the ability to generate free

radicals intranuclearly for direct DNA damage which is known to cause G2 arrest and accumulation. Here we

studied the consequential phenomena in a time-response, demonstrating a transient G2 arrest followed by a 'release

Figure 1 Quantitation of vanadium internalization by nuclear microscopy. Particle Induced X-ray Emission (PIXE) elemental spectra of a representative control (without V(4) prepulse) human Chang liver cell (A and C). No vanadium (V) peak is found in either (A) nucleus or (C) cytoplasm of the control. On the other hand, the vanadium peak (V) is clearly evident 3 min after a 30 min V(4) prepulse (B and D). The V(4) peak is higher in (B) the nucleus, than in (D) the cytoplasm. The calcium (Ca) peak is also raised.

Figure 2 Cellular localization of vanadium in control cells by nuclear microscopy. Scanning Transmission Ion Microscopy (STIM) defined the cell and its nucleus which is outlined. Bitmapping of PIXE spectral energies shows the element phosphorus having a high concentration in the nucleus, thereby helping to define the nucleus in conjunction with STIM. Calcium and vanadium elements are at low levels in the control cells.

Figure 3 Cellular localization of vanadium in V(4)-prepulsed cells by nuclear microscopy. The nucleus is defined by STIM and PIXE bitmap of phosphorus. Here the cells are seen loaded with vanadium and calcium, concentrating particularly in the nucleus, the areas outlined by STIM and PIXE.

phase' abolishing G2M and releasing not only apoptotic cells and nuclear ghosts, but also mitotic cells, and cells expressing both mitotic chromosomes and apoptotic condensations.

Results and Discussion

Vanadyl(4) internalization

Hydroxyl radicals (OH^*) were generated inside human Chang liver cells by inducing an intracellular loading of vanadyl(4), (V4), verified by nuclear microscopy (Figures 1–3). Nuclear

microscopy incorporated (a) particle-induced X-ray emission (PIXE) to measure directly total (free and bound) elemental concentrations in the *as-is* state of single cells, (b) Rutherford backscattering spectrometry (RBS) which provided information on the matrix elements C, N and O, and (c) scanning transmission ion microscopy (STIM) which provided information on the structure and density to image the cell and its nucleus through transmitted proton energy loss (Sit *et al.*, 1993). V(4) internalization was produced by a 30 min acidification prepulse in V(4) buffer, pH 5.5. Following V(4) prepulsing the cells were reincubated in normal DMEM growth medium containing 10% FBS, for realkalinization back to

Figure 4 (A) and (B) are the control and V(4)-prepulsed Chang liver cells, respectively, showing their oxidative states highlighted by the redox indicator methylene blue. Methylene blue is colourless in the reduced state but metachromatic (darker) when oxidized. The nuclei of V(4)-prepulsed cells have high oxidative reactivity, suggesting direct oxidative damage to genomic DNA. (C) Glutathione (GSH) content of control cells, evaluated by GSH-monochlorobimane (MCB) adduct fluorescence at the excitation/emission wavelength of 395/470 nm. (D) V(4)-prepulsed cells showing poor GSH-MCB fluorescence from GSH depletion.

physiological levels. At pH 7, the internalized V(4) readily converted with oxidation (even molecular oxygen) to V(5), the yellow dioxovanadium ions. In the V(4)-to-V(5) conversion, superoxides were generated and these underwent spontaneous dismutation to form hydrogen peroxide (Hamada, 1994; Keller *et al*, 1988). Hydrogen peroxide and superoxides were sources of hydroxyl free radicals (OH^{*}) generated via the transition metal-ion driven Fenton and Haber-Weiss reactions (Halliwell and Gutteridge, 1984). An OH^{*} burst was triggered with realkalinization in normal growth medium after the V(4) prepulse (Sit *et al*, 1996a). OH^{*} could react with most cellular biomolecules of which DNA is a known target molecule (Farber *et al*, 1990). However the high reactivity of OH^{*} imposes strict limitations on its diffusion distance, estimated to be less than 7 nm from the site of generation (Byrnes, 1996). Thus unless OH^{*} was generated intranuclearly, it would be quite unlikely to have a direct effect on genomic DNA.

Intranuclear oxidative damage

High oxidative reactivity localized particularly in the nucleus after the V(4) prepulse was demonstrated by the redox indicator methylene blue which became a deep metachromatic (purple) colour from oxidation (Figure 4A and B). Methylene blue is colourless in the reduced state. The OH^{*} burst (Sit *et al*, 1996a) coincided with depletion of glutathione (GSH), the main reducing agent of the cell (Figure 4C and D), thereby enhancing the oxidative stress. Flow cytometric cell-by-cell evaluation of GSH content (measuring GSH-Mono-chlorobimane (GSH-MCB) adduct fluorescence) corroborated the cytological demonstration of GSH decline with realkalinization after a V(4) prepulse (Figure 5A). Without realkalinization, the V(4)-prepulsed cells had pHi (intracellular pH)=4.3, and mean GSH-MCB=226.9 PMT4 channel units (Figure 5A(i)). V(4)-prepulsed cells that had a 15 min recovery incubation in normal DMEM were realkalinized back to physiological levels (pHi=7.1) and overshoot slightly the resting level (Figure 5A(ii)). At this realkalinized state, the mean GSH-MCB=109.4 units, representing a reduction by more than 50% of the original value. V(4)-prepulsed cells with a 2 h recovery in normal DMEM had pHi=6.9 and mean GSH-MCB=108.7 units thereby indicating that GSH was already depleted to basal limits immediately upon realkalinization. GSH depletion coincided with (a) the triggering of an OH^{*} burst from alkalinization of V(4) (Sit *et al*, 1996a), and (b) the demonstration of high oxidative reactivity in the nuclei (see Figure 4B). GSH depletion would enhance the oxidative stress, and contribute to the oxidative genomic damage (Kane *et al*, 1993; Dawson and Dawson, 1996). DNA fragmentation was confirmed by flow cytometric evaluation of specific terminal deoxynucleotidyl transferase (TdT)-mediated labeling of 3'OH ends of DNA breaks (Figure 5B).

G2M transient and release sensitive to Zn²⁺

Within 30 min after V(4) prepulsing, a significant increase in the proportion of cells in the G2M peak was detected (Figure 6A(ii) cf. Figure 6A(i)). However this was associated with an obvious right-shifting of the peak suggesting cellular

hyperdiploidy from G2 arrest blocking mitotic division (Figure 6A(ii-iv)). This was consistent with G2 accumulation due to G2/M transition arrest consequential to DNA damage from the OH^{*} burst released from internalized V(4). Concurrently there was sub-G1 accumulation. Cells with sub-G1 DNA were those having gross genomic DNA fragmentation with or without apoptotic chromatin morphologies (Tounekti *et al*, 1995). After

Figure 5 (A) Flow cytometric quantitation of GSH-MCB adduct fluorescence in a time-response after V(4) prepulse. Insets show the intracellular pH (pHi) of cells at each stage, measured by ratiometric (FL1/FL3) BCECF evaluation in flow cytometry. GSH depletion to basal levels occurred 15 min after V(4) prepulse. (C) Terminal deoxynucleotidyl transferase (TdT) mediated end-labelling of 3'OH ends of DNA fragments evaluated by flow cytometry confirms that genomic DNA fragmentation characteristic of apoptosis has occurred.

2 h, the G2 arrest was 'released', as shown by reduction of the G2M peak. G2M flattened out by 4 h (Figure 6A(v)). By 8 h, the G2M became virtually abolished, while sub-G1 dominated as the only peak in the cell cycle (Figure 6A(vi)). Addition of 1 mM ZnCl_2 (Figure 6B) into the incubations completely prevented any G2M accumulation or destruction. It also prevented sub-G1 accumulation and apoptotic condensations in cytological profiles.

Cytological profile of 'release' from G2 arrest

Release from G2 arrest produced mitotic and apoptotic cell clusters (Figure 7B). The cell suspension that gave the flow cytometry profile shown in Figure 6A(v) produced the Cytospin cytological profile shown in Figure 7B. This contrasted with the control untreated profiles (Figures 7A and 6A(i)) where neither mitotic nor apoptotic cells were

prominent features. The mitotic figures however were not altogether normal. In the cluster of five mitotic cells (indicated by thin arrows in Figure 7B) one had a triradial metaphase plate (arrow-(i)) and the other had disorientated chromosome distribution (arrow-(ii)). Intermingled with the mitotic cells were apoptotic cells with 'lumpy' apoptotic chromatin condensations (arrowheads). Fat arrow points to a dense 'single-ball' chromatin condensation which is also typical of apoptosis (Earnshaw, 1995). Even when genomic degradation had affected virtually the entire population (indicated by the solitary sub-G1 peak in Figure 6A(vi)), there were mitotic cells accompanying apoptotic cells (Figure 7C). These cells were distinctively highlighted by their bright fluorescence while the rest of the cells were non-fluorescent ghost-like images from severe degradation. In addition, there were conjoint expressions of both mitotic chromosomes and apoptotic condensation in the same cell (Figure 7C, fat

Figure 6 (A) Flow cytometric DNA cell cycle profiles of Chang liver cells in normal DMEM growth medium after a V(4) prepulse, in a time-response evaluated by the Multicycle DNA cell cycle analysis programme version 2.53 (Phoenix Flow Systems, Sorrento Valley, San Diego, USA). In the first 2 h after V(4) prepulsing, the G2M peak increased concomitantly with increase in the sub-G1 or apoptotic peak (A(i)-(iv)). Thereafter the G2M peak decreased and flattened out with continued sub-G1 accumulation which reached 92% of the population at 8 h after V(4) prepulsing (A(v)-(vi)). (B) DNA cell cycle profiles of parallel cultures of Chang liver cells in DMEM growth medium supplemented with 1 mM ZnCl_2 , after a V(4) prepulse which also contained 1 mM ZnCl_2 supplement. All profiles were normal.

arrow). The presence of even a single mitotic cell in such devastated circumstances would be striking. Presence of clusters of these mitotic cells became therefore a stunning sight, providing irrefutable evidence of cell division occurring with cell death.

Mitotic cells have been noted in G2-arrested leukaemic HL-60 cells from genistein treatment, although they appear to be from continuing cell division because of surrounding normal cells (Traganos *et al*, 1992). In contradistinction, our mitotic cell clusters were in the exclusive company of either apoptotic cells or nuclear ghosts only (Figure 7C). The

entire cell population at this stage did not have any normal cell that could sustain cell cycling. It was total population cell suicide with the paradoxical presence of mitotic cells, albeit not altogether normal. Mouse fibrosarcoma L929 cells and human leukaemic HL-60 cells have also been shown to undergo transient G2 arrests after γ -irradiation (Abend *et al*, 1996). There the release from G2 arrest is seen associated with either apoptosis or formation of micronucleated cells which must develop from aberrant mitosis, and suggests therefore a similar path of concomitant apoptosis and mitosis.

Figure 7 (A) PI-DNA binding cytological profile of the control state without V(4) prepulsing (obtained by Cytospinning an aliquot of the cell suspension that gave the cell cycle profile of Figure 6A(i)). (B) Experimental state 2 h after V(4) prepulse (obtained by Cytospinning an aliquot of the cell suspension that gave the cell cycle profile of Figure 6A(v)). Mitotic cells are clustered with apoptotic cells. Five mitotic cells are indicated by thin arrows. Arrow-(i) points to a triradiate metaphase plate and arrow-(ii) points to disorientated distribution of metaphase chromosomes. Arrowheads point to nuclei with characteristic 'lumpy' apoptotic chromatin condensations. Fat arrow points to a dense 'single-ball' chromatin condensation which is also typical of apoptosis (Earnshaw, 1995). (C) Cytological profile of the experimental state shown in Figure 6A(vi). The majority of cells are ghost-like with poor PI-DNA binding consistent with the solitary sub-G1 peak seen in flow cytometric DNA profiling. Apart from the ghost-like cells, however, the population was characterized by two other morphologies, viz., (1) apoptotic cells with 'lumpy' chromatin condensations (arrowhead), and (2) mitotic cells. Fat arrow points to an abnormal anaphase cell containing a dense chromatin clump suggesting conjoint expression of mitotic and apoptotic condensations in the same cell.

Materials and Methods

Cell culture

Monolayer cultures of Chang liver cells (ATCC CCL 13) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, USA) with 10% fetal calf serum (Biological Industries, Kibbutz, Israel). Parallel cultures were seeded in 25 cm² flasks (Costar) at 3×10^5 cells/flask from stirred stock suspension and grown to near confluency. Protocol was as previously described (Sit and Wong, 1989).

Nuclear Microscopy

For nuclear microscopy, cultures were seeded on serum-prepulsed Pioloform film anchored by Permout over an aluminium target holder and processed as previously described by us (Sit *et al*, 1993).

Vanadyl(4) prepulse

4 mM vanadyl(4) oxide sulphate (Fluka, Switzerland) in an NH₄Cl buffer (50 mM NH₄Cl, 50 mM glucose) was prepared by dissolving first in H₂O and adjusted to pH 5.5 using 4N NaOH. Cells were given a 30 min prepulse (37°C) in the V(4) buffer, before reincubation in normal DMEM growth medium containing 10% foetal bovine serum. Protocol was as previously described (Sit *et al*, 1996a).

Cellular localization of oxidative reactivity using redox indicator methylene blue

After V(4) prepulsing, cells were fixed in 100% methanol and then stained for 3 min in 3 ml aqueous methylene blue (Gurr, BDH, UK, 0.5 g mg/ml) and air dried. In the reduced state methylene blue is colourless. When oxidized, methylene blue becomes metachromatic and appears purple. Protocol was as previously described (Sit *et al*, 1996b).

Glutathione (GSH) evaluation

Cells prepulsed with V(4) with or without recovery in normal DMEM growth medium had monochlorobimane (MCB, Molecular Probes, Eugene, Oregon, USA) added to their respective incubation medium to a final concentration of 40 μ M (stock solution 40 mM in DMSO) and incubated at 37°C for 15 min. MCB readily entered the cell to form GSH-MCB adducts, a reaction mediated by intracellular glutathione-S-transferase (GST). GSH-MCB fluoresces at the excitation/emission wavelength of 395/470 nm. By itself, MCB is non-fluorescent. Protocol was as previously described (Sit *et al*, 1996b, Kane *et al*, 1993).

Ratiometric BCECF measurement of intracellular pH (pH_i)

For measurement of pH_i, the cells were loaded with 10 μ M BCECF-am (Molecular Probes) with detailed protocol as previously described (Sit *et al*, 1994).

Detection of genomic DNA fragmentation by TUNEL

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) highlights 3'OH termini of DNA fragmentation which are characteristic of apoptotic DNA fragmentation. The manufacturer's protocol (ApopTag, Oncor, USA) was followed. The principle involved

was template-independent addition of dUTP-digoxigenin to 3'OH ends of the fragmented DNA catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT), which was followed by binding with fluorescein-labelled antidigoxigenin antibody for detection. Procedure was previously described (Sit *et al*, 1996b).

Permeabilization of cells for propidium iodide binding and micrococcal nuclease digestion

Single cell suspension of the monolayer culture was obtained by scraping with a Cell Scraper (Costar) and triturated using a 5 ml syringe and a 21 gauge needle. Permeabilization was achieved by resuspension in 1% Bouin's fluid (15 parts of 1.2% aqueous picric acid, five parts of formalin and one part of glacial acetic acid) in Dulbecco's phosphate buffered saline (PBS) for 1 min, and then washed twice with 10 ml of PBS. The permeabilized cells were still alive and when reseeded into the culture flask will grow to confluency, with neutral red viability test (Sit *et al*, 1992) showing increased uptake (27.4% higher than the controls, as evaluated by flow cytometry) which was a reflection not of higher viability but of permeabilization without compromising viability.

Propidium iodide-DNA binding

Cells were permeabilized as described above, and incubated in 1 ml of freshly prepared propidium iodide (PI, Sigma, St Louis, USA) solution (Tris 1.21 g/l, NaCl 0.589 g/l, PI 0.050 g/l) which contained 30 mg ribonuclease A (RNase A, Type IIA, Sigma) per 5 ml PI solution, added just before incubation. Incubation time was 45 min over an ice bath. Cells were pelleted by centrifugation and washed with PBS. For cytological profiling, a 50 μ l aliquot of the cell suspension was Cytospinned onto slides (Cytospin-2, Shandon, UK) for examination under the Reichert-Jung Univar fluorescence microscope at excitation/emission wavelength of 510/630 nm. The rest of the cell suspension was used by the Coulter EPICS ELITE ESP flow cytometer for cell cycle evaluation. The Coulter Elite Workstation programme version 4.0 was used. For DNA cell cycle analysis the bundled Multicycle software version 2.53 was used.

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