Flux of intracellular labile zinc during apoptosis (gene-directed cell death) revealed by a specific chemical probe, Zinquin

PD Zalewski1*, IJ Forbes1, RF Seamark2, R Borlinghaus3, WH Betts1,4, SF Lincoln5 and AD Ward5

[1]Department of Medicine, University of Adelaide, The Queen Elizabeth Hospital, Woodville, South Australia, 5011, Australia, [2]Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, South Australia, 5005, Australia, [3]Division of Microscopy, Carl Zeiss, 73446 Oberkochen, Germany, [4]Rheumatology Unit, The Queen Elizabeth Hospital, Woodville, South Australia, 5011, Australia and [5]Department of Chemistry, University of Adelaide, Adelaide, South Australia, 5005, Australia

Background: The transition metal Zn(II) is thought to regulate cell and tissue growth by enhancing mitosis (cell proliferation) and suppressing the counterbalancing process of apoptosis (gene-directed cell death). To investigate the role of Zn(II) further, we have used a UV-excitable Zn(II)-specific fluorophore, Zinquin. The ester group of Zinquin is hydrolyzed by living cells, ensuring its intracellular retention; this allows the visualization and measurement of free or loosely-bound (labile) intracellular Zn(II) by fluorescence video image analysis or fluorimetric spectroscopy.
Results: Here we show that in cells undergoing early events of apoptosis, induced spontaneously or by diverse agents, there is a substantial increase in their Zinquin-detectable Zn(II). This increase occurred in the absence of exogenous Zn(II) and before changes in membrane permeability, consistent with a release of Zn(II) from intracellular stores or metalloproteins rather than enhanced uptake from the medium. We propose that there is a major redistribution of Zn(II) during the induction of apoptosis, which may influence or precipitate some of the later biochemical and morphological changes.
Conclusions: The phenomenon of Zn(II) mobilization, revealed by Zinquin, presents a new element in the process of apoptosis for investigation and may permit rapid and sensitive identification of apoptotic cells, particularly in those tissues where their frequency is low.

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Introduction

Despite the importance of intracellular Zn(II) in the regulation of diverse cellular events and as an essential cofactor in many metalloenzymes and DNA-binding transcription factors [1], very little is known about how Zn(II) is taken up and used by cells and to what extent intracellular Zn(II) fluxes influence cellular events. Zn(II) is critical in tissue growth and repair [2]. The important pool of Zn(II) is probably a minor, relatively labile fraction, since in Zn(II) deficiency there is only a small change of total cellular Zn(II), as measured by atomic absorption spectroscopy, when growth is severely affected [3]. The bulk of cellular Zn(II) is very tightly bound in metalloenzymes and not readily depleted in Zn(II) deficiency [1–3]. To monitor the more labile Zn(II) pool, we have used a Zn(II)-specific fluorophore, Zinquin [4,5], in a manner analogous to the use of Ca(II)-specific fluorophores (such as Quin-2 and Fura-2) which have enabled changes in intracellular free Ca(II) to be correlated with physiological events [6]. Zinquin is related to the sulphonamidoquinoline TS-Q which forms UV-excitable fluorescent complexes with Zn(II) and has been used as a histochemical stain for Zn(II), largely in tissue sections [7,8]. To use this probe to monitor Zn(II) in unfixed viable cells under physiological conditions, we have synthesized and screened various sulphonamidoquinoline esters. Ester groups have been incorporated so that the probe, following cleavage by intracellular esterases, will carry a net negative charge and be trapped within the cell. One of these esters, ethyl (2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetate, designated Zinquin (Fig. 1 inset), has been used to reveal spatial and temporal changes in labile Zn(II) that accompany mitogenesis of lymphocytes, insulin secretion by pancreatic islet cells and redistribution of Zn(II) from plasma to liver cells in inflammation [4,5,9]. Zinquin has excitation and emission fluorescence peaks at 364 and 485 nm, respectively. It is sensitive to nanomolar concentrations of Zn(II). Fluorescence is specific for Zn(II), apart from a weak reaction with Cd(II), and Zn(II)-dependent fluorescence is unaffected by the presence of mM concentrations of the important biological divalent metal cations Ca(II) and Mg(II) (Fig. 1).

Studies from our laboratory and others (reviewed in [10]) have pointed to a critical role for zinc in the suppression of apoptosis. Apoptosis is a highly regulated active mechanism of cell death that is important in tissue development and homeostasis [11]. Suppression of apoptosis contributes to tumour growth, and many anti-cancer

*Corresponding author.

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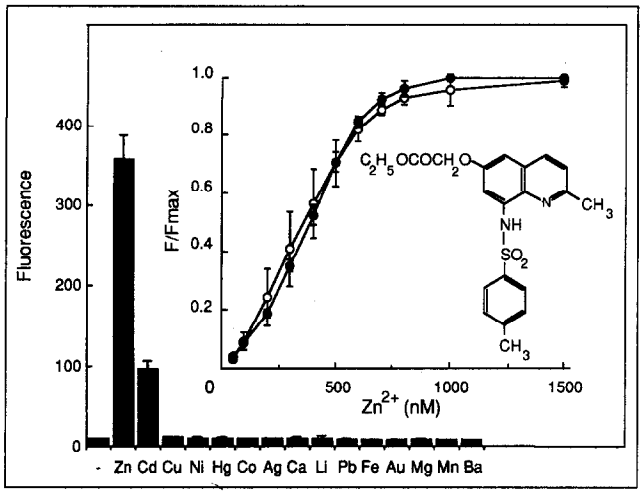

Fig. 1. Structure and metal specificity of Zinquin. Columns show fluorescence of 1.25 μM Zinquin in spectrofluorimeter with 1μM metals (II or III) in HBSS. Line graph shows fluorescence (F) of Zinquin with varying [Zn(II)] as a proportion of maximal fluorescence (F_{max}) in absence (o) or presence (•) of 1mM Ca(II) plus 1mM Mg(II). Inset shows structure of Zinquin.

agents act by inducing tumour cells to apoptose [12]. Apoptosis is distinguished from necrosis (passive cell death) by a unique series of morphological changes including a decrease in cell volume, membrane blebbing, dramatic condensation of chromatin and cytoplasm and budding of the cell contents into membrane-enclosed vesicles (apoptotic bodies) which are eventually phagocytosed [13]. A major biochemical marker of apoptosis is fragmentation of the DNA, first into very large fragments, and later into small fragments which appear as a ladder pattern on gel electrophoresis and correspond to fragmentation of the chromatin at inter-nucleosomal sites [14,15]. Apoptosis is induced in cells by a diverse range of physiological and pharmacological agents. It may also arise spontaneously in cultured cells [16,17], possibly by deprivation of essential growth factors [12]. Supplementation of Zn(II) in cell cultures suppresses apoptosis, while removal of Zn(II), *in vivo* or *in vitro*, enhances apoptosis [10].

Using Zinquin, we have shown an inverse correlation between the level of labile Zn(II) in cells, varied by pretreatment of cells with Zn(II) ionophore or chelator, and their susceptibility towards apoptosis in culture [4]. Here we report that once cells begin to apoptose, there is a substantial increase in their fluorescence with Zinquin, even in the absence of exogenous Zn(II), suggesting a major change in intracellular labile Zn(II) homeostasis.

Results and discussion

Bright Zinquin-fluorescent cells arise spontaneously during culture

A small proportion (usually between 2 % and 10 %) of the cells in growing populations of a range of cell lines fluoresced much more intensely than the rest, when loaded with Zinquin and examined by UV fluorescence microscopy. These brightly-fluorescing cells were seen in all cultured cell populations tested, including pre-B lymphoid Raji cells, B lymphoid SKW.6.4 cells, Jurkat T lymphoid cells, promyelocytic HL60 cells, Lewis lung carcinoma cells and fibroblastoid 3T3 cells. Fig. 2a shows one of these intensely fluorescent cells with other cells fluorescing only weakly in a population of Jurkat cells at day two of subculture.

To estimate the levels of labile Zn(II) in these brightly-fluorescent cells, Jurkat cells were loaded with Zn(II) until they fluoresced with Zinquin to a similar intensity. Loading with Zn(II) was performed by pretreating cells with varying concentrations of the Zn(II) ionophore sodium pyrithione and $ZnSO_4$, as described in Materials and methods. In the absence of $ZnSO_4$, pyrithione did not increase fluorescence, but in the presence of excess Zn(II) there was a linear relationship between the concentration of pyrithione and the average cellular fluorescence intensity as determined by either image analysis or spectrofluorimetry. Cells treated with 1 μM pyrithione

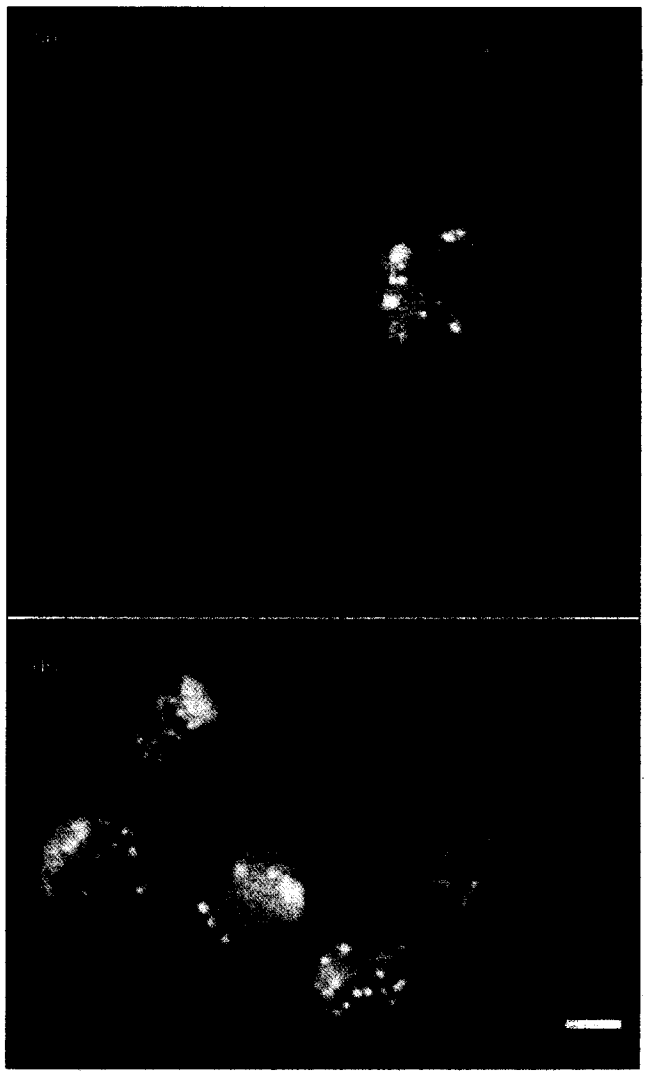

Fig. 2. Zinquin fluorescence of apoptotic and Zn(II) ionophore-loaded cells. **(a)** A brightly-fluorescent apoptotic Jurkat T lymphoblast amongst weakly fluorescent normal cells. **(b)** Brightly-fluorescent normal Jurkat cells after increasing intracellular Zn(II) using sodium pyrithione. Bar represents 10 μm.

had a fluorescence intensity (Fig. 2b) similar to that of the brightly-fluorescing cells occurring in culture (Fig. 2a). Fluorescence of the cells was equated to an amount of Zn(II) by the calibration procedure described in Materials and methods. We calculate that pyrithione-treated cells contain a mean of 202 ± 51 pmol Zn(II) per 10^6 cells, about 14-fold the concentration seen in untreated cells. Therefore, the brightly-fluorescing cells which arise in culture are likely to have a similar level of labile Zn(II), which would be equivalent to about 0.2 fmol per cell.

Apoptotic morphology of Zn(II)-rich cells

When Zinquin-loaded HL60 cells were examined simultaneously by UV fluorescence and phase-contrast microscopy, the brightly-fluorescent cells appeared a vivid blue and one or more morphological features of apoptosis were readily discernible, including membrane blebbing, decrease in cell volume, pyknotic nuclei and presence of apoptotic bodies (Fig. 3a). Although most shrunken apoptotic cells were brightly fluorescent, there was no simple correlation between degree of shrinkage and fluorescence intensity (as determined by image analysis, data not shown). Therefore, the increase in fluorescence does not reflect the labile Zn(II) content of the cell concentrated within a smaller volume. Occasional brightly-fluorescent cells had normal volume, implying that the flux of Zn(II) may begin very early in apoptosis before cell shrinkage.

Brightly-fluorescent apoptotic cells were also seen in other tissues examined. For example, mouse and sheep 32-cell or 64-cell blastocysts often contained between one and four brightly-fluorescent degenerate cells corresponding to the reported frequency of apoptotic cells at this stage of development [18]. Culture of these early embryos and blastocysts with different media that do not sustain normal growth, and in which many of the embryos die, increased the frequency of brightly-fluorescent and apoptotic cells. Under these conditions, brightly-fluorescent degenerating cells were seen even as early as the four-cell embryo (Fig. 3b). Brightly-fluorescent degenerating neurons have also been reported in fixed sections of ischemic, but not normal, rat brain labelled with TS-Q [19]. Whether these neurons were undergoing apoptosis was not studied.

Dual labelling with Zinquin and vital dyes

Brightly-fluorescent apoptotic cells that arose early in culture (for example, at 6 h), still excluded the vital dyes trypan blue or propidium iodide, indicating that increase in Zinquin fluorescence preceded changes in membrane permeability. Later in culture, however, (for example, at 48 h) most of the apoptotic cells failed to exclude vital dyes and the same cells had bright Zinquin fluorescence. Fig. 3c shows a high magnification image of a brightly-fluorescent B lymphoblastoid Raji cell failing to exclude the vital dye propidium iodide. The Zinquin fluorescence (which appears white) is largely in packets around the propidium iodide-labelled (pink–orange) nucleus. When Zn(II) was loaded into chronic lymphocytic leukaemia (CLL) cells or HL60 cells by treatment with the Zn(II) ionophore pyrithione plus $ZnSO_4$, the fluorescence was also patchy (see, for example, Fig. 2b). Nuclei with attached cytoskeletal compo-

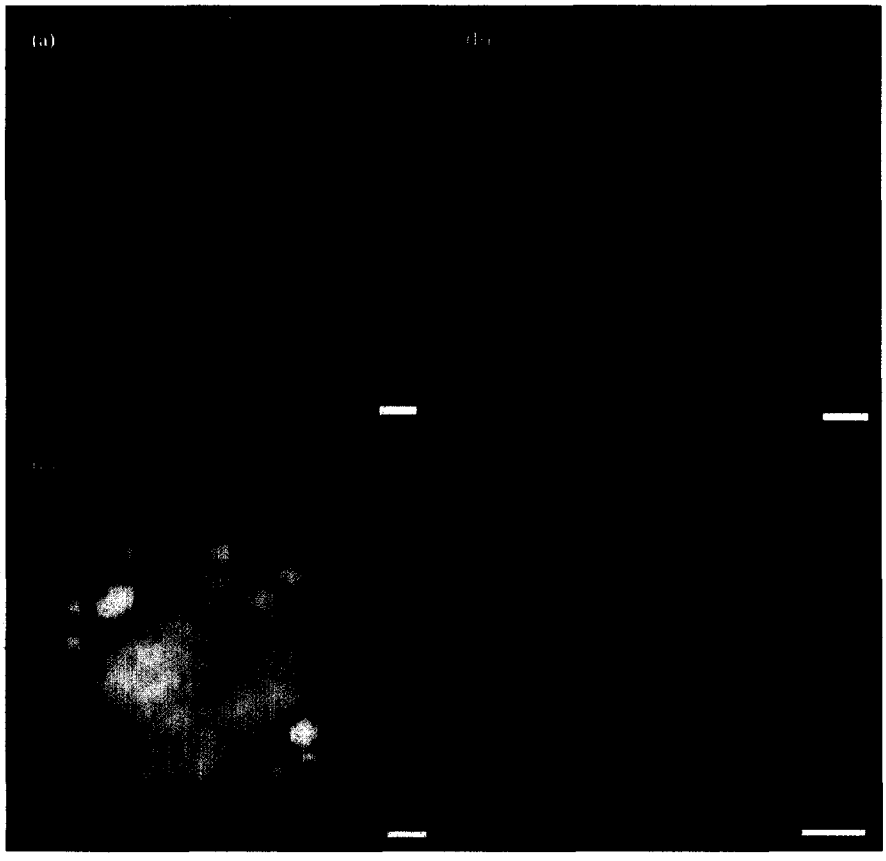

Fig. 3. Morphological features of brightly-fluorescent apoptotic cells. **(a)** A photomicrograph taken by simultaneous illumination of Zinquin-loaded HL60 cells with UV fluorescence and transmitted light. Brightly-fluorescent cells appear blue and have the morphology of apoptotic cells. Bar represents 10 μm. **(b)** Brightly-fluorescent degenerate cell in an *in vitro* fertilized four-cell mouse embryo, cultured under sub-optimal conditions. Bar represents 5 μm. **(c)** Apoptotic Raji B lymphoblastoid cell showing patches of bright Zinquin fluorescence (white) around a propidium-iodide-labelled (orange) nucleus. Bar represents 2 μm. **(d)** Confocal scanning UV-laser fluorescence microscopy of a group of three normal SKW.6.4 B lymphoblastoid cells (left) and two apoptotic brightly-fluorescent cells (right). The figure is a pseudo-coloured superimposed image: green depicts morphology (by differential interference contrast microscopy) and orange–yellow shows Zinquin fluorescence. Note the absence of labelling in nuclei and blebs and the intense punctate cytoplasmic fluorescence. Bar represents 10 μm.

nents, isolated from these fluorescent cells by a detergent lysis technique, also had patches of fluorescence around their periphery. The patchy nature of the cytoplasmic fluorescence and its persistence even after loss of membrane integrity in apoptotic cells indicate either a reasonably tight association of the labile Zn(II) with a macromolecular complex (such as the cytoskeleton) or localization of Zn(II) within membrane-enclosed vesicles.

Confocal scanning UV-laser microscopy of Zn(II)-rich cells

A confocal scanning laser fluorescence microscope fitted with a UV-laser (Carl Zeiss) [20] was used in further investigations. Fig. 3d shows a pseudo-coloured image taken with this microscope of two brightly-fluorescent (yellow–orange colour) apoptotic B lymphoblastoid cells on the right and three normal cells on the left; the morphology of the cells (by differential interference contrast microscopy) is depicted in green. While the cytoplasm of the apoptotic cells was intensely fluorescent, both the nuclei and the membrane blebs were devoid of fluorescence. The lack of fluorescence in the nuclei indicates that either there is no loosely bound Zn(II) in the nuclei available to bind with Zinquin, or that Zinquin does not reach the cell nucleus because it is hydrolyzed by the cytoplasmic esterases and thus unable to penetrate intracellular membranes such as the nuclear membrane [4,5]. The image in Fig. 3d corresponds to one of the middle of the series of the optical sectioning study shown in Fig. 4. The increase in fluorescence intensity as the laser was scanned deeper into the cells and the subsequent decrease in fluorescence as the other side of the cells was approached, indicate that the Zinquin fluorescence of the apoptotic cells was indeed intracellular rather than representing Zn(II) associated with the plasma membrane.

Increase in frequency of Zn(II)-rich cells during induction of apoptosis

Confirmation that increase in Zinquin fluorescence is a feature of apoptosis was provided by studies in which Zinquin fluorescence was measured before and after induction of apoptosis in cells *in vitro*. Human CLL cells have a relatively high rate of spontaneous apoptosis when cultured *in vitro* [17]. There was a similar increase in Zn(II)-rich cells. Thus, a population of freshly isolated CLL cells, prepared by a Ficoll–Hypaque density centrifugation technique which removes dead cells, lacked brightly-fluorescent and apoptotic cells; when the cells were cultured for 22 h, brightly-fluorescent cells appeared in substantial numbers (37 % of the total cells in a typical experiment); these cells had morphological features of apoptosis. Culture in the presence of 100 nM 12-*O*-tetradecanoyl phorbol 13-acetate, a potent suppressor of apoptosis in these cells [17], resulted in only 7 % of the cells being strongly fluorescent. On the other hand, the apoptosis-enhancing agent colchicine (50 μM) [17] induced 88 % of the cells to become brightly fluorescent with Zinquin.

Similarly, treatment of HL60 cells overnight in culture with the apoptosis-inducing agent etoposide (50–100 μM), an inhibitor of topoisomerase II [21], increased brightly fluorescing cells (≥ 30 grey scale units

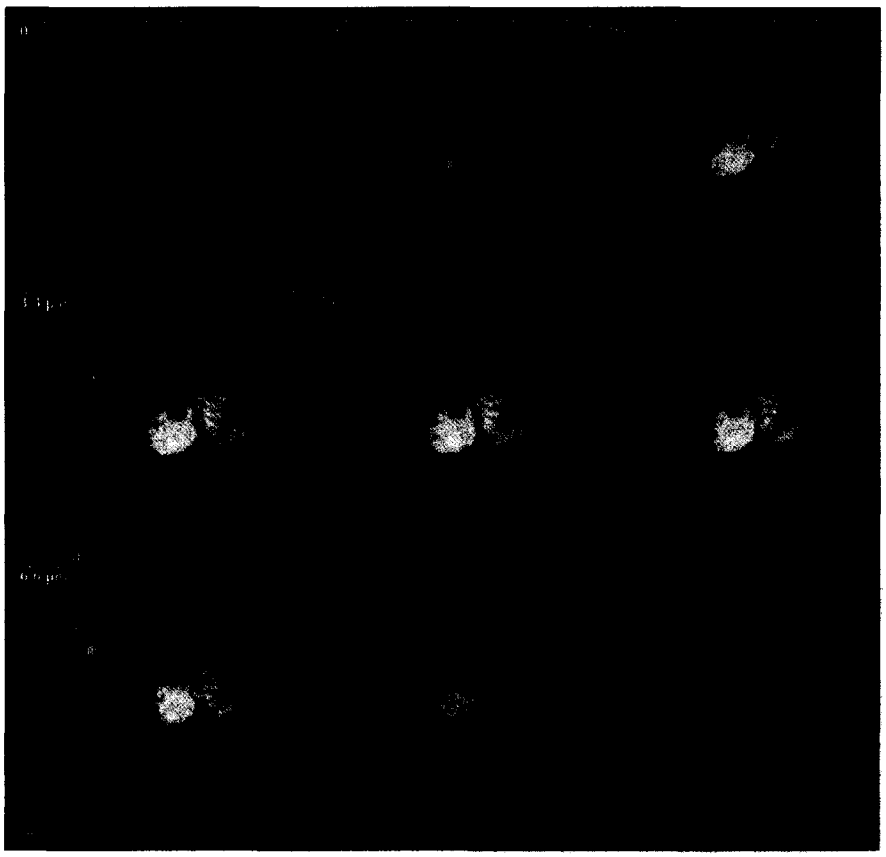

Fig. 4. Optical sectioning of fluorescent cells by scanning confocal UV-laser microscopy. Sequential fluorescent images of the same cells as Fig. 3d taken at focal planes separated by 1.1 μm. Note the increased fluorescent signal at sections midway through cells. Bar represents 10 μm.

by image analysis) from a mean of 4 % to 46 % of the cells; average cellular fluorescence intensity increased from a mean of 13.7 to 30.6 grey scale units as determined by image analysis (Fig. 5b) and from 101.3 to 131.9 fluorescence units as determined by spectrofluorimetry of cells in cuvettes. These values represent substantial increases in fluorescence (significant at $p < 0.001$) on their respective scales. There was a wide spread of intensities (Fig. 5b), consistent with a gradual accumulation of intracellular labile Zn(II). A smaller, although significant ($p < 0.01$), increase in fluorescence was obtained with 25 μM etoposide. Labile Zn(II)-rich cells coincided with cells with apoptotic morphology. Another apoptosis-inducing agent, H_2O_2 (1 mM), also increased the proportion of Zn(II)-rich cells from a mean of 4 % to 28 %; average cellular fluorescence intensity was increased from a mean of 13.7 to 25.1 grey scale units (by image analysis, significant at $p < 0.001$, Fig. 5c) and from 101.3 to 131.2 fluorescence units (by spectrofluorimetry, significant at $p < 0.001$). With 0.1 mM H_2O_2, mean cellular fluorescence increased to 20.0 grey scale units and 117.6 spectroscopic fluorescence units ($p < 0.01$). Similar results were obtained with other types of cells (for example, Raji cells, thymocytes, CLL cells) treated with the apoptosis-inducing agents etoposide (50–100 μM), H_2O_2 (0.1–1 mM), colchicine (100 μM) or dexamethasone (3 μM).

By contrast, cell necrosis induced by a high concentration (80 mM) of H_2O_2 or by 50 μM digitonin was not accompanied by increased Zinquin fluorescence; rather, the fluorescence declined to values below controls. For example, 80 mM H_2O_2 caused a 65.7 % decrease in average cellular fluorescence intensity from 13.7 to 9.0 grey scale units. Furthermore, these cells lacked the morphological features of apoptosis, failed to exclude trypan blue and did not contain fragmented DNA.

Effect of the Zn(II) chelator TPEN

To confirm that the bright fluorescence was due to Zn(II), we examined the effect of the intracellular Zn(II) chelator TPEN (N,N,N',N'-tetra*kis*-(2-pyridylmethyl)-ethylenediamine), which has a much higher affinity for Zn(II) than Zinquin [4]. Rat thymocytes were treated overnight with dexamethasone (3 μM) to induce apoptosis. One aliquot of cells was then treated with TPEN (100 μM) for 2 h before loading with Zinquin. The remaining cells were incubated without TPEN and then loaded with Zinquin. TPEN eliminated the bright Zinquin fluorescence, decreasing the proportion of bright cells (≥ 30 grey scale units) from 36 % to 0 % and the mean cellular fluorescence intensity from 29.7 ± 14 to 16.8 ± 1 grey scale units. Therefore, the enhanced fluorescence is due to Zn(II) rather than to some altered behaviour of the probe in these cells.

Enhanced Zn(II) uptake or release?

Except for the experiment described in Fig. 2, the medium in these experiments contained no added Zn(II) and the only Zn(II) present was that due to the 10 % foetal calf serum (about 2 μM). Supplementation of the medium with 25 μM $ZnSO_4$ did not further enhance the fluorescence intensity or the frequency of brightly-fluorescent cells. This suggests that the appearance of labile Zn(II)-rich cells was not the result of an influx of Zn(II) from the medium.

A labile Zn(II) content of about 200 pmol/10^6 apoptotic lymphoid cells (see above) would be similar in magnitude to the total Zn(II) content of lymphoid cells (~150–200 pmol/10^6 cells as measured by atomic absorption spectroscopy [4,22]). The increase in fluorescence in apoptosis could therefore represent a mobilization or release of the major pools of intracellular Zn(II) that were previously

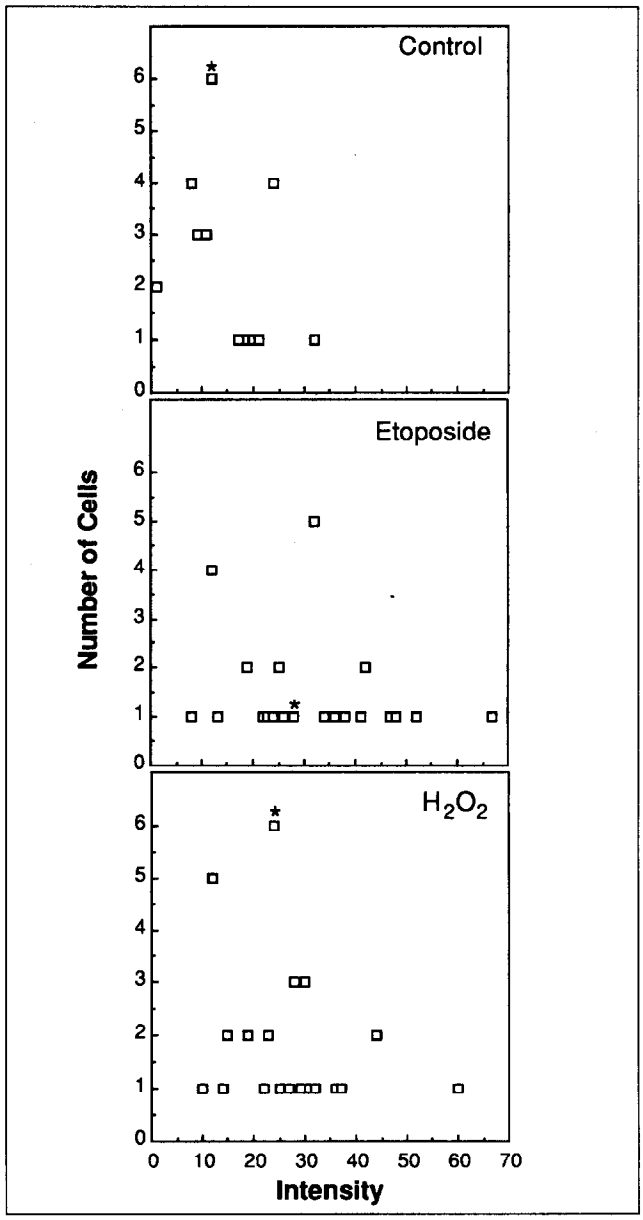

Fig. 5. Measurement of increase in fluorescence of cells during apoptosis. Graph shows frequency of HL60 cells with given Zinquin fluorescence intensity after incubation overnight in medium without addition (Control) or with etoposide (50 μM) or H_2O_2 (1 mM). Ten images (each containing five to ten cells) were analyzed. The asterisk indicates median fluorescence intensity. The median for the control group was significantly different ($p < 0.001$) from the medians of treated samples.

unavailable for interaction with Zinquin. Zinquin detects only about 10 % to 25 % of total cellular Zn(II) in healthy lymphocytes [4]. The remaining Zn(II) will include that sequestered in intracellular compartments that are impermeable to Zinquin (such as the nucleus [4,5]), as well as Zn(II) bound to metalloproteins. Metalloproteins have much higher affinities for Zn(II) (with K_ds typically ranging from 10^{-12} to 10^{-13} M) than does Zinquin (Zinquin forms both 1:1 and 2:1 Zinquin–Zn(II) complexes with respective stepwise K_ds of $3.7 \pm 0.6 \times 10^{-7}$ and $8.5 \pm 1.6 \times 10^{-8}$ M in physiological media). Cellular Zinquin fluorescence could thus be increased to the extent that it is in apoptotic cells if there was a substantial release of Zn(II) from metalloproteins.

Such a release could be triggered by a change in the redox state of the cell, since Zn(II) is most often bound to protein via Zn–S thiolate bonds [23,24], which are highly labile to oxidation, releasing Zn(II) [25,26]. Oxygen activation via redox cycling has been implicated as a central event in apoptosis caused by diverse stimuli including several cytotoxic drugs used in chemotherapy [27,28]. The protein product of the *bcl-2* proto-oncogene, which is involved in physiological suppression of apoptosis, appears to act by blocking either the generation or action of oxyradicals that are generated early in apoptosis [29]. In support of this hypothesis, H_2O_2 caused a rapid increase in Zinquin fluorescence (Fig. 5).

Relationship between release of Zn(II) and other events in apoptosis

It is not yet clear whether the changes in Zn(II) are a cause or consequence of apoptosis. This question could be answered by an exploration of the temporal relationship between alteration in intracellular labile Zn(II) and the onset of apoptotic cell death. High concentrations of exogenous Zn(II) suppress apoptosis at a stage beyond the early chromatin cleavage to high molecular weight (50 and 300 kbp) fragments but before the late chromatin cleavage associated with internucleosomal DNA fragmentation [15]. Therefore, we examined the temporal relationship between increase in fluorescence and inter-nucleosomal DNA fragmentation. When HL60 cells were treated with etoposide, the kinetics of the rise in average cellular fluorescence paralleled the increase in DNA fragmentation (Fig. 6). Since DNA fragmentation is due to a nuclear endonuclease which is normally suppressed by binding of Zn(II) [10,30,31], the release of Zn(II) from the endonuclease early in apoptosis may facilitate DNA fragmentation. This may also provide an explanation for the apparent contradiction between our findings of increased intracellular Zn(II) in apoptosing cells, and the previous finding that exogenously added Zn(II) inhibits apoptosis [10,30,31]. Addition of exogenous Zn(II) may prevent the release of Zn(II) from the endonuclease, as well as from other important Zn(II)-dependent enzymes and macromolecules (as discussed below), and hence prevent the initiation of apoptosis. Whether this explanation is correct remains to be explored.

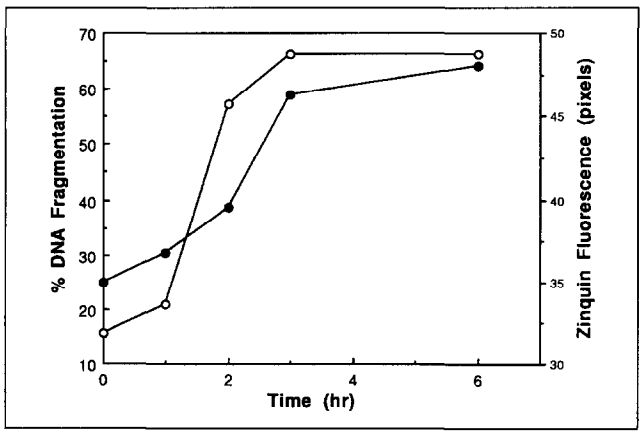

Fig. 6. Kinetics of increase in Zinquin fluorescence and DNA fragmentation. Graph shows similar kinetics of increase of average fluorescence intensity (solid circles; determined by fluorescence spectroscopy) and % DNA fragmentation (open circles) in cells treated with 50 μM etoposide. Cells with apoptotic morphology at different times constituted 10 % (zero time), 21 % (1 h), 48 % (2 h), 69 % (3 h) and 54 % (6 h) of total cells.

Other components of the apoptotic cascade which may be influenced by release of Zn(II) include a cytosolic factor that accumulates in cells arrested in mitosis [32] and poly(ADP-ribose) polymerase, a Zn(II)-dependent negative regulator of apoptosis [33]. Recently, Rice and colleagues [33] showed that treatment of cells with C-nitrosoligands will cause both apoptosis and the ejection of Zn(II) from poly(ADP-ribose) polymerase. Such ejected Zn(II), detected by NMR spectroscopy, may be part of the flux of Zn(II) detectable by Zinquin in apoptotic cells.

Released Zn(II) may also constitute a flux sufficient to disturb other cellular structures and processes. For example, it may contribute to the changes in the cytoskeleton and membrane during apoptosis, since Zn(II) binds to and stabilizes these structures [3]. Treatment of cells with high concentrations of Zn(II) causes extensive cross-linking of the membrane/cytoskeletal proteins and renders them insoluble in non-ionic detergents [34], reminiscent of the detergent-insoluble, cross-linked shells that enclose apoptotic bodies and preserve their integrity prior to phagocytosis [35].

Significance

The use of chemical fluorophores for studying Zn(II) in living cells under near-physiological conditions is expected to advance our understanding of the normal role of this metal ion, much as the Ca(II) fluorophores have revolutionized our understanding of the physiology of this cation. This study may represent the first example in which an intracellular labile Zn(II) flux is correlated with a major pathological event. Apoptosis or programmed cell death is a key event in the development of organisms and tissue homeostasis, and is characterized by the fragmentation of DNA, condensation of chromatin and loss of nucleoli. It requires the active participation of

intracellular components such as enzyme synthesis. Apoptosis is distinctly different from necrosis or accidental cell death, where cell membranes are often damaged first, resulting in cell lysis. In view of the crucial, yet poorly understood, role of Zn(II) in regulation of apoptosis and the potential for Zinquin to identify very early stages in apoptosis, the intracellular fluxes of Zn(II) described here offer a new parameter for study of this fundamental cellular event. It will be important to determine whether there are instances in which apoptosis proceeds in the absence of a Zn(II) flux. This is particularly relevant to current investigations that aim to determine whether similar mechanisms mediate the diverse forms of physiological, immune and pathological apoptosis. The studies in this paper focus largely on apoptosis in cultured cell populations. It is now necessary to look for similar Zn(II) flux in cells undergoing apoptosis in vivo, such as in the prostate after androgen ablation, and in intestinal epithelial cells as they migrate up the colonic crypt. Zinquin may also prove to be a useful reagent in identification of early apoptotic cells, particularly in tissues where the frequency of these cells is very low. Finally, the identification of a substantial efflux of Zn(II) in apoptosis has important implications for recent studies (see, for example, [36]) that have attempted to correlate Ca(II) flux with apoptosis using Fura-2, Quin-2 and some other fluorophores that are also influenced by Zn(II).

Materials and methods

Materials

Zinquin (A.D. Ward, Dept of Chemistry, University of Adelaide, South Australia 5005) was stored at 5 mM in DMSO at –20 °C. Other reagents and their suppliers were $ZnSO_4$, colchicine, sodium pyrithione, 12-O-tetradecanoyl phorbol 13-acetate, NP40, digitonin, H_2O_2 and TPEN (Sigma), propidium iodide (Molecular Probes), etoposide and dexamethasone (David Bull Labs). All other compounds used were reagent-grade. Ultra pure water (Permutit) was used for making solutions.

Preparation of cultured cells

Thymocytes were prepared from Sprague Dawley rats [4]. All animal procedures were approved by The University of Adelaide Animal Ethics Committee. Human CLL cells were obtained from patients undergoing leukapheresis [4]. Before use, cells were thawed, washed and dead cells removed by Ficoll-Hypaque density centrifugation. Cells were washed and suspended in Hanks' balanced salt solution (HBSS) pH 7.4, containing 1.3 mM Ca^{2+}, 0.9 mM Mg^{2+} and 4.2 mM sodium bicarbonate (Gibco). Cells were cultured in RPMI 1640 supplemented with 10 % foetal bovine serum (ICN Biologicals), 2 mM L-glutamine, 24 mM sodium bicarbonate (BDH), 25 mM Hepes (BDH) and 160 μg ml^{-1} gentamycin sulphate (Delta West). Cell viability was monitored by trypan blue or propidium iodide exclusion and phase contrast microscopy. Propidium iodide and trypan blue were added to final concentrations of 32 μM and 0.1 % (w/v), respectively.

Preparation of embryos and blastocysts

Mouse oocytes were collected from superovulated 6-week-old (CBA x C57F1) females. Sheep oocytes were recovered from ovaries obtained from a local abattoir. Oocytes were matured *in vitro* in groups in 15-mm plastic culture dishes containing 400 μl of medium (TCM 199) supplemented with 10 % foetal calf serum (FCS), and gonadotrophins (5 μg ml^{-1} FSH, Folltrophin, 5 μg ml^{-1} LH, Vetrapharm, Canada), 35 mg ml^{-1} sodium pyruvate (Sigma) and 1 μg ml^{-1} estradiol-17β (Sigma) at 38.5 °C in 5 % CO_2 in air atmosphere for 24–26 h. *In vitro* fertilization was achieved by transferring 10–15 *in vitro* matured oocyte cumulus complexes to 50 μl drops of bicarbonate-buffered Tyrode's salt solution supplemented with 2 % sheep serum under light paraffin oil (BDH) and inseminating with 50–70 x 10^3 mobile spermatozoa. The insemination droplets were then incubated at 38.5 °C in 5 % CO_2 in air atmosphere for 24 h. Embryos and blastocysts were hand-picked and aliquotted into fixed volumes of buffer.

Loading of cells with Zn(II) using pyrithione

Cells (5 x 10^6 ml^{-1}) were treated for 40 min at 37 °C with 25 μM $ZnSO_4$ and different concentrations of sodium pyrithione (0.1–5 μM) [4], then washed 3x with HBSS to remove extracellular Zn(II) before labelling with Zinquin.

Spectrofluorescence measurements with Zinquin

For studies of interaction of Zinquin with metal ions in solution, Zinquin was added to cuvettes containing known amounts of Zn(II) or other metal ions. For measurements of cellular Zinquin fluorescence, cells in suspension (5–10 x 10^6 cells ml^{-1}) were incubated with Zinquin (final concentration 25 μM) in HBSS for 30 min at 37 °C. Cells were washed 3x with HBSS and suspended at 2–5 x 10^6 ml^{-1} in HBSS in cuvettes. Fluorescence was measured at room temperature in a Perkin Elmer LS 50 luminescence spectrophotometer at excitation and emission wavelengths of 370 nm and 490 nm, respectively (slit widths 10 nm). Fluorescence of unloaded cells (due to autofluorescence and light scattering) was subtracted from readings to derive Zinquin-dependent fluorescence. In some experiments, cells were lysed in the cuvettes by addition of digitonin to 50 μM and the released Zinquin was saturated with $ZnSO_4$. This fluorescence was used to determine the amount of Zinquin loaded into the cells [4], which was calculated to be 3 μM. It was then possible to calculate the amount of labile Zn(II) in cells using a standard curve derived by titration of increasing amounts of $ZnSO_4$ into a solution of 3 μM Zinquin until the fluorescence was equivalent to that obtained with Zinquin-labelled cells. The medium for this titration was a solution with an ionic constitution resembling that of lymphocyte cytosol (125 mM K(I), 20 mM Na(I), 1 mM Mg(II), in Hepes buffered to pH 7.05) and supplemented with 0.1 mg ml^{-1} bovine serum albumin.

Fluorescence microscopy and image analysis

Fluorescence of individual cells was visualized using an Olympus microscope with a UVB dichroic mirror (Olympus, Tokyo) for low wavelength excitation and connected to a CCTV video color camera and computer work station. Images were captured and fluorescence was quantified using the Video Pro Image Analysis System (Leading Edge Pty Ltd, S. Australia). Photomicrographs were taken directly or from computer images. Mean fluorescence intensity of individual cells was computed with the Video Pro program. Both fluorescence and corresponding transmitted light images were captured. On the latter, the perimeters of individual cells (at least 50 per group on

5–10 individual images) were outlined as an overlay. The overlay was superimposed on the corresponding fluorescence image and the fluorescence intensity of the outlined areas was measured; background illumination was subtracted. The program was previously calibrated by comparing increase in average cellular fluorescence intensity, determined as above, with increasing concentration of intracellular Zn(II), using lymphocytes treated with varying concentrations of Zn(II) plus ionophore [4].

Confocal UV-laser scanning fluorescence microscopy

Confocal fluorescence microscopy studies were done at the Carl Zeiss factory (Oberkochen, Germany), using a standard Zeiss confocal scanning laser microscope (LSM 410 UV). In addition to the standard laser lines (488, 543, 568 and 633 nm), this instrument was equipped with an argon ion UV laser (wavelength of 364 nm), with variable output power of 20-100 mW (Spectra Physics, Type 2016 UV, USA). This laser was used for excitation of Zinquin. Unlike a former model [20], this inverted microscope was equipped with a motorized and computer-controlled device to influence the UV beam to compensate for different UV-focus deviations with different objective lenses.

Isolation of nuclei/cytoskeleton

A crude nuclei/cytoskeletal fraction was prepared by NP40 extraction of HL60 cells (slightly modified from the method in [37]). Cell pellets (~2 x 10^7 cells) were resuspended in 25 mM Tris pH 8.0 containing 25 mM KCl, 7.5 mM $MgSO_4$ and 30 % w/v sucrose (TKM buffer). NP40 was then added to a final concentration of 0.25 %. After 15 min at 4 °C, detergent-insoluble material (nuclei + cytoskeletal components) was pelleted at 1 000 g, washed 3x and resuspended in TKM buffer.

DNA fragmentation

DNA fragmentation was assayed in supernatants of cell lysates spun at 15 000 g by detection of DNA fragments using diphenylamine as described in [17]. Ladder patterns of DNA fragmentation were examined by ethidium bromide after extraction of DNA and electrophoresis on 1 % agarose [17].

Expression of results

Data are expressed as means of replicate samples plus or minus standard deviations. Statistical significance was determined with the *t*-test.

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