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Manganese induces apoptosis of human B cells: caspase-dependent cell death blocked by Bcl-2

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Abstract

Manganese ions block apoptosis of phagocytes induced by various agents. The prevention of apoptosis was attributed to the activation of manganous superoxide dismutase (Mn-SOD) and to the antioxidant function of free Mn^{2+} cations. However, the effect of Mn^{2+} on B cell apoptosis is not documented. In this study, we investigated the effects of Mn^{2+} on the apoptotic process in human B cells. We observed that Mn^{2+} but not Mg^{2+} or Ca^{2+}, inhibited cell growth and induced apoptosis of activated tonsilar B cells, Epstein Barr virus (EBV)-negative Burkitt's lymphoma cell lines (BL-CL) and EBV-transformed B cell lines (EBV-BCL). In the same conditions, no apoptosis was observed in U937, a monoblastic cell line. Induction of B cell apoptosis by Mn^{2+} was time- and dose-dependent. The cell permeable tripeptide inhibitor of ICE family cysteine proteases, zVAD-fmk, suppressed Mn^{2+}-induced apoptosis. Furthermore, Mn^{2+} triggered the activation of interleukin-1β converting enzyme (ICE/caspase 1), followed by the activation of CPP32/Yama/Apopain/caspase-3. In addition, poly-(ADP-ribose) polymerase (PARP), a cellular substrate for CPP32 protease was degraded to generate apoptotic fragments in Mn^{2+}-treated B cell lines. The inhibitor, zVAD-fmk suppressed Mn^{2+}-triggered CPP32 activation and PARP cleavage and apoptosis. These results indicate that the activation of caspase family proteases is required for the apoptotic process induced by Mn^{2+} treatment of B cells. While the caspase-1 inhibitor YVAD was unable to block apoptosis, the caspase-3 specific inhibitor DEVD-cmk, partially inhibited Mn^{2+}-induced CPP32 activation, PARP cleavage and apoptosis of cells. Moreover, Bcl-2 overexpression in BL-CL effectively protected cells from apoptosis and cell death induced by manganese. This is the first report showing the involvement of Mn^{2+} in the regulation of B lymphocyte death presumably via a caspase-dependent process with a death-protective effect of Bcl-2.

Keywords: Apoptosis; manganese; human B cells; caspase

Abbreviations: PI, propidium iodide; DMSO, dimethyl sulfoxide; PFA, paraformaldehyde; ICE, interleukin-1β-converting enzyme; PARP, poly-(ADP-ribose) polymerase; zVAD-fmk, Z-Val-Ala-DL-Asp-fluoromethylketone; DEVD-cmk, Asp-Glu-Val-Asp-chloromethylketone; BL-CL, Epstein Barr virus negative Burkitt's lymphoma cell lines; EBV-BCL, Epstein Barr virus (EBV)-transformed B cell lines; ROS, reactive oxygen species; SAC, staphylococcus aureus cowan 1 strain (pansorbin)

Introduction

Apoptosis or programmed cell death is a physiological process leading to methodical destruction of the cell. This phenomenon is observed in various situations and is critical during organ development and tissue homeostasis.[1,2] It can be initiated by several physiological and pathological processes or in response to chemical or physical treatments.[3] Morphologically, apoptosis is characterized by loss of cell volume, plasma membrane bleeding, cell shrinkage, expression of phosphatidylserine on the outer leaflet of the cell membrane, chromatin condensation and DNA fragmentation into oligo-nucleosome-sized fragments.[4,5]

Apoptosis plays a key role in the maintenance of cell population homeostasis including B lymphocytes.[6] Diverse stimuli, including cytokines, soluble factors, oxidative stress, drugs and ions can modulate lymphocyte activation and lead to apoptosis (reviewed in[7]). Apoptosis of B cells can be spontaneous[8] or it can be induced by several mechanisms: Ag receptor cross-linking,[9–11] APO-1 (CD95) ligation,[12] increase of the intracellular Ca^{2+} by ionomycin,[13] cytokines such as IL-10 and IL-5[14,15] and chemotherapic agents.[16,17] Survival factors, particularly interleukins IL-2, IL-4, IL-6, IL-8, IL13, INF-α and INF-γ,[18–24] and CD6 and CD40 antigens[25,26] prevent apoptosis of B leukemia cells. Among these stimuli, CD40 increases the survival of B cells by upregulating the expression of bcl-2 protein.[27] There is accumulating evidence suggesting that members of the ICE/CED-3 protease family are involved in the execution phase of apoptosis.[28,29] These cysteine-related proteases, named caspases, are synthesized as inactive proenzymes which are activated by cleavage at specific aspartate sites. Activation of the caspase cascade results in changes in the plasma-membrane, mitochondria and nucleus.[7] Apoptosis is a multistep process involving the sequential activation of different caspases. In the Fas activation system, such a multistep process has been proposed: caspase-8 initiates the activation of caspase-1 which potentiates the activity of the protease machinery including caspase-3 and caspase-7.[30]

Various lines of evidence suggest that divalent cations are involved in the regulation of apoptosis in mammalian

cells. For example, depletion of Zn^{2+} enhances apoptosis[31] while its supplementation inhibits internucleosomal fragmentation and apoptosis *in vitro* and *in vivo*.[32,33] Recent studies have shown the contribution of Zn^{2+} to the inhibition of caspase-3 activity.[34,35] Another divalent cation Mn^{2+} down-regulates apoptosis activation in some cell types and up-regulates it in others. Mn^{2+} at low concentration ($<10^{-7}$ M), interfering with oxidative products, acts as an antioxidant and inhibits the apoptosis of neutrophils.[36,37] In contrast, at high concentration (>0.5 mM), Mn^{2+} induces apoptosis in cultured cerebellar granular neurons and in PC12 cells.[38] Thus Mn^{2+} appears to be able to counteract oxidative stress and modulate apoptosis depending on the cell type and concentration used.

In this report we show that 100 μM Mn^{2+} suppresses proliferation and induces apoptosis of human B cells. This effect was observed with normal tonsillar B cells and cell lines such as EBV-negative Burkitt lymphoma B cells and EBV-transformed B cells. Mn^{2+}-induction of apoptosis was dose-dependent and triggered the activation of ICE followed by CPP32 activation and *in vivo* cleavage of poly (ADP-ribose) polymerase (PARP). Our results suggest that Mn^{2+} initiates a death signaling caspase cascade leading to B cell apoptosis.

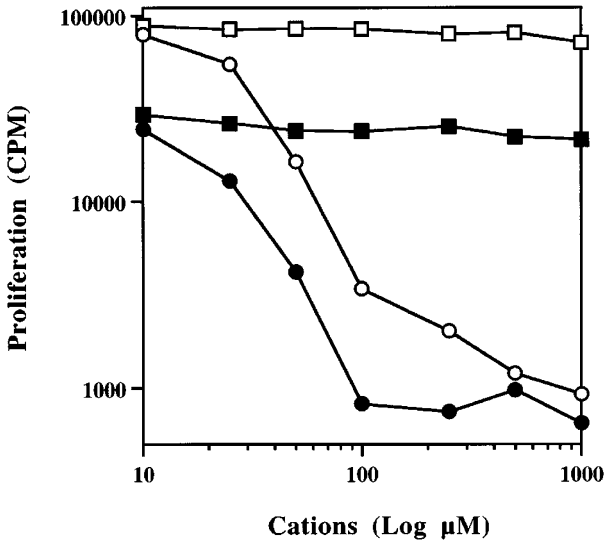

Figure 1 Effects of Mn^{2+} and Mg^{2+} on proliferation of normal B cells and the EBV-B cell line. Tonsillar B cells (2×10^5 per well; filled symbols) stimulated with 1/10000 SAC were cultured for 72 h and DN1 cell line (5×10^4 per well; open symbols) for 48 h at 37°C in the presence of various concentrations of divalent cations (Mn^{2+}, circle and Mg^{2+}, square). [^{3}H]thymidine incorporation was assessed during the last 6 h of culture. All determinations were made in triplicate. The data shown are representative of three separate experiments

Results

Manganese induces growth inhibition and apoptosis in human B cells

To examine the effect of divalent cations on B lymphocyte proliferation and apoptosis, we cultured B cells in the presence of different cations. The data in Figure 1 indicate that Mn^{2+} but not Mg^{2+} dose-dependently inhibited the proliferation of SAC-activated normal B cells and DN1 EBV-BCL. The growth inhibition of tonsillar B cells and the DN1 cell line was almost complete at 100 μM $MnCl_2$. We subsequently investigated whether Mn^{2+}-mediated growth inhibition correlated with an induction of apoptosis. We treated BL41 BL-CL with various cations for 48 h and apoptosis was determined by three different methods to assess whether the cell death observed was indeed apoptosis (Figure 2). Analysis of cell dot-blot light scatter profiles by flow cytometry showed that 100 μM $MnCl_2$ induced a cell shrinkage (63 *vs* 19% in control cells, Figure 2A) whereas $MgCl_2$ at the same concentration had no effect. We also assessed chromatin condensation in the presence and absence of Mn^{2+} following the hypodiploid DNA peak by flow-cytometry: 35% of the nuclei in Mn-treated cells but only 2–3% in control and Mg-treated cells were hypodiploid (Figure 2B). Finally, $MnCl_2$ induced phosphatidylserine expression on the outer leaflet of the cells (67 *vs* 15% in control or Mg-treated cells; Figure 2C). To establish more apoptotic criteria in Mn^{2+}-induced programmed cell death, we next analyzed other morphologic nuclear manifestation of apoptosis: nuclear fragmentation and internucleosomal DNA cleavage. Figure 3 shows the typical condensed and fragmented nuclear morphology accompanied by the characteristic ladder pattern of DNA fragmentation of BL41 cells undergoing apoptosis after 48 h treatment with 100 μM $MnCl_2$. Similar results were observed with other cell lines as DN1 or Ramos (data not shown). Thus, inhibition of human B cell proliferation by Mn^{2+} is clearly associated with an apoptotic process.

Apoptosis of DN1 cells induced by Mn^{2+} was dose- and time-dependent as detected by flow cytometric analysis of hypodiploid nuclei (Figure 4A and B). Treatment of DN1 EBV-BCL with $MnCl_2$ 48 h induced apoptosis with maximum effect at doses (100–500 μM) close to those giving complete growth inhibition. $MnCl_2$-induced apoptosis was first observed at 15 h and reached a plateau at 24 h.

We next analyzed the effect of Mn^{2+} on a panel of seven human B cell lines, tonsillar B cells and a monoblastic cell line U937 (Table 1). In BL-CL (BL41, Ramos, CA46 Raji and Daudi), EBV-BCL (DN1 and GL1) and normal B cells, Mn^{2+} (250 μM) inhibited growth, decreased cell viability and induced apoptosis at 48 h. In contrast, Mn^{2+} had no effect on proliferation or apoptosis of the monoblastic cell line U937.

Apoptosis triggered by Mn^{2+} involves activation of ICE, CPP32 and cleavage of PARP

Activation of the cascade of proteolytic caspases is a pathway of apoptosis in diverse biological systems. Members of the ICE caspase family are synthesized as proforms, which are proteolytically cleaved and activated during apoptosis. We investigated whether caspases,

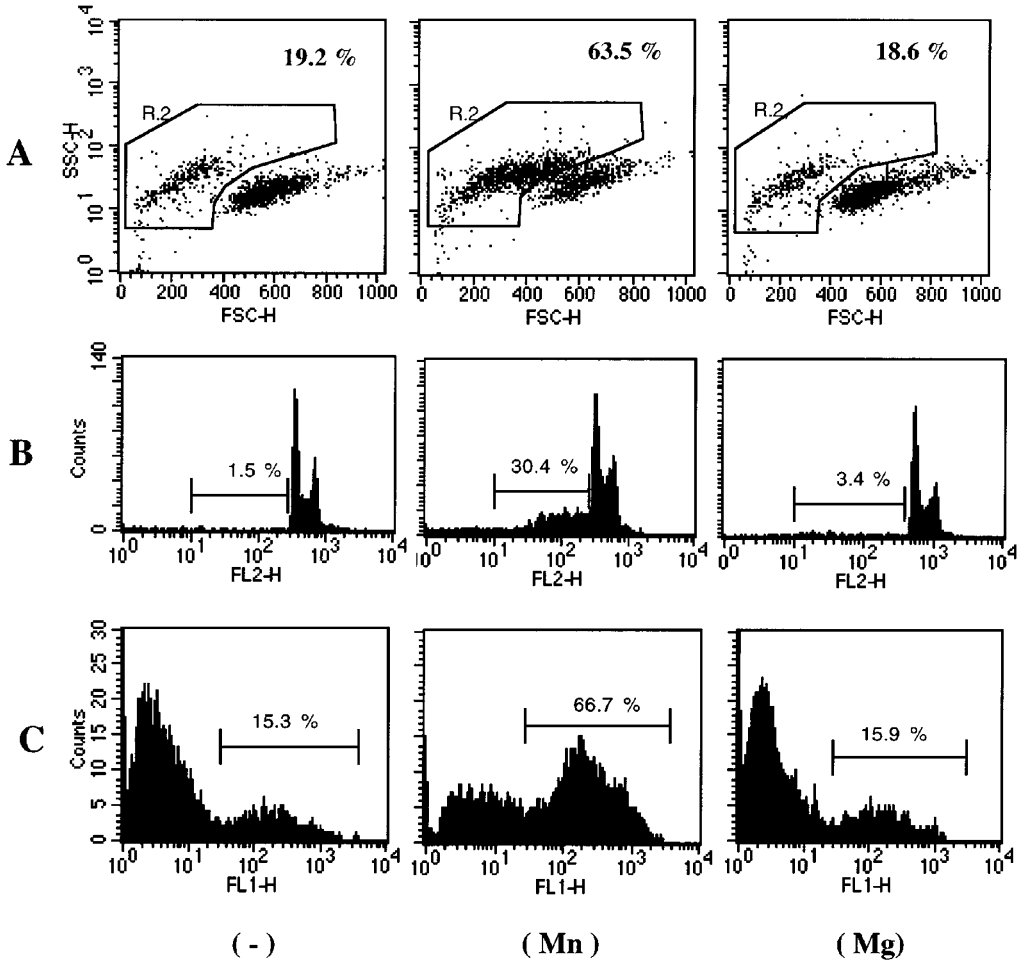

Figure 2 Apoptosis analysis of BL41 cells treated with Mn^{2+} and Mg^{2+}. Cells were cultured for 48 h without (−) or in the presence of $MnCl_2$ (100 μM) (Mn), or $MgCl_2$ (100 μM) (Mg). Cells were analyzed by flow cytometry. (**A**) Apoptotic cells having a relatively high side-scatter (SSC) and a low forward-scatter (FSC) properties were enumerated as a percentage of total population. (**B**) Cell nuclei were stained with PI and the hypodiploid DNA peak corresponding to apoptotic nuclei was quantified. (**C**) Cells were stained with Annexine-V-FITC and apoptotic cells having phosphatidylserine on the outer leaflet of the cell membrane were quantified. These data represent three independent experiments

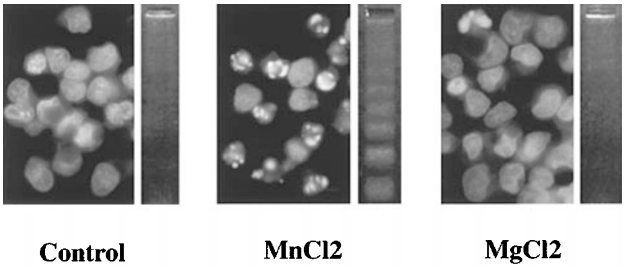

Figure 3 Nuclear morphology and internucleosomal DNA cleavage in BL41 cells treated with Mn^{2+} and Mg^{2+}. Cells were cultured for 48 h with 100 μM $MnCl_2$ or $MgCl_2$. Nuclear DNA of cells was stained with DAPI and subsequently analyzed under a fluorescence microscope. DNA fragments were analyzed as described in Materials and Methods

particularly caspase-1/ICE and caspase-3/CPP32, which are highly expressed in cell lines of lymphocytic origin, are components of the Mn^{2+}-triggered pathway leading to apoptosis in the BL41 cell line. BL41 cells were treated with 100 μM $MnCl_2$ and harvested at various times during the treatment (Figure 5). Each sample was tested for active caspases by Western blotting with appropriate specific antibodies. Processing of ICE was first detected as the appearance of p10 fragment after 30 min with its disappearance after 48 h (Figure 5A). We next analyzed the expression of CPP32 (Figure 5B). Western blot analysis showed that CPP32 on p17 and p23 subunits appeared after 24 h of stimulation. The p23 fragment (also referred to as p19 in the literature) appeared after 24 h with a decrease after 48 h. The amount of p17 subunit was maximal 48 h after induction. These results suggest that p17 fragment may be a product of p23 proteolysis.[39] These data show that apoptosis of B cells induced by Mn^{2+} is associated with activation of ICE and CPP32. These activations are sequential: ICE is processed first giving the characteristic p10 activated subunit after 30 min of incubation; CPP32 is then activated as shown by the appearance of the p23-p17 doublet after 24 h coinciding with the detection of apoptosis.

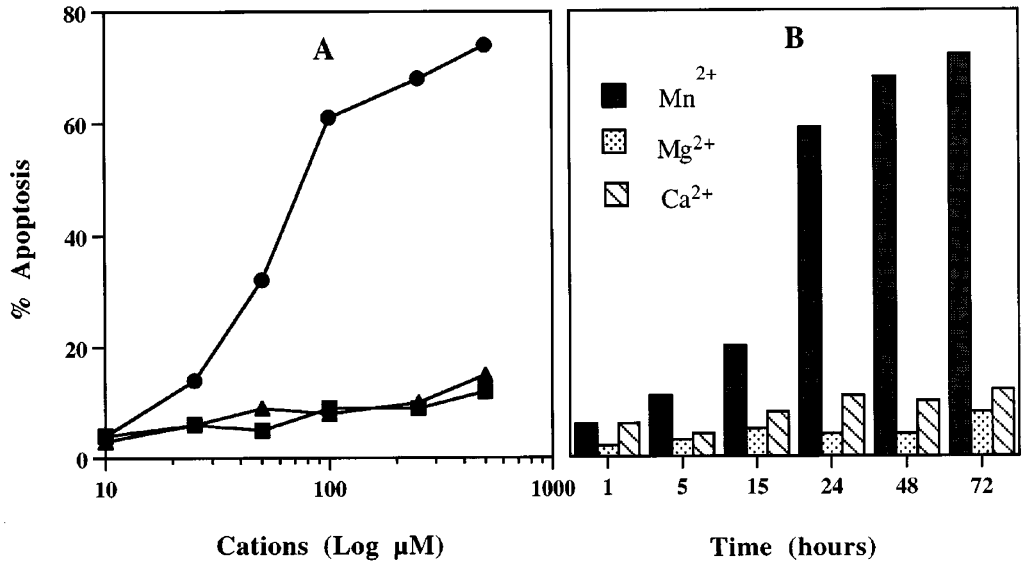

Figure 4 Time course and dose-dependent effect of divalent cations on B cell apoptosis. (**A**) DN1 cells (5×10^5 per ml) were incubated with medium alone or exposed to one of a series of concentrations of Mn^{2+} (●), Mg^{2+} (■) or Ca^{2+} (▲) and hypodiploid nuclei were evaluated by cytometry after lysis in hypotonic propidium iodide. (**B**) Time-course experiments of apoptosis induced by Mn^{2+}, Mg^{2+} and Ca^{2+} (250 μM) in DN1 cells. Cations-induced apoptosis was evaluated as described in Materials and Methods. Data are representative of four independent experiments

Table 1 Induction of apoptosis and growth inhibition by Mn^{2+} in various human B cells

	Inhibition of proliferation (%)	Viability (%)	Apoptosis (%)
EBV-B cell line			
GL1	87±6.5	9	41±3.5
DN1	97±2.5	3	59.5±14.8
Burkitt lymphoma			
BL41	95±5.1	7	67.5±6.4
Ramos	97.3±4.6	5	60±8.5
CA46	90.6±4.1	8	49±4.2
Raji	95.6±2.1	6	49±4.6
Daudi	92±4	10	ND
Tonsillar B cells	98.7±1.1	3	71±4.2
U937 (monoblastic cell line)	19.3±3.8	75	11.5±4.9

All cell lines were cultured for 24 h (apoptosis) or 48 h (proliferation) and tonsillar B cells were stimulated by 1/10000 SAC for 24 h (apoptosis) and 72 h (proliferation) in the presence of 250 μM $MnCl_2$ or in medium alone as described in Figure 1. Cell viability was measured by trypan blue exclusion after 24 h. Values are means±S.E.M. of three experiments. The percentage of apoptosis was calculated as described in Materials and Methods. ND, not done

To confirm that Mn^{2+} triggers activation of CPP32, we investigated the expression of PARP, one of the endogenous substrates of CPP32. PARP is a 110-kDa original molecule and is proteolyzed by caspase-3 to give two apoptotic fragments of 87-kDa and 24-kDa. We examined the cleavage of PARP in Mn^{2+}-treated BL41 cells by Western blotting using anti-PARP polyclonal antibody which recognizes both the 110-kDa and 87-kDa polypeptides (Figure 5C). PARP breakdown, with complete disappearance of the 110-kDa form, was observed after 24 h in cells treated with 100 μM $MnCl_2$ but not in $MgCl_2$ treated cells.

Apoptosis and PARP cleavage induced by Mn^{2+} is inhibited by the tripeptide inhibitor of the ICE-like protein zVAD-fmk

The above results clearly indicated that CPP32 is activated in B cells in response to Mn^{2+} treatment. To test whether the activation of caspase-3 and PARP cleavage is required for the apoptotic process in Mn^{2+}-treated B cells, we examined the effects of zVAD-fmk, a broad inhibitor of the ICE family of cysteine proteases, and DEVD-cmk, a more specific inhibitor of the caspase-3 (CCP32) family of cysteine proteases. Apoptosis and PARP cleavage induced by Mn in BL41 cells were followed. After 48 h of culture with Mn, zVAD-fmk (10–100 μM) induced a dose-dependent decrease in the percentage of apoptotic BL41 cells (Figure 6). This inhibition correlated with a decrease in the expression of 87-kDa fragment and with a complete inhibition of PARP proteolysis at 25 μM zVAD-fmk. In contrast, DEVD-cmk partially inhibited Mn^{2+}-induced apoptosis and PARP cleavage even at the high concentration of 100 μM. Experiments of cell viability measured by trypan blue exclusion showed that 100 μM zVAD-fmk was also able to almost completely block cell death (85% inhibition) induced by 48 h manganese treatment of BL41 cells (data not shown). These results indicate that apoptosis induced by Mn^{2+} is more efficiently blocked by the broad spectrum caspase inhibitor than by the relatively caspase-3 specific inhibitor DEVD-cmk. Presumably, other members of the caspase family, sensitive to zVAD-fmk, are involved in the apoptotic process. In conclusion, our results indicate that Mn^{2+}-induced apoptosis of BL41 cell involves the sequential activation of several caspases leading to a caspase-dependent cell death process.

Overexpression of Bcl-2 inhibited manganese-mediated apoptosis

The Bcl-2 protein is a potent inhibitor of both apoptosis and caspase-3 activation in different cells including Burkitt cell lines. To better understand the apoptotic pathways triggered by manganese, we further investigated whether overexpression of the proto-oncogene Bcl2 in BL-CL Ramos could counteract Mn-induced apoptosis. We transfected Ramos cells with the human *Bcl-2* gene (Ra-bcl2) and isolated a subclone based on its Bcl-2 overexpression. FACS analysis revealed the overexpression of Bcl-2 in stable Ramos-Bcl-2 subclone in contrast to Ramos control cells (Figure 7A). Ramos and Ramos-Bcl-2 cells were treated with different concentrations of manganese and viability and features of apoptosis were analyzed. We observed in Figure 7B that overexpression of Bcl-2 completely rescued Ra-Bcl-2 cell viability (trypan-excluding) even at high manganese concentrations (up to 250 μM). Other apoptotic features, induced with

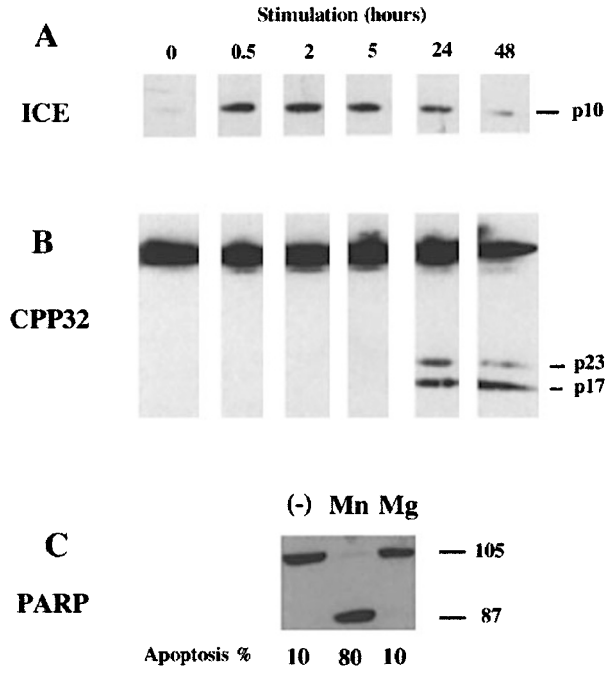

Figure 5 Analysis of caspases and PARP expression in stimulated BL41 cells. (**A** and **B**) Cells were cultured with $MnCl_2$ (100 μM) and the activated fragment of ICE, the p10 subunit (**A**) and CPP32, the p23 and p17 subunits (**B**) were analyzed after various times (0, 0.5, 2, 5, 24, 48 h) by Western blotting. The membrane from ICE analysis was stripped and reprobed for CPP32 detection. After 48 h of Mn^{2+} treatment, apoptotic cells were quantified by flow cytometric analysis of dot-blot light scatter profiles (9% of apoptosis in control and 59% in $MnCl_2$-stimulated cells). (**C**) Cells were cultured for 24 h in medium alone or with 100 μM $MnCl_2$ or 100 μM $MgCl_2$ and PARP cleavage was analyzed by Western blotting with specific anti-PARP antibodies. The proportion of apoptotic cells in each sample was quantified after 48 h by flow cytometric analysis of dot-blot light scatter profiles

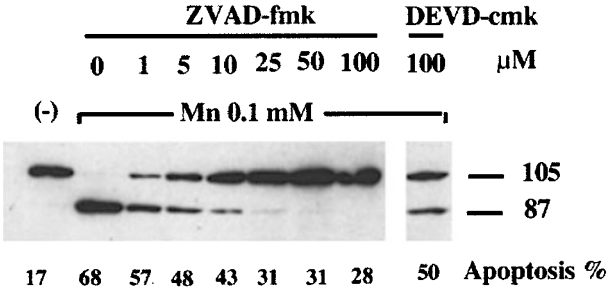

Figure 6 Inhibition of Mn^{2+}-induced apoptosis and PARP cleavage in BL41 cells by zVAD-fmk, a tripeptide inhibitor of ICE like protein. Cells were cultured for 48 h in the absence (−) or in the presence of 100 μM $MnCl_2$ and various concentrations of zVAD-fmk (0, 1, 5, 10, 25, 50, 100 μM) or DEVD-cmk (100 μM). The cleavage of PARP was determined by immunoblotting and the amount of apoptotic cells in each sample evaluated after 48 h of stimulation by flow cytometry. Cell morphology established by flow cytometry measurement of shrunken cells was correlated to cell viability measured by trypan blue exclusion

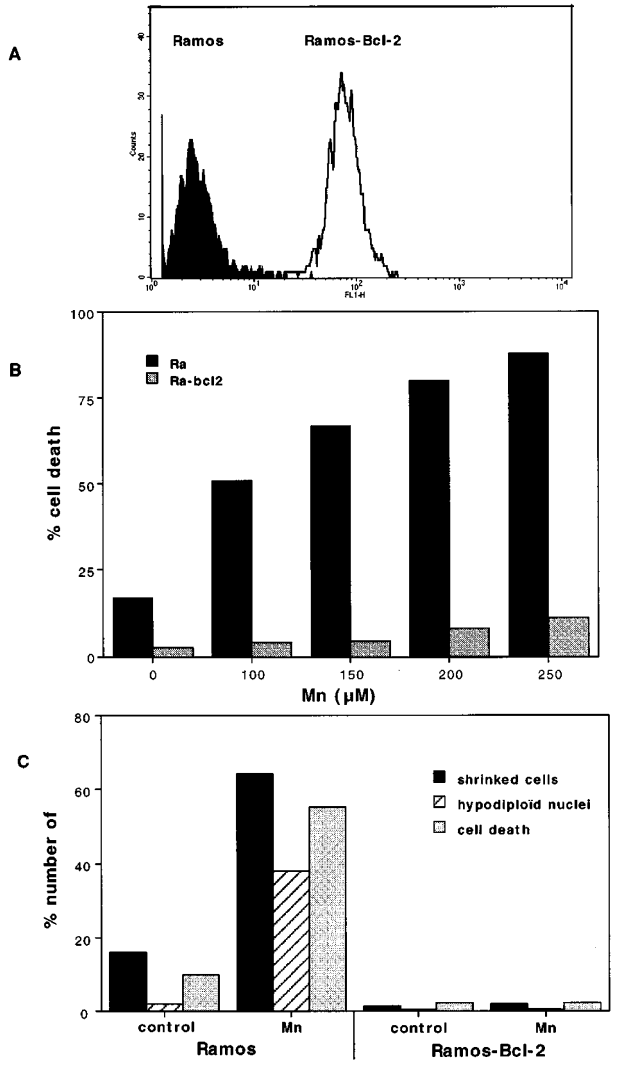

Figure 7 Overexpression of Bcl-2 counteracts Mn^{2+}-induced apoptosis and cell death. (**A**) Overexpression of Bcl-2 was quantified in Ramos and Ramos-Bcl-2 cells by flow cytometric analysis using FITC-labelled anti-human Bcl-2 monoclonal antibody. (**B**) Ramos and Ramos-Bcl-2 cells were cultured with various concentrations of $MnCl_2$ for 48 h and viable cells were counted using trypan blue exclusion. (**C**) After 48 h of treatment with 100 μM $MnCl_2$, cells were analyzed for cell shrinkage, chromatin condensation and viability. Here is shown a representative experiment done in triplicate

100 μM $MnCl_2$, including cell shrinkage and chromatin condensation, were also reversed in Ramos cells overexpressing Bcl-2 as compared to Ramos control cells. These results indicate that overexpression of Bcl-2 protects Ramos cells from Mn-mediated apoptosis and cell death and that manganese activates an apoptotic pathway sensitive to Bcl-2 regulation.

Discussion

In this report, we provide the first demonstration that the divalent cation Mn^{2+} induces growth inhibition and cell death of human B cells (Figure 1). Other cations including Mg^{2+} and Ca^{2+} had no effect on B cell apoptosis. Mn^{2+} effect was observed on normal and various malignant B cells. In all cases (normal-activated B cells, BL-CL and EBV-BCL), Mn^{2+} blocked the proliferation and induced apoptosis of B cells (Table 1). This Mn^{2+}-mediated cell death displayed characteristic features of apoptosis: cell shrinkage, chromatin condensation, expression of phosphatidylserine on the outer leaflet of the cell, nuclear fragmentation and DNA laddering (Figures 2 and 3). To our knowledge, this is the first report of the involvement of Mn^{2+} in the apoptosis of B cells.

The mechanisms by which Mn^{2+} mediates B cell apoptosis remain unclear. The neurotoxic effect of Mn^{2+} associated with the formation of reactive oxygen species[40] and the subsequent oxidative stress has been suggested to be responsible for neural damage[41] and apoptosis of PC12 cells.[38] A recent report has shown that U937 TNF-induced cell death is blocked by N-acetylcysteine, implying the involvement of ROS in apoptosis.[42] We observed that Mn^{2+} treatment had no effect on the proliferation or apoptosis of the monocyte cell line U937. Moreover, the addition of antioxidants (N-acetylcysteine, L-NAME or catalase) did not prevent the apoptosis of B cells after Mn^{2+} treatment. Moreover, Mn^{2+} pretreatment of SAC- or PMA-stimulated normal B cells or EBV-BCL completely abolished the generation of O^{2-} (data not shown). Thus, these observations appear to exclude the role of ROS in Mn^{2+}-induced B cell apoptosis.

Activation by divalent cations and cationic polypeptides has been extensively studied for the insulin[43,44] and EGF[45] receptors. Cations activate protein tyrosine kinases by facilitating kinase-kinase interactions thereby resulting in an increase of kinase activity. In fact, Mn^{2+} strongly enhances EGF receptor kinase activity by promoting the aggregation of kinase domains of the receptor, thus mimicking the effect of an effector ligand.[46] Similar observations have been reported for the insulin receptor.[47] The putative target molecules activated by divalent cation Mn^{2+} remain to be identified. Possibly kinases, associated with cell surface receptors and/or involved in the regulation of apoptosis pathway, could be activated by Mn^{2+}. We previously reported[9] that apoptosis triggered through AgR or its associated surface molecules, CD19 or CD22, was dependent on the degree of cross-linking of these receptors. Moreover, the appearance of apoptotic features and the kinetics of ICE and CPP32 activation after AgR cross-linking in B cells are similar to those observed after Mn^{2+} treatement (A. Vazquez *et al*, manuscript in preparation). In addition, z-VAD and DEVD inhibited apoptosis and caspase-3 processing mediated by AgR cross linking or Mn^{2+}. These results suggested that Mn^{2+}-mediated apoptosis in B cells could involve surface receptor aggregation leading to kinase activation. However, in our experimental conditions, Mn^{2+} did not trigger major tyrosine kinase activation even after 1 h of stimulation, when ICE was already activated (data not shown). This does not exclude the possibility that Mn^{2+} mobilized other kinases involved in AgR-triggered apoptotic pathway. Work is in progress to verify this hypothesis and try to characterize the potent targets of manganese.

To elucidate the apoptotic pathway following Mn^{2+} treatment of B cells, we attempted to block cell death with the inhibitor zVAD-fmk (a broad spectrum caspase inhibitor). ZVAD-fmk blocked morphological changes (Figure 6) and disorganization of plasma membrane phospholipids but not cell proliferation inhibition associated with Mn^{2+}-induced apoptosis (data not shown). This finding suggests that Mn^{2+} growth inhibition does not involve the ICE family protease family. It is possible that Mn^{2+}-mediated inhibition of cell proliferation and induction of apoptosis involve distant activation pathways. Another interpretation could be that high intracellular levels of Mn^{2+} causes irreversible damage to the cell, which usually triggers the apoptotic pathway for efficient cell destruction and removal. After blocking the caspases, the apoptotic manifestations of cell death are suppressed, yet the cell is damaged beyond repair and ultimately dies of necrosis. To investigate the biochemical mechanism by which Mn^{2+} induces B cell apoptosis, we examined the activation and the processing of the caspase cascade. Mn^{2+} (100 μM) activated ICE then CPP32 in specific temporal sequence. This activation was characterized by the production of their catalytic subunits: p10 for ICE and the doublet p17-p23 for CPP32. ICE was activated during the first 30 min of treatment and CPP32 later, after 24 h. Moreover, appearance of the CPP32 catalytic subunits P17 and p23 was associated *in vivo* by PARP cleavage, one of the CPP32 substrates (Figure 5). This cleavage (Figure 6) was completely inhibited by a caspase family inhibitor zVAD-fmk (25 μM) and to a lesser extent by DEVD-cmk (100 μM) a more restricted caspase-3 inhibitor. The same concentration of zVAD-fmk almost completely suppressed Mn^{2+}-mediated apoptosis (80% inhibition) whereas DEVD-cmk at doses up to 100 μM partially blocked B cell death (37% inhibition). These data demonstrate that Mn^{2+} activates the caspase pathway for inducing cell death. The remaining cleavage of PARP in DEVD pre-treated cells was probably due to caspases other than caspase-3 also being able to cleave PARP *in vivo* albeit with a lower efficiency.[48] The recruitment of this pathway might depend on a caspase, other than CPP32, and which is sensitive to zVAD-fmk but not to DEVD-cmk inhibitors. Finally, the induction of apoptosis and cell death by manganese was completely abolished in BL-CL Ramos which overexpress Bcl-2. Bcl-2 is an anti-apoptotic protein upregulated after stimulation by several physiological factors which increase the survival of B cells. Like the germinal center B cells, Burkitt lymphoma B cells which have a germinal center origin do not express

the bcl-2 protein. After stimulation by anti-CD40 antibody, these cells upregulated bcl-2 protein and were more resistant against apoptosis.[27] These findings indicate that manganese induces a caspase-dependent process of cell death in B cells which can be negatively regulated by survival factors such as Bcl-2.

It is clear that divalent metal ions play a major role in the regulation of cellular apoptosis. For instance, Zn^{2+} influences the regulation of apoptosis in various cell types. Unlike Mn^{2+}, Zn^{2+} prevents apoptosis in both *in vitro* and *in vivo* models.[32,49,50] Ca^{2+}/Mg^{2+}-endonucleases have been proposed as possible candidates for Zn^{2+} anti-apoptotic activity.[33,51] Caspase-3/CPP32 was identified as a target of Zn^{2+} inhibition in T cell apoptosis.[34] Our study shows that Mn^{2+} triggers B cell apoptosis which is associated with the differential activation of caspase-1 and caspase-3. This observation supports the importance of divalent cations in the induction and/or regulation of programmed cell death of human B lymphocytes. Recently, investigators reported that low doses of arsenic trioxide, another heavy metal ion, could induce complete remissions in patients with acute promyelocytic leukemia who have relapsed after extensive prior therapy.[52] Clinical responses were associated with induction of apoptosis through caspase activation.[53] Arsenic trioxide affected only partially proliferation and did not induce apoptosis of B lymphoma cell lines such as Raji and Daudi.[54] The ability of manganese to induce cell death of EBV^+- and EBV^--B cells such as Burkitt's lymphoma may be applied to other B-cell tumors (chronic lymphocytic leukemia and follicular non-Hodgkin's lymphomas). These observations could provide an experimental basis and a new approach for B cell tumor therapy.

Materials and Methods

Reagents

$MnCl_2$, $MgCl_2$, $CaCl_2$ and DAPI (4,6-diamidino-2-phenylindole) were obtained from Sigma (St. Louis, MO, USA). Z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk), a cell permeable irreversible tripeptide inhibitor of the ICE family of cysteine proteases and Z-Asp-Glu-Val-Asp-chloromethylketone (DEVD-cmk), a cell permeable inhibitor of the caspase 3 cysteine protease were supplied by Bachem Biochimie SARL (Voisin le Bretonneux, France). Stock solutions of zVAD-fmk and of DEVD-cmk, 100 mM each in DMSO were kept at $-20^{\circ}C$ and appropriate dilutions prepared immediately prior to use. SAC, staphylococcus aureus cowan 1 strain (pansorbin) was from Calbiochem (France Biochem).

BL cell lines

The Burkitt lymphoma cell line Ramos, BL41, Raji, CA46 and Daudi and the monoblastic leukemia line U937 were obtained from ATCC (Rockville, MD, USA). DN1 and GL1 B cell lines were obtained by EBV infection of normal B cells *in vitro* as previously described.[55]

Cell culture and bioassays

BL cells were cultured in RPMI 1640 medium (Seromed, Biochrom, Berlin, Germany) supplemented with 1% glutamine, 1% antibiotic and 5% fetal bovine serum (Gibco, Grand Island, NY, USA). B cell proliferation was studied in the presence of various concentrations of $MnCl_2$ or $MgCl_2$ in 96-well flat-bottom microtest plates (Falcon, Oxnard, CA, USA). Cells were cultured for 2 days and then pulsed with $[^3H]$Thymidine (0.5 μCi per well) (CEA, Saclay, France) for an additional 16 h. The cells were then harvested and radioactivity counted. For cytometric analysis, BL cells were recovered at the end of treatment and the percentage of apoptotic cells was determined as described below.

Cell transfection

The pSFFV-bcl2-neo vector bearing the human bcl-2 cDNA was transfected into Ramos cells by electroporation and stable transfectant was isolated. After selection, overexpression of Bcl2 protein by Ramos-bcl-2 cells was verified by flow cytometry analysis using an anti-human Bcl-2 monoclonal antibody FITC-labeled (DAKO A/S, Denmark).

Determination of apoptosis

B cell apoptosis was detected first by cytometric analysis of dot-blot light scatter profiles. The cells (10^6) were washed in PBS and resuspended in 1% PFA (paraformaldehyde) in PBS. After 1 h of incubation at room temperature, apoptotic cells were analyzed for their dot-blot light scatter profile by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Apoptotic cells having relatively high light side-scatter and a low light forward-scatter properties were enumerated as a percentage of the total population.

For the DNA labeling flow cytometric analysis, hypodiploid DNA was analyzed by flow-cytometry as previously described.[56] Briefly, cells (10^6) were washed in PBS and resuspended in 1 ml hypotonic fluorochrome solution (50 mg ml^{-1} propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) (Sigma, St. Louis, MO, USA). Samples were incubated at room temperature for 1 h before flow-cytometry analysis of PI fluorescence of individual nuclei using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) as described elsewhere.[57] Cellular debris were excluded from analysis by raising the forward scatter threshold and the DNA content of the intact nuclei was registered on a logarithmic scale. Apoptotic cell nuclei having hypodiploid DNA emitting fluorescence in channels 10–200 were enumerated as a percentage of the total population. The percentage of Mn^{2+}-induced apoptosis (Figure 3 and Table 1) was calculated as follows: (% of hypodiploid nuclei in test sample $-$% of spontaneous hypodiploid nuclei)/(1$-$% of spontaneous hypodiploid nuclei) $\times$ 100.

Phosphatidylserine on the outer leaflet of the cells was also analyzed as a marker of apoptosis. Cells (10^6) were washed in PBS and resuspended in incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and Annexine-V-FITC (Boehringer Mannheim). After 15 min of incubation, the fluorescence emitted by cells was analyzed using a FACScan flow cytometer (Becton-dickinson, Mountain View, CA, USA). Apoptotic cells having phosphatidylserine on the outer leaflet of the cell membrane and positively stained by annexine V were enumerated as a percentage of the total population.

Morphological assessment of nuclear fragmentation was determined microscopically. Cells were collected, washed with PBS and fixed for 10 min in PBS containing 4% paraformaldehyde. After centrifugation, cells were resuspended in 5 μl of 1 μg ml^{-1} DAPI. Nuclear morphology was monitored under a fluorescence microscope (Leica DM RB).

Analysis of DNA fragmentation

DNA fragments were extracted and separated by electrophoresis in agarose gels according to Duke *et al*[58] with minor modifications. Briefly, 2×10^6 cells were incubated with cations for defined periods, washed twice in PBS and then lysed with 30 μl of a buffer containing 10 mM Tris (pH 7.5), 5 mM EDTA, and 0.5% Triton X-100 for 30 min at room temperature. Cell lysates were centrifuged at 15 000 $\times$ *g* for 20 min and the supernatants incubated with proteinase K (0.2 mg ml^{-1}) and ribonuclease A (0.1 mg ml^{-1}) at 42°C for 1 h. The samples were electrophoresed in a 2% agarose gel in 0.5 $\times$ TBE running buffer (4.5 mM Tris, 4.5 mM boric acid, 62.5 μM EDTA) containing ethidium bromide (10 μg ml^{-1}).

Western blotting analysis

Cells were lysed by incubation for 1 h on ice in lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% Nonidet P-40, 2 mM Pefablock from Interchim (Montluçon, France), 1 μM ml^{-1} Aprotinine, 10 μg ml^{-1} leupeptine, 2 μg ml^{-1} Pepstatin A and 100 μg ml^{-1} Soybean Trypsine inhibitor all from Sigma (St. Louis, MO, USA). The lysate was centrifuged for 30 min at 13 000 $\times$ *g* at 4°C and the protein concentration of the supernatant determined (micro-BCA protein assay, Pierce Chemical Co., Rockford, IL, USA). Cell lysate (40 μg of the protein) was boiled for 5 min in 1 $\times$ sample buffer and resolved by 7.5 or 15% SDS–PAGE. Proteins were then electroblotted onto 0.45 μm pore size nitrocellulose filters, and the filters were blocked for 1 h with 5% non-fat milk in PBS, 0.1% Tween-20. The filters were then incubated 1 h at room temperature with anti-ICE mAb (2 μg ml^{-1}, ICE p10 (C20) Santa Cruz Biotechnology, Santa Cruz, CA, USA) or CPP32 mAb (1 μg ml^{-1}, polyclonal Rabbit Anti-Caspase-3 antiserum, Pharmingen, San Diego, CA, USA) or PARP mAb (1 μg ml^{-1}, C2.10 was obtained from Dr G. Poirier, Quebec, Canada). The blots were washed three times for 10 min with 0.2% Tween 20 in PBS and incubated for 1 h with peroxidase-labeled anti-mouse or rabbit immunoglobulin (1/5000). Blots were then developed using an enhanced chemiluminescence detection system (ECL, Amersham Corp., UK).

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