BIOCHEMICAL PHARMACOLOGY 72 (2006) 671–680

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Oxidative stress by ascorbate/menadione association kills K562 human chronic myelogenous leukaemia cells and inhibits its tumour growth in nude mice

Julien Verrax [1,*], *Julie Stockis, Aurélie Tison, Henryk S. Taper, Pedro Buc Calderon*

Unité de Pharmacocinétique, Métabolisme, Nutrition et Toxicologie, Département des sciences pharmaceutiques, Université Catholique de Louvain, Belgium

ARTICLE INFO

Article history:
Received 10 March 2006
Accepted 30 May 2006

Keywords:
Ascorbate
Menadione
Oxidative stress
Cancer
Apoptosis
necrosis

ABSTRACT

The effect of oxidative stress induced by the ascorbate/menadione-redox association was examined in K562 cells, a human erythromyeloid leukaemia cell line. Our results show that ascorbate enhances menadione redox cycling, leading to the formation of intracellular reactive oxygen species (as shown by dihydrorhodamine 123 oxidation). The incubation of cells in the presence of both ascorbate/menadione and aminotriazole, a catalase inhibitor, resulted in a strong decrease of cell survival, reinforcing the role of H_2O_2 as the main oxidizing agent killing K562 cells. This cell death was not caspase-3-dependent. Indeed, neither procaspase-3 and PARP were processed and only a weak cytochrome *c* release was observed. Moreover, we observed only 23% of cells with depolarized mitochondria. In ascorbate/menadione-treated cells, DNA fragmentation was observed without any sign of chromatin condensation (DAPI and TUNEL tests). The cell demise by ascorbate/menadione is consistent with a necrosis-like cell death confirmed by both cytometric profile of annexin-V/propidium iodide labeled cells and by light microscopy examination. Finally, we showed that a single i.p. administration of the association of ascorbate and menadione is able to inhibit the growth of K562 cells by about 60% (in both tumour size and volume) in an immune-deficient mice model. Taken together, these results reinforced our previous claims about a potential application of the ascorbate/menadione association in cancer therapy.

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1. Introduction

Since many years, the reactivation of the apoptotic program appears as a major target to eliminate cancer cells and therefore, is being a current goal of both chemotherapy and radiotherapy. However, defects in signaling pathways leading to apoptosis are common in cancers [1]. A well-known case is the alteration of the p53 pathway, occurring in the majority of invasive cancers [2], thus allowing genotypic drift and resistance to most of the cytotoxic agents due to their mechanism of action generally based on DNA damage [3]. Instead of a frontal confrontation with tumour cell biology, a more elegant strategy would consist in taking an advantage of its features, e.g. by inhibiting tumour angiogenesis [4] which must ultimately result in cancer cell apoptosis by oxygen and essential nutrients starving [5]. In a similar way,

* *Corresponding author at*: Unité PMNT 7369, 73, avenue E. Mounier, 1200 Bruxelles, Belgium. Tel.: +32 2 764 7366; fax: +32 2 764 7359.
E-mail address: julien.verrax@uclouvain.be (J. Verrax).

[1] Julien Verrax is an FRIA recipient.

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doi:10.1016/j.bcp.2006.05.025

we noted that cancer cells usually exhibit a poor antioxidant status [6–8], thus raising the possibility to kill cancer cells through an oxidative stress. To do that, we used a synergistic association of ascorbate and menadione (also known as Vitamins C and K_3). Actually, menadione is reduced by ascorbate to form the dehydroascorbate and the semiquinone free radicals. The semiquinone radical is rapidly reoxidized to its quinone form by molecular oxygen thus generating reactive oxygen species (ROS). Among them hydrogen peroxide (H_2O_2) is likely the major oxidizing agent involved in the cytolytic process [9,10]. Based mainly on histological analysis, some authors have called this cell death ''autoschizis'' [11], but the molecular mechanisms of cell killing by ascorbate/menadione observed in several cell lines [12–14] are not yet totally clarified.

The aim of this work was to examine, in K562 human chronic myelogenous leukaemia cells, the way by which this association is killing the cells in both in vitro conditions as well as in a tumour-bearing mice model. The following parameters were measured: processing of both procaspase-3 and PARP and cytochrome *c* release from mitochondria as well (western blots), changes in mitochondrial membrane potential ($\Delta\psi_m$) and intracellular ROS presence by using respectively JC-1 and dihydrorhodamine-123 (DHR123) as fluorescent probes (flow cytometry), flow cytometry of annexin-V-labeled cells, as well as morphological studies. The results we obtained indicated that cell death by ascorbate/menadione redox cycling is more related to necrosis than apoptosis. Most probably as a consequence of such a cytotoxicity, we also show that the ascorbate/menadione association reduces the growth of subcutaneously-implanted K562 cells in nude female mice, demonstrating the in vivo antitumoural capacity of such an association.

2. Materials and methods

2.1. Cell line

The K562 cell line was a gift of Dr. F. Brasseur (Ludwig Institute for Cancer Research, LICR-Brussels). They were cultured in DMEM/F12 (Dulbecco's modified eagle medium, Gibco) supplemented with 10% foetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml). The cultures were maintained at a density of (1–2) $\times 10^5$ cells/ml. The medium was changed at 48–72 h intervals. All cultures were maintained at 37 °C in a 95% air/5% CO_2 atmosphere with 100% humidity.

2.2. Chemicals

Menadione bisulfite (Vitamin K_3), sodium ascorbate (Vitamin C), sanguinarine, *N*-acetylcysteine (NAC), aminotriazole, dihydrorhodamine 123 and dimethylsulfoxide (DMSO), were purchased from Sigma (St. Louis, MO). Complete Mini protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1) was purchased from Fluka Chemie (Buchs, Switzerland). All other chemicals were ACS reagent grade.

2.3. Cell survival assay

The viability of cells was estimated at different times of incubation by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue [15], both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as 100 minus the ratio of released activity to the total activity.

2.4. Immunoblotting

At the indicated times, cells were washed twice with ice-cold PBS and then resuspended in a lysis buffer containing 0.1% phenylmethylsulfonyl fluoride, 0.1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 100 μM sodium vanadate in PBS supplemented with one tablet of Complete Mini protease inhibitor cocktail. The samples were kept on ice for 20 min, centrifuged at 13,000 × *g* for 20 min at 4 °C and then stored at −20 °C. Equal amounts of proteins were subjected to SDS-PAGE (6–15% separating gel) followed by electroblot to nitrocellulose membranes. The membranes were blocked 1 h in TBS buffer (pH 7.4) containing 5% powdered milk protein followed by an overnight incubation with diluted antibodies in a fresh solution of powdered milk protein (1%, w/v) in TBS buffer. The membranes were washed and incubated for 60 min with a dilution of secondary antibody coupled to horseradish peroxidase or alkaline phosphatase. Anti-cytochrome c, anti-procaspase-3 and anti-PARP were all rabbit polyclonal antibodies diluted 1:200. They were purchased respectively from Cell Signalling (Beverly, MA, USA) and Santa-Cruz (Santa-Cruz, CA, USA). Anti-rabbit goat antibodies were purchased from Chemicon International (Temecula, CA, USA).

2.5. Translocation of cytochrome c

5×10^6 cells were washed in phosphate-buffered saline and lysed by incubating for 30 s in lysis buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, and 350 μg/ml digitonin). After centrifugation at 13,000 × *g* for 2 min, the supernatant (cytosolic fraction) was collected in an equal volume of 2× sample buffer, and the pellet (mitochondria-rich fraction) was lysed by sonication in 1× SDS sample buffer. For both cytosolic and pellet fractions, the proteins were quantified, and 5 μg of protein per condition was used for immunoblotting.

2.6. DNA fragmentation

The *in situ* nuclear DNA fragmentation was determined according to a method based on 3′-OH end labeling of DNA breaks with deoxyuridine terminal deoxynucleotidyl transferase (TUNEL). The fragmented DNA was detected by the ''*In Situ* Cell Death Detection'' kit (Boehringer, Mannheim, Germany). At the indicated times, cells were washed three times with cold PBS before being deposited on poly-L-lysine-coated slides. Cells were fixed by using a 4% paraformaldehyde solution before their permeabilisation with a 0,2% Triton X-100 solution. The treated slides were incubated in terminal deoxynucleotidyl transferase buffer containing fluorescein-dUTP and terminal deoxynucleotidyl transferase for 1 h at 37 °C. Then, slides were washed twice in PBS before mounting

with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Finally, cells were examined with a Zeiss Axioskop microscope equipped for fluorescence.

2.7. Annexin-V/Propidium iodide staining

Cells were harvested at different times of incubation and 1×10^6 cells were stained with the Roche Annexin-V-FLUOS Staining kit (Mannheim, Germany) following the manufacturer's instructions. 1×10^4 cells were analysed using a Becton-Dickinson FACScan apparatus. The data were collected, stored and analyzed using CellQuest software (Becton Dickinson, Mountain View, CA).

2.8. Assessment of mitochondrial membrane potential ($\Delta\psi_m$)

Mitochondrial membrane potential was monitored using a fluorescent cationic dye known as JC-1. In healthy cells JC-1 enters the negatively charged mitochondria where it aggregates and fluoresces red. In apoptotic cells, where the mitochondrial potential has collapsed, JC-1 exists as monomers throughout the cell. When dispersed in this manner JC-1 fluoresces green. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. After 3 h of incubation with the different compounds, cells were harvested and, for each conditions,

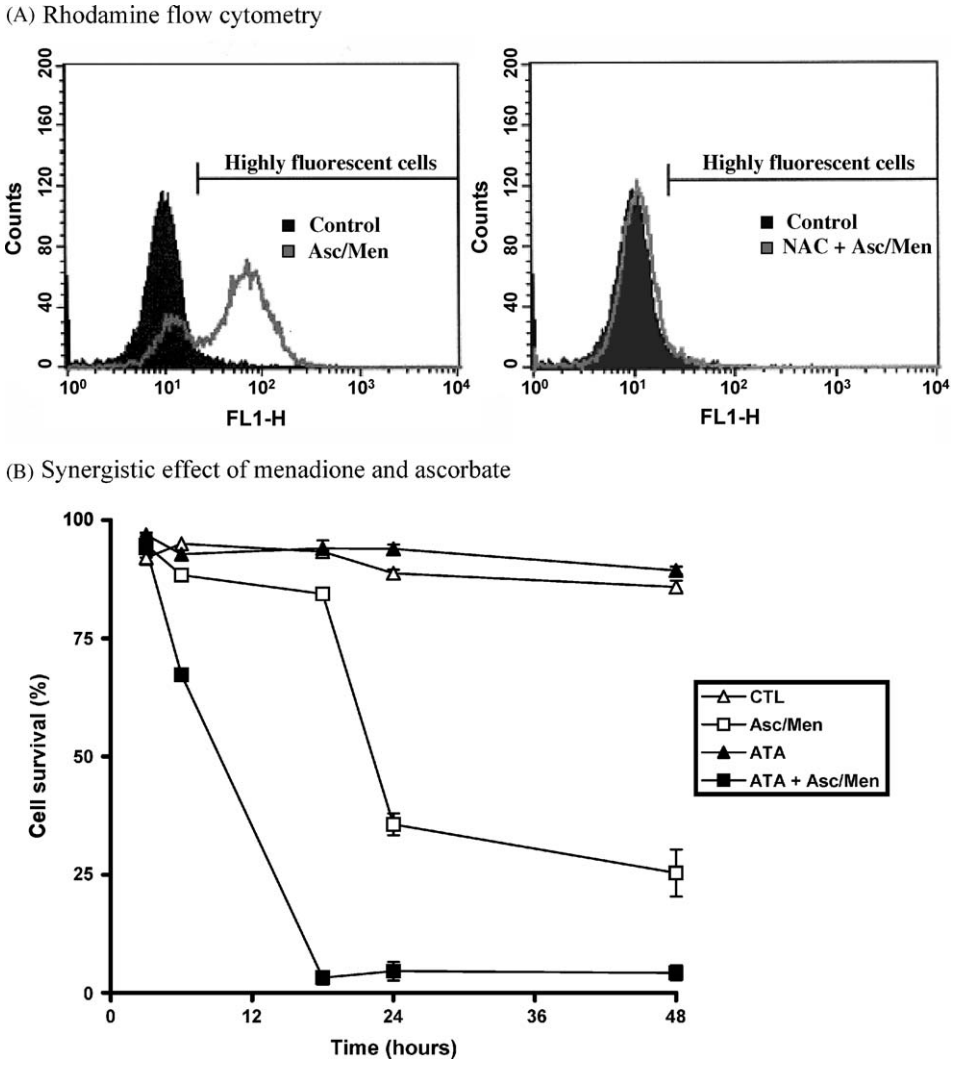

Fig. 1 – Cell death by ascorbate/menadione redox cycling. (A) K562 cells were incubated for 3 h at 37 °C in the absence (control) or in the presence of a mixture of ascorbate and menadione (2 mM and 10 μM, respectively). When used, *N*-acetylcysteine (NAC) was incubated at 3 mM without preincubation. In the left panel, it is shown that in the presence of ROS (ascorbate/menadione-treated cells), DHR 123 is oxidized to the highly fluorescent rhodamine 123. In the right panel, the antioxidant NAC suppresses the formation of ROS by ascorbate/menadione. A typical result out of three separate experiments is presented. (B) Cells were incubated as previously reported for 48 h at 37 °C. When present, aminotriazole (ATA) was used at 5 mM and preincubated for 1 h. In ascorbate/menadione-treated cells, the addition of ATA induces a loss of cell survival that becomes evident before 18 h, underlining the cytotoxic effect of H_2O_2. The results represent the mean ± S.E.M of at least three separate experiments. The effects of ascorbate/menadione and aminotriazole + ascorbate/menadione were significantly different ($p = 0.001$, using two-way ANOVA) as well as to compare to control group ($p < 0.001$, using two-way ANOVA).

1×10^6 cells were incubated at 37 °C for 15 min in 1 ml of 10 μg/ml of JC-1. 1×10^4 cells were subsequently analyzed using a Becton-Dickinson FACScan apparatus with excitation and emission settings of 488 and 525/595 nm. The data were collected, stored and analyzed using CellQuest software. Control experiments documenting the loss of $\Delta\psi_m$ were performed by exposing cells to 50 μM carbamoyl cyanide *m*-chlorophenylhydrazone, an uncoupling agent that abolishes the mitochondrial membrane potential.

2.9. Assessment of ROS formation

DHR 123 was used to measure ROS. Cells were incubated in the presence or the absence of ascorbate/menadione. After 3 h of incubation, cells were harvested and, for each condition, 1×10^6 cells were incubated at 37 °C for 30 min in 1 ml of 1 μM of DHR. 1×10^4 cells were analyzed using a Becton-Dickinson FACScan apparatus with excitation and emission settings of 488 and 525 nm. The data were collected, stored and analyzed using CellQuest software.

2.10. Cell morphology

Cells were observed after various incubation times under an inverted microscope from Optika (Ponteranica, Italy). Pictures were taken with a Coolpix 4500 from Nikon (Nikon Corporation, Tokyo, Japan). Light microscopic evaluation was performed using a magnification of 400×.

2.11. K562 xenograft in nude mice

Approximately 10^7 K562 cells resuspended in 200 μL of a serum-free culture medium/Matrigel mixture (1:1) were injected s.c. into the right flank of female BALB/c nude mice. Forty-eight hours after implantation, ascorbate/menadione-treated group were injected i.p. with a mixture of ascorbate and menadione sodium bisulfite (1 g/kg and 10 mg/kg, respectively). Control mice were injected i.p. with vehicle only. All solutions were extemporaneously prepared in saline and sterilized by filtration through a 0.2 μm filter (Millipore, Billerica, MA, USA). Tumour-bearing mice were sacrificed after

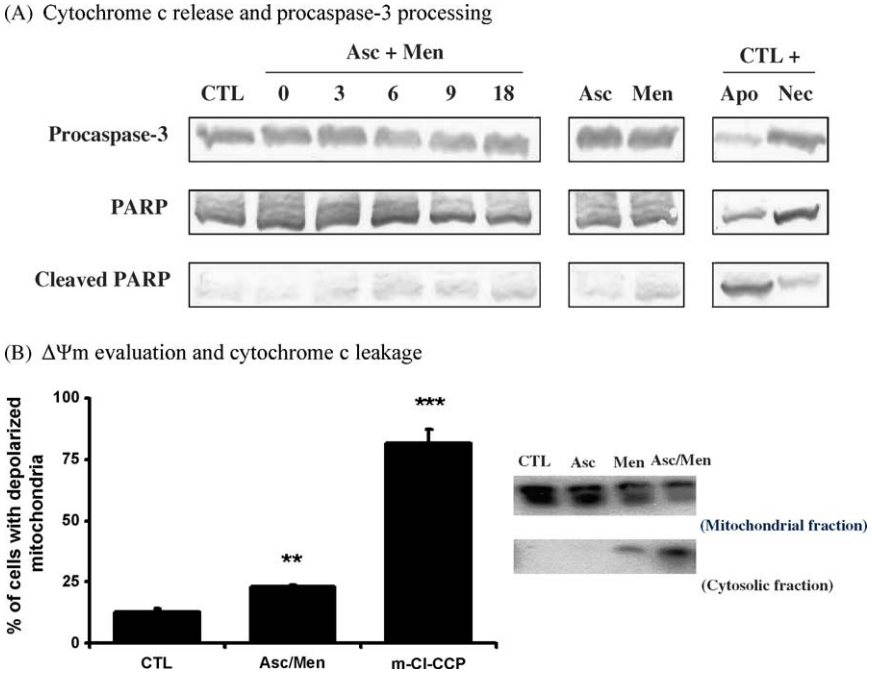

Fig. 2 – A caspase independent cell death. (A) Cells were incubated in the absence (control) or in the presence of a mixture of ascorbate/menadione (2 mM and 10 μM, respectively) for different times ranging from 0 to 18 h. The absence of procaspase-3 and PARP processing was also assessed for ascorbate and menadione alone (18 h of incubation). A positive control of apoptosis (Apo) was performed by incubating the cells with 10 μM of sanguinarine for 5 h. In addition, a positive control of necrosis (Nec) was also performed by incubating the cells at 55 °C for 15 min. Afterwards, cells were washed with PBS and lysed according to the procedures described under Section 2. Twenty micrograms of protein for each condition was subjected to Western-blot. As expected, sanguinarine induces a strong release of cytochrome *c* leading to procaspase-3 and PARP processing. In the other groups, neither procaspase-3 nor PARP processing was observed. Typical results out of three separate experiments are presented. (B left panel) Flow cytometric analysis of mitochondrial transmembrane potential in K562 cells treated or untreated with ascorbate and menadione (2 mM and 10 μM respectively). M-Cl-CCP was used at 50 μM as positive control. Histograms show the mean values ± S.E.M of cell percentage derived from three independent experiments. In ascorbate/menadione-treated cells, 23% of cells lost their mitochondrial membrane potential. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with control values, using Student's *t*-test. (B right panel) Cytochrome *c* release from mitochondria was assessed after 3 h of incubation in the absence (control) or in the presence of 2 mM ascorbate, 10 μM menadione and a mixture of both compounds.

4 weeks and tumour masses were measured before excision and weighing. Tumour volumes were calculated according to the following formula: (length × width^2 × π)/6.

3. Results

3.1. *ROS formation during ascorbate/menadione redox-cycling*

Ascorbate/menadione-treated cells were loaded with dihydrorhodamine 123, which was oxidized into rhodamine 123, leading to the apparition of a population of highly fluorescent cells (Fig. 1A). Since this fluorescence can be inhibited by incubating cells in the presence of the antioxidant *N*-acetylcysteine (NAC), this indicates the intracellular presence of ROS. On the other hand, the incubation of cells in the presence of both ascorbate/menadione and aminotriazole (ATA), a catalase inhibitor, resulted in a strong decrease of cell survival (Fig. 1B). Indeed, after 24 h cell survival was decreased by 95% as compared to 64% observed with ascorbate/menadione alone. It should be noted that ascorbate and menadione when added separately to cell suspensions did not induce a loss of cell survival. Actually, after 48 h of incubation,

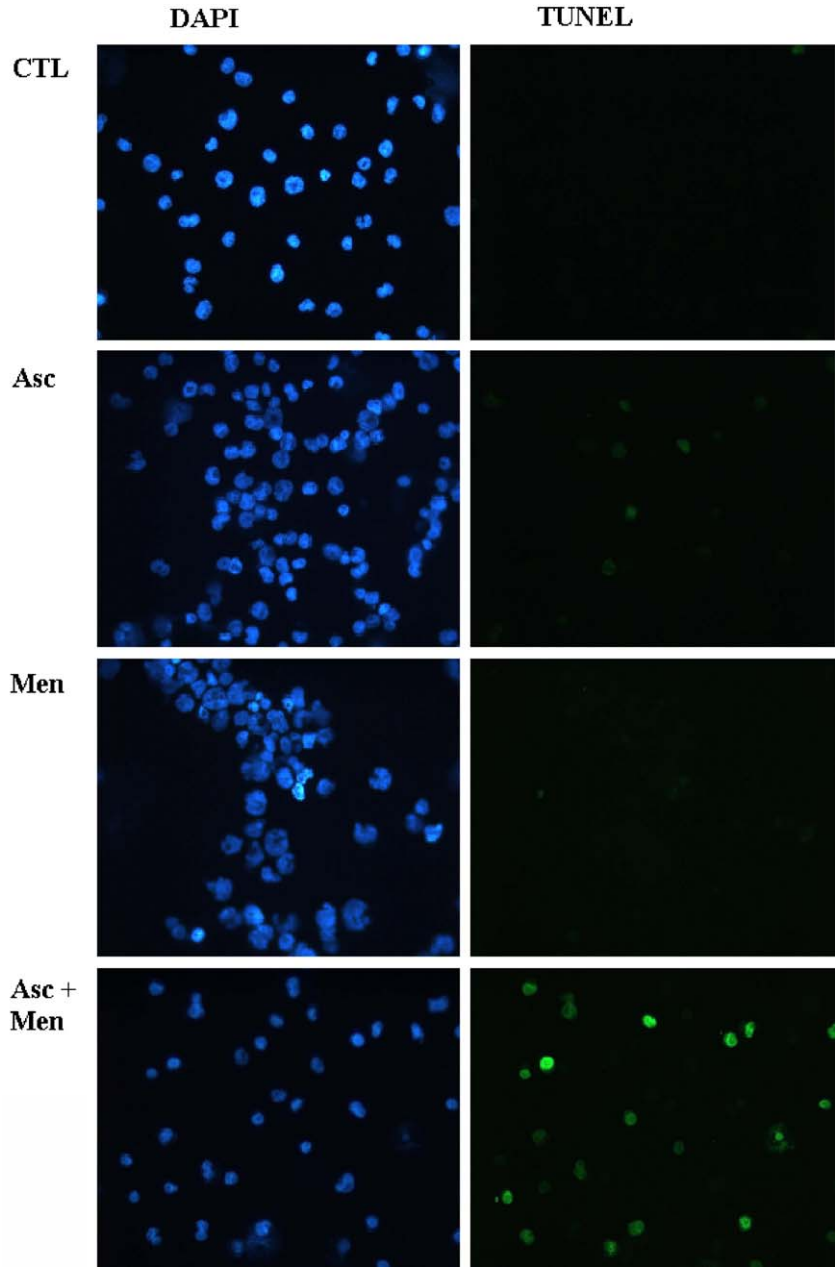

Fig. 3 – DNA fragmentation. K562 cells were incubated for 24 h at 37 °C in the absence (control) or in the presence of 2 mM ascorbate, 10 μM menadione and a mixture of both compounds. DAPI counterstaining is presented in the left panel showing no chromatin condensation in any group. TUNEL staining is shown on the right, showing DNA fragmentation in ascorbate/menadione-treated cells. Note that ascorbate and menadione when added separately to cell suspensions do not induce DNA fragmentation. Typical results out of three separate experiments are presented.

the cell survival was 86% and 85%, respectively. These results reinforced the major role of H_2O_2 among the ROS produced by ascorbate/menadione redox-cycling.

3.2. *Cell death by a caspase-independent process*

Since cell death induced by ascorbate/menadione in a murine liver hepatoma (TLT cells) was caspase-3 independent [16], we investigated whether a similar process was occurring in K562 cells that were more resistant than TLT cells [10]. Therefore, we measured different markers reflecting caspase-3 activity. A lack of DEVDase activity was observed in cells incubated in the presence of ascorbate/menadione (data not shown). This lack of DVEDase activity was further confirmed by the absence of cleavage between 0 and 18 h of incubation of both procaspase-3 and one of the main substrate of caspase-3, namely the poly(ADP-ribose) polymerase (PARP) protein (Fig. 2A). As expected, both the procaspase-3 and the PARP proteins were cleaved when cells were incubated in the presence of a positive control of apoptosis (sanguinarine, [17]) while no cleavage was observed with a positive control of necrosis (cells heated at 55 °C for 15 min). It should be noted that a broad caspase inhibitor, Z-VAD-fmk, failed to protect against cell death (data not shown).

The release of cytochrome *c* from mitochondria and the modification of mitochondrial membrane potential were also determined (Fig. 2B). After 3 h of incubation in the presence of ascorbate/menadione, a certain amount of cytochrome *c* is released from mitochondria (Fig. 2B right panel). This cytochrome *c* release seems to be correlated to a loss of mitochondrial membrane potential, as measured by the percentage of cells with depolarized mitochondria (Fig. 2B left panel). In cells incubated in the presence of ascorbate/menadione, only a weak effect was observed since this percentage reaches about 23% while in control cells such a value was of 12%. In the presence of *m*-Cl-CCP, an uncoupling

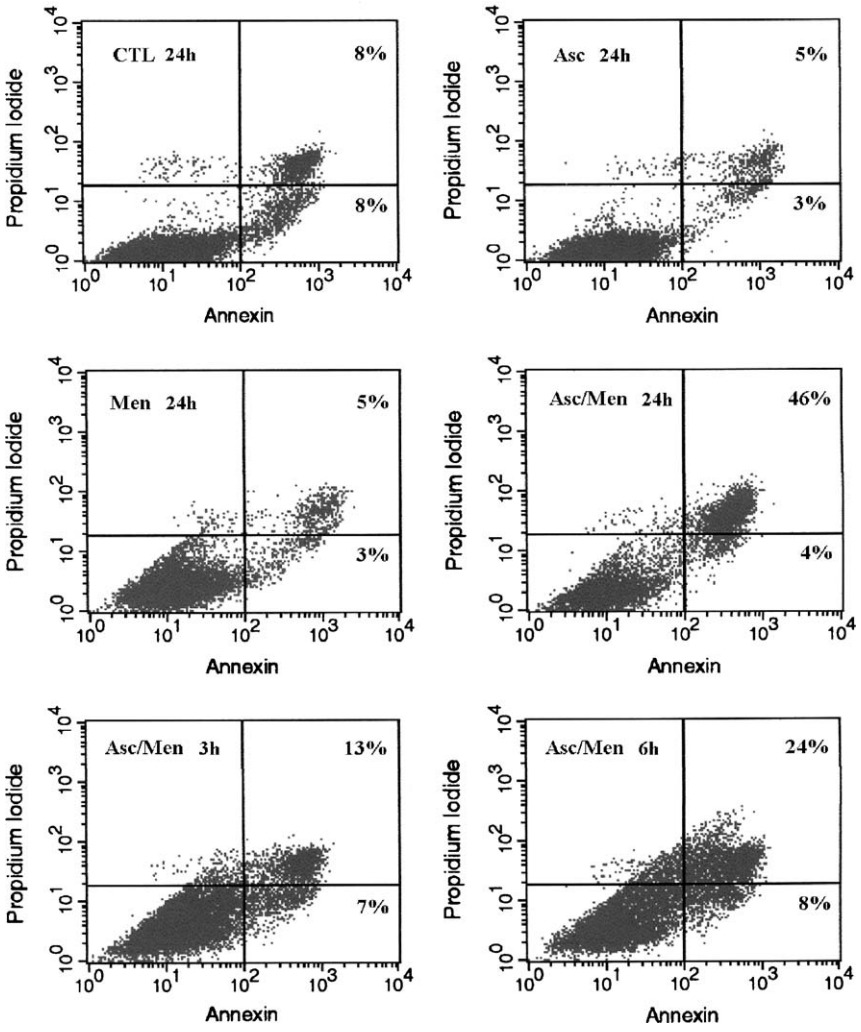

Fig. 4 – Annexin-V/propidium iodide staining. K562 cells were incubated in the absence (control) or in the presence of 2 mM ascorbate, 10 μM menadione and a mixture of both compounds. After 24 h of ascorbate/menadione treatment, a necrotic-like cell death becomes evident as shown by the increase in the upper right quadrant from 8% in control condition to 46% in these treated cells. Since the necrotic population already reaches 13 and 24% after 3 and 6 h respectively, the possibility of an early apoptotic population that degenerates in necrosis was excluded. Neither ascorbate nor menadione alone induce any sign of apoptosis or necrosis. A typical result out of three separate experiments is presented.

agent, the percentage of cells with depolarized mitochondria was more than 80%.

3.3. *Induction of a necrotic-like cell death by ascorbate/menadione*

Figs. 3 and 4 show that cell demise by ascorbate/menadione is consistent with a necrosis-like cell death. Indeed, DAPI and TUNEL staining procedures (Fig. 3) show that cells incubated in the presence of ascorbate/menadione have some DNA breaks. Once again, ascorbate and menadione added separately did not induce a DNA fragmentation. Since we have previously reported that the fragmentation profile of genomic DNA exhibited a DNA smear rather than a laddering profile [16], this appears to be a necrotic type of cell death. When cells were analysed by flow cytometry, those incubated in the presence of ascorbate/menadione show a necrotic profile as evidenced by the double staining Annexin-V and propidium iodide. Indeed after 3, 6 and 24 h of incubation, necrotic cells reached 13%, 24% and 46% respectively (Fig. 4). As expected, ascorbate and menadione added separately did not induce any sign of apoptosis nor necrosis even after 24 h of incubation. Fig. 5 shows the morphological changes of cells incubated in the absence (Fig. 5A) or in the presence of the association ascorbate/menadione (Fig. 5B). Actually, by using light microscopy, it can be observed that most cells exposed to ascorbate/menadione exhibited large blisters of empty cytoplasm coupled with a relocation of organelles around the nuclei. These cells are reduced to a perikaryon where the remaining cytoplasm is excised continuously and the nuclear chromatin undergoes further digestion.

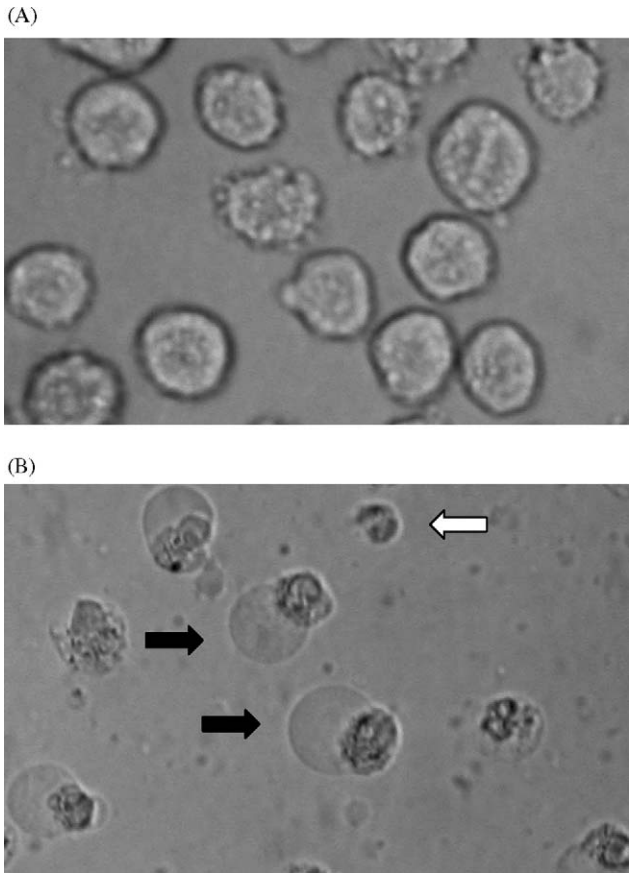

Fig. 5 – Morphological studies by light microscopy. K562 cells were incubated at 37 °C in the absence (control cells) (A) or in the presence of a mixture of ascorbate and menadione (2 mM and 10 μM respectively) (B). Pictures were taken after 24 h. Small dark arrows indicate some cells presenting large blisters of empty cytoplasm and a relocation of organelles around the nucleus. The small white arrow points to a cell reduced to a perikaryon.

3.4. *Ascorbate/menadione growth-inhibitory effect in a mouse xenograft model*

Finally, the potential inhibitory effect of ascorbate/menadione association on K562 cell growth was investigated in a tumour-bearing mice model. The leukaemia K562 cells were injected into female BALB/c nude mice and 48 h later, a single intraperitoneal injection was given to the animals of either saline or a combination ascorbate/menadione (1 g/10 mg/kg body weight, respectively). Four weeks later, in all the untreated-mice, the injected cells were developed thus reaching a considerable volume as compared to ascorbate/menadione-treated mice (Fig. 6A). The animals were killed and tumours from each mouse were excised (Fig. 6C), measured (Fig. 6B) and weighed (Fig. 6B). Fig. 6B shows that the mean tumour volume was significantly reduced by 65% in ascorbate/menadione-treated mice (210 ± 86 mm^3, $n = 9$) as compared to control mice injected with saline (594 ± 163 mm^3, $n = 7$). A similar reduction (60%) was observed when the weight of the tumours was compared: 311 ± 111 mg versus 764 ± 196 mg for ascorbate/menadione- and untreated-mice, respectively.

4. Discussion

In a wide variety of cells including murine hepatomas such as TLT [16], human leukaemias such as Molt4 [14] and urological cell lines such as T24 and DU145 [13], the association ascorbate/menadione has been shown to induce a time- and dose-dependent cytotoxic effect. This is because ascorbate enhances menadione redox cycling thus mainly generating H_2O_2 as has been highlighted by the enhanced cytotoxicity induced by aminotriazole (a catalase inhibitor). This is in agreement with previous results showing that the cytotoxicity by menadione/ascorbate was totally abolished by the addition of catalase (CAT), the enzyme that destroys the H_2O_2, and by N-acetylcysteine (NAC), an antioxidant acting as precursor for the biosynthesis of GSH [9,14,16]. Taken together, these data reinforce the key role of hydrogen peroxide as main oxidizing agent generated during the ascorbate/menadione redox cycling.

The results presented here confirm a previous report [16] showing that cell death is independent of caspases activation. One may speculate that oxidative stress is inducing apoptosis but caspases are not activated because critical SH residues are oxidized during the oxidant insult [18,19]. To check this possibility, we examined whether procaspase-3 was processed or not in ascorbate/menadione-treated cells. Our results show that neither procaspase-3 nor PARP were cleaved

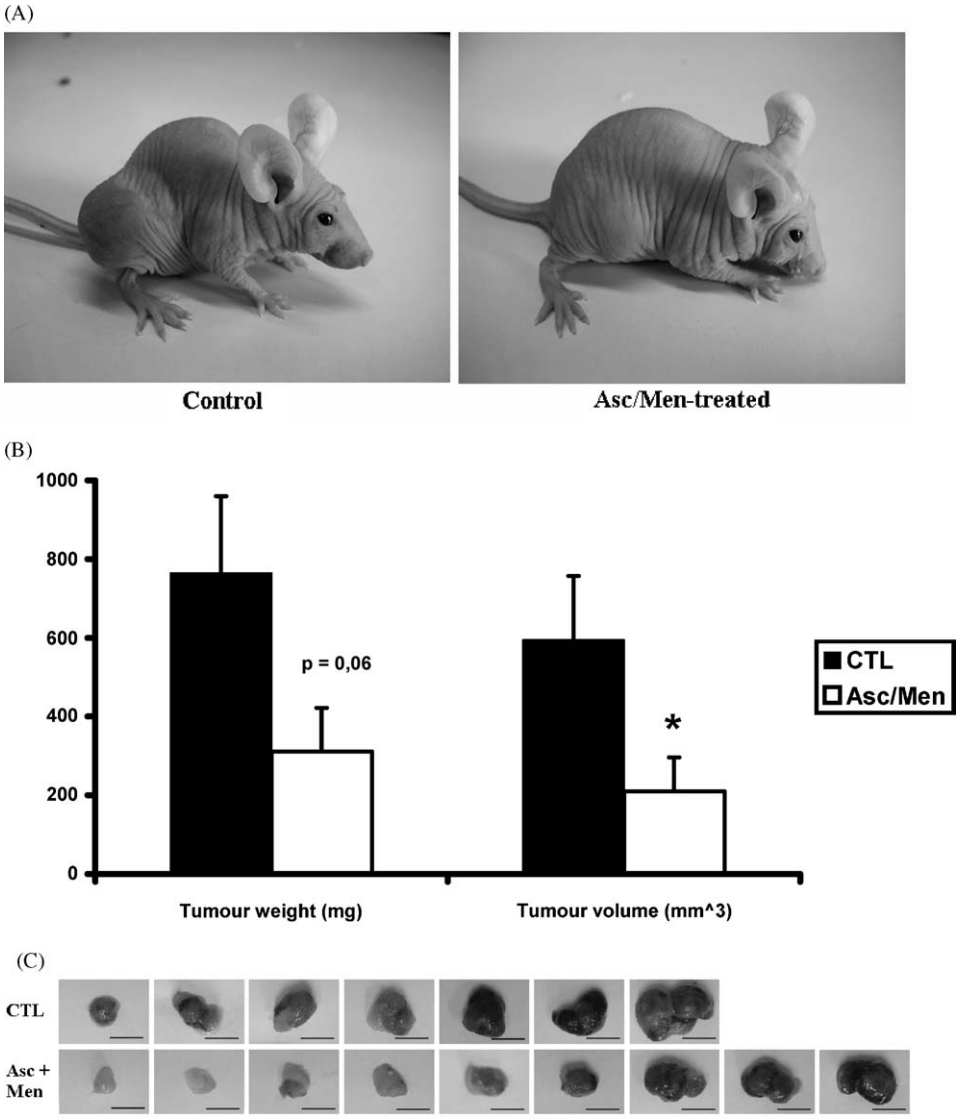

Fig. 6 – Reduction of tumour growth. (A) Examples of nude mice injected with saline or a combination of ascorbate and menadione. Treatment of immunocompetent mice with K562 flank tumours by a single injection of ascorbate/menadione association led to marked growth inhibition as shown by the mean tumour volume and weight (B). Tumour pictures of each group were taken after excision and are presented in (C) (scale bar = 1 cm). Histograms show the mean values ± S.E.M. $^{*}p < 0.05$ when compared with control values, using Student's *t*-test.

in ascorbate/menadione-treated cells. It should be noted that a weak release of cytochrome *c* was observed, suggesting that an incomplete apoptotic program was triggered in cells incubated in the presence of ascorbate/menadione. This cytochrome *c* release may be associated to a loss of mitochondrial membrane potential. Since procaspase-3 and PARP were not cleaved, the fact that cytochrome *c* was partially released from mitochondria suggests that apoptotic signals, if any, are being blocked upstream in the caspase activation pathway. Nevertheless, since in ascorbate/menadione-treated cells, both ATP and the reduced glutathione (GSH) are depleted by 70% and 50%, respectively [9], another possibility to explain the release of cytochrome *c* in the absence of a subsequent apoptotic process has been provided by Ghibelli et al. [20]. These authors reported that the release of cytochrome *c* is a cellular response to the depletion of GSH independently from the destiny of the cells, i.e. apoptosis or survival. Furthermore, the absence of apoptosis, even in the presence of cytochrome *c* leakage, could also be explained by the ATP-dependence of downstream steps like the apoptosome formation [21,22]. Moreover, a depletion of ATP by 50–70% is sufficient to change the mode of cell demise from apoptotic to necrotic [23]. Sanguinarine, a well-known inducer of apoptosis in K562 cells [17], was able to induce cytochrome *c* release, PARP cleavage and cell death by apoptosis. These results show that the lack of apoptosis was not due to an incomplete cell machinery. The absence of chromatine condensation (Fig. 3) as well as the DNA fragmentation profile showing no sign of DNA laddering but rather a smear profile as we have previously reported [16], suggests that cell demise was redirected to another form of

cell death. In addition, the morphology of dying cells, when incubated in the presence of ascorbate/menadione, is definitely excluding cell death by apoptosis. Indeed, our results show segregation of organelles in the perikaryon, blebs and signs of the self-excision of organelle-free cytoplasm on the cell surface, features that are closely related to necrosis. These morphological characteristics correspond to that reported by Gilloteaux et al. as autoschizis [11], a name coined by these authors to describe cell death on human bladder carcinoma T24 cells by ascorbate/menadione.

Finally, by keeping in mind that killing cancer cells is ultimately the key issue in anticancer therapy, the results reported here show that the association of ascorbate/menadione is doing exactly that in a very efficient way. This oxidative injury is most probably the main mechanism by which an antitumoural effect has also been observed in vivo. Indeed, after a single injection of ascorbate/menadione the growth of K562 cells in a model of immune-compromised mice was reduced by about 60–65%. Therefore, this association could be of particular interest in cancer therapy, and several observations argue for its potential use in human cancers. First, many tumour cells can survive the activation of caspases due to defects in signaling pathways [24], possibly leading to the acquisition of unexpected physiologic functions such as invasiveness [25]. Therefore, there is a need for additional death pathways, and especially those that are caspases-independent [26]. Second, since cancer cells are lacking antioxidant defenses [6–8] they are expected to be more sensitive towards oxidative stress by ascorbate/menadione redox than healthy cells. This difference of sensitivity between cancer and healthy cells was previously reported by us and others [9,27]. Third, the association of ascorbate/menadione could represent a new non-toxic auxiliary cancer therapy without any supplementary risk for the patients and could be easily included in the classical anticancer protocol.

Acknowledgements

The authors thank Isabelle Blave for her excellent technical assistance and Dr. Louahed (LICR-Brussels) for her advise during the FACScan experiments. This work was supported by grants from the Belgian Fonds National de la Recherche Scientifique (FNRS-FRSM Grant 3.4594.04.F and Télévie Grant 7.4520.04) and by the Fonds Spéciaux de Recherche (FSR) Université Catholique de Louvain.

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