Autoschizis: a new cell death induced found in tumour cells induced by oxidative stress mechanism

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1. Introduction

The incidence of carcinomas in the digestive system [1], the central nervous system [2] or in carcinogenesis [3-5] was found to be in direct relationships with the repressed activity of nucleases (DNAses and RNases) in tumors. Hence, a possible reactivation of both alkaline and acid nucleases could be envisaged to decrease the resistance of those cancer cells to radiation and chemotherapy. Taper and his collaborators [6-9] found that the co-admistration of ascorbate (VC) and menadione (VK3) resulted to a variety of carcinoma cell lines resulted in tumor specific antitumor activity of those aforementioned therapies at doses that were 10-50 times lower when either vitamin was administered alone. Additionally studies using a murine acites transplantable liver tumor model have shown that the VC: VK3 combination is an effective chemosentisizer that induces little systemic or major organ pathology [9-10]. Similarly, a study done in vitro providing similar data as in vitro allowed us to reinforce this contention when a combined vitamin treatment allowed us to show the degradation of the proliferating malignant tumors in nude mice [10, 11].
From an original study dealing with bladder carcinoma cells treated by CK3, scanning electron micrographs showed peculiar cell excisions [12]. From which data emerged the conviction that a special way of cell death occurred in tumor cells. Verified in 1998 and later by a series of publications summarizing our observations in human carcinoma cell lines, bladder, prostate, ovarian, etc and other carcinoma cell lines [13-22] as well as in vivo [10, 11]. One summarizes in this contribution some of the main cytotoxic changes observed in those cancer cells obtained as a result of treatments by VC alone, VK3 alone, combined VC+VK3 [23] as well as in vivo studies in the aim to support the use of this vitamins' combination as adjuvant therapy or treatment strategy against some forms cancers [24]. As shown in the report those vitamins, used against cancer cells, were able to trigger and induce characteristic cell injuries that lead toward a new form of cell death we have called autoschizic cell death or death by autoschizis, different tham necrotic or oncotic cell death or apoptotic cell death [12-22].

2. Material and methods

Carcinoma cell lines: Human MDAH2770 (ovarian), RT4 and T24 (both bladder) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in McCoy's 5A medium (M5A, Gibco, Grand Island, NY) supplemented with 10% bovine serum (FBS, Gibco) and 50 mg/mL gentamicin sulphate (Sigma St Louis, MO) as 1.0 x 10^6 cells grown in 12 mm diam titer dishes, incubated at 37° C with 5% CO_2. Chemicals: sodium ascorbate (VC), menadione bisulfite (VK_3) were purchased from Sigma and dissolved in the culture medium to final concentrations of 2,032 µM and 20.32 µM VK_3. To prevent photodegradation of the vitamins, all the vitamins' solutions and experiments were performed in a darkened laminar flow hood. Following overlaying the cells' monolayers to 1, 2, 4, 6h with 2 ml M5A containing phosphate buffer saline (PBS) as Control-Sham or to each of the vitamin alone, and to the combined VC+VK_3 vitamins at their 99% cytotoxic doses (CD_{99}).
Microscopy: Treated cells were then washed with phosphate buffer saline (PBS), harvested altogether and processed for light (LM), transmission (TEM) and scanning (SEM) electron microscopy. In this report, in order to reveal and verify subcellular changes associated with cell damages and cell deaths resulting from these treatments, sets of cells treated 2- hour long were prepared for TEM. For TEM cells were fixed in 2.5% phosphate-buffered glutaraldehyde solution (with 0.13 M sucrose-phosphate buffer (SPB), pH 7.35 for 30 min. After fixation, pellets obtained at less than 500 rpm were 2 x 10-min washed in SPB, and postfixed 30 min in a 1% osmium tetroxide solution. The fixed specimens were then washed twice for 10 min in SPB before they were dehydrated and processed into PolyBed epoxy resin (Polysciences, Warrington PA) in the titer dishes. After polymerization, 1-µm thick sections cut and stained with Toluidine blue were used to select areas with LM without overlapping cells. Ultrathin sections were spread on 50 or 75- meshed grids (SPI, West Chester PA), contrasted they were examined in a Zeiss EM-10 TEM (Carl Zeiss, Thornwood NY) using digitized images captured with an analySIS 2.1 software system © (Soft Imaging System GmbH, Lakewood CO and Münster, Germany) in the Pathology Department of Childrens' Hospital Medical Center of

Akron, OH. Scanning microscopy (SEM) examination was achieved by underwent similar treatment than the TEM samples. On titer plates cuts, cells were washed with SPB, fixed with the same glutaraldehyde solution as TEM and examined with an Hitachi S-570 model (Mountain View, CA.) at the Department of Biology, Behrend College (Erie, PA); micrographs were collected on Polaroid 55 films (Polaroid, Cambridge, MA) as in [14].
DNA fragmentation assay:- **Gel electrophoresis**: Tumor cells treated with the vitamins CD_{90} doses for 2 h, then cells were trypsinized, washed with ice-cold PBS, and resuspended at a density of 2 x 10^6 cells/mL in cell lysis buffer (5 mM Tris, pH 7.4, 5 mM EDTA, and 0.5% Triton X-100). After 2h on ice, the lysate was centrifuged at 27,000g for 20 min. The supernatant was exposed to proteinase K for 1 hr at 50° and extracted with phenol–chloroform. The aqueous layer was treated with 0.13 M NaCl and the DNA precipitated overnight at -20° C with 2 vol. of ethanol. Following treatment with boiled bovine pancreatic RNase for 1h at 50° C, the DNA concentration was determined spectroscopically and 10 µg per lane of DNA was loaded into the wells of 1.3% agarose gels. Electrophoresis was carried out for 3h at 10 mA with TBE buffer (89 mM Tris–HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.0). A HaeIII digest of X174 DNA was applied to the gel as markers. DNA was visualized by ultraviolet illumination of ethidium bromide-treated gels [25].

3. Results

Fine alterations induced in tumour cells by VC alone, VK_3 alone and combination of VC+ VK_3 versus Sham-treatment were surveyed. They include: overall cell morphology, changes affecting plasma membrane and superficial cytoplasm, nucleus and nucleolus, organelles and also focusing on injuries or damages progressing toward cell death, especially those leading toward autoschizis and autoschizic cell death.

3.1. Sham- treated cells (Fig 1 A-B):

All carcinoma cells, here exemplified by MDAH 2770 cell line, commonly appear pleiomorphic with a polyhedral-like profile in most sections. They usually range from 15-25 µm in diameter and display delicate and short filopodia or microspikes. Viewed by SEM or LM with random sectioning, most tumor cells usually possess a main, ovoid-shaped, euchromatic nucleus with a variable sized but typical, shallow, indented groove that makes the nucleus resembling in a thick section as a wide, coffee bean profile. One large, reticulated and branching nucleolus, usually adjacent or associated with the nuclear indented zone forms strands and anastomoses into a network-like pattern containing the dense and fine fibrillar components making several nucleolar organizer centers (NORs), the granular component, interstices and the associated heterochromatin; this later is usually more electron densely contrasted than all other components. Abundant ribosomes or polysomes admixed with glycogen-like particles or often aggregates make most of the fine inclusions distributed quasi evenly throughout the cytosol among all the other organelles of these active, tumor cells. Mitochondria with elongated profiles appear dispersed in the perikayon and often occupy a large cytoplasmic region spreading outwardly in a fan-like fashion facing the nuclear indent with all other cell organelles. Their distribution can be suggested by cytoskeletal components originating from a centrosome core, located adjacent to the nuclear concave zone. Rough endoplasmic reticulum (RER) and smooth ER (SER) components are found amongst the other organelles, sometime closely associated with mitochondria with small, narrow, elongated or even with sinuous, curved cistern shapes. The Golgi apparatus is not prominent and appears in the form of small dictyosomes. Lysosomal activities are suggested by viewing small onion complexes, small heterogeneous vacuoles or membranous components encircling organelles confirming small, but non-deleterious, cell maintenance as autophagocytotic events occur in the cytoplasm of these active Sham-treated tumor cells [15, 18, 22]. Those are similarly to those that would be found in untreated tumor cells but also in normal, active transitional epithelial cells. It is also common to detect short, annulate lamellae among the perikaryal cytoplasm.

3.2. Morphological similarities in treatment with either VC or VK_3

Individually VC (Fig. 2A-B) or VK_3 (Fig. 3A-B) treatments cause significant reduction in the cell diameter compared to the Sham treated cells. Additionally, the VC treated cells showed a decreasing size in a time dependent manner [12 - 23]. Both treatments affect the cytoskeleton as they favor self-excisions or superficial bursts of the cytoplasm free of organelles. These excisions are confirmed by cell distorted lamellipodia, edges and all sized debris noticed throughout most intercellular spaces, whether viewed by either SEM or TEM (Figures 2A and 3B). After each treatment alone nuclei display a narrow 50 to 150 nm heterochromatic, electron dense layer covering of their inner envelope. Prolonged treatment of either VC or VK_3 leads to resolving later into a condensation of the nucleolus into one or more than one compact, osmiophilic masses and a segregation of their components. **I**t is also possible to count between a few large to as much as 20 nucleoplasm aggregates of separated, chromatic dots creating ovoid to circular patches of 0.3-0.6 µm diameter circumscribing both the remaining small and large nucleolar masses ; those can be called satellite nucleoli [22]. Their presence alongside the compacted, altered nucleoli, indicate both or either that the nucleolus is remodelled as some of its protein components can migrate (i.e. fibrillarin [23)] and without polymerases or even transcripts decreasing or totally shutting all cell synthesis or repair mechanisms [26]. All of the aforementioned nuclear events with nucleolar fibrillarin migration [23] are indicators of irreversible cell injuries preceding cell death of the tumour

cells by autoschizis**.** With immunolabelling and other biochemical investigations, specific steps and differences could be further elucidated.

Again, in both treatment regiments most organelles are segregated into a large cytoplasmic sector facing the concave side of the indented nucleus. Among them mitochondria, SER and RER depict swelling and other damage associated with intracellular lysosomal leakage.

3.2.1. Ascorbate-treated cells (Figs 2 A-B):

After 2 hour treatment, most of the treated cells showed the chromatin-associated (DNA) separates or even leaves the nucleolar mass. The remaining nucleolar structure results in a dense aggregate of its granular component (ribonuclear proteins). A prolonged ascorbate treatment up to 4 hours exacerbates the number of cells with the changed morphology as described earlier [15-18, 22, 23] and discussed later in this contribution.

3.2.2. Menadione-treated cells or VK_3 treatment: (Figure 3 A-B)

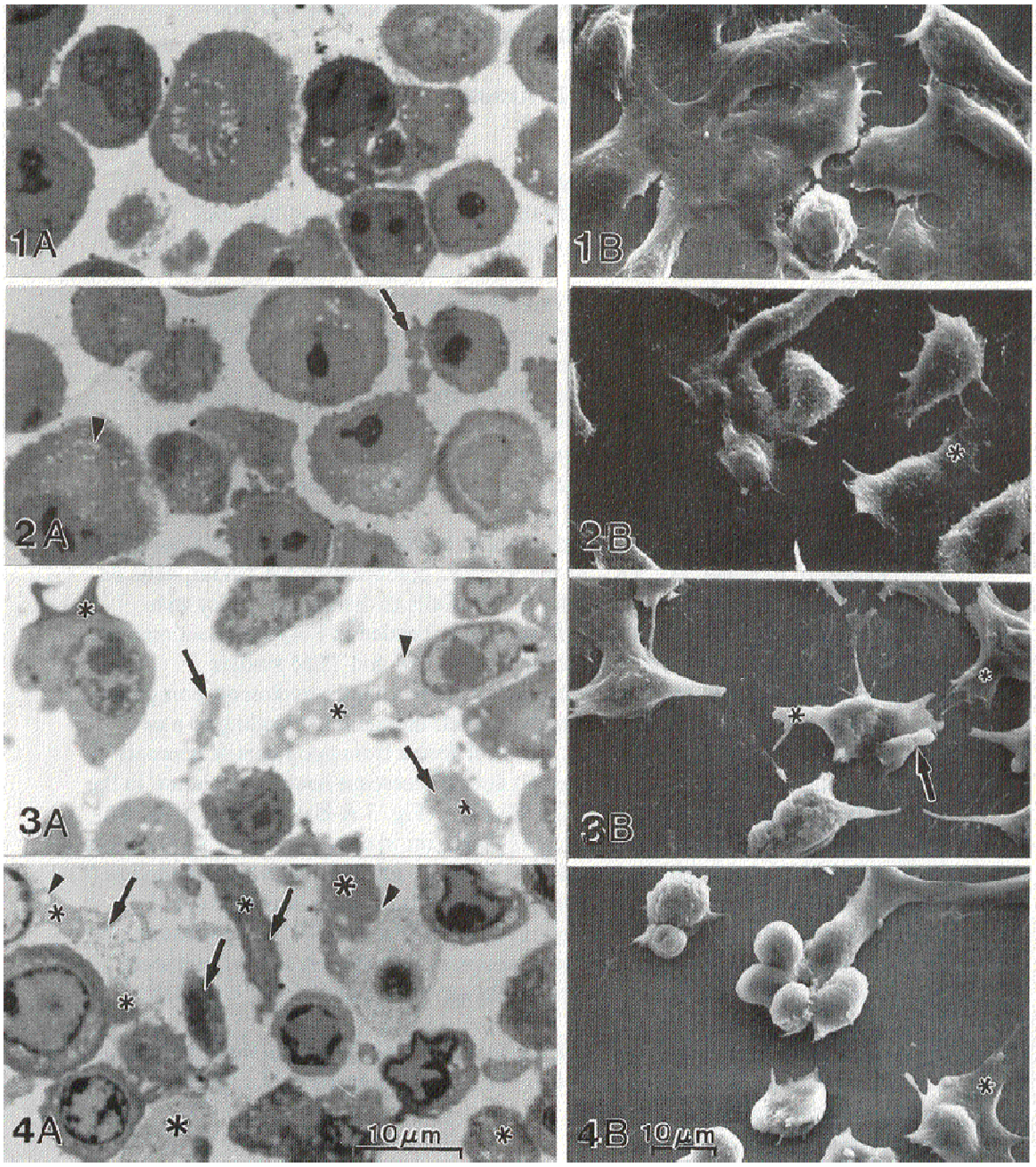
After 2-hour duration treatment by menadione, the nucleoli become separated into small, associated chromatin. It is quite characteristic to detect the chromatin after this treatment as it appears more densely contrasted than after ascorbate treatment alone. Minute, peripheral aggregates of heterochromatin can be seen throughout the nucleoplasm in addition to the inner surface of the nuclear envelope. Dilated SER and RER, Golgi apparatus, swollen mitochondria (with damaged matrices and cristae), heterogeneous lysosomal bodies accumulated in a narrow 1-um perikaryal zone as if corralled by reorganized cytoskeletal filaments while cytoplasm pieces were removed peripherally, as best suggested and viewed in [17].

3. 3. Combined Ascorbate: Menadione ($VC+VK_3$) treatment (Figs. 4 A-B, 5, and 6)

As a result of 2hours of this combined VC+VK3 action, most treated cells are depicting a series of cell's alterations similar to those described the aforementioned paragraphs but with an accelerated pace as one can observe similar changes as described above after 1hour of treatment. Most specific changes are: significant decreased cell diameter and rounding morphology, all proportional to the length of treatment, as well as accompanied by the number of tumor cell demises. The cell samples, slowly centrifuged, have cell remnants and debris that would have been collected among the supernatant removed during processing the samples as they would offer a slow sedimentation process and, if remained the same kind of corpses would be similarly collected in the buffered, washing solutions, again, during the samples' processing. Hence in the LM and TEM observed samples show remaining or surviving cells as obtained after a long cytotoxic treatment. .

After this combined treatment, nuclei are usually eccentrically located, of small diameter and their poorly contrasted nucleoplasm encompasses one or more oblong to spheroid, highly contrasted nucleolar masses caused by their extreme compaction (Figs 4 A-B - 6). A thin, heterochromatic layer is laid on the internal envelope and often shows a fragmented, thick to fine marbled appearance. The nucleoli and heterochromatin goes from a somewhat reduced contrast to clusters of fine chromatic, punctate dots to an uniformly, meltdown into a fine to undistinguishable poorly contrasted, homogeneous aspect accompanied by a wide range of nucleus shrinkage aspects. However, until ultimate demise the dying cells maintain their nuclear envelope integrity without showing any compaction on nucleoids as in apoptosis or apoptotic bodies (Figs 4 - 6). In the smallest cells, the nucleus remains in a central location and the cytoplasm is reduced to a rim of less than two micrometers, as comforted by LM, TEM and SEM views (Figs 4 - 6). In those diminished and dying cells, the narrow perikaryal, cytoplasmic sector is filled with innumerable strings of ribosomal particles remaining attached as beads on strings to mRNAs shaped as short winding, straight to sinuous hair loops. There most of the injured organelles, aggregated or congregated with an irradiating, fan-like spread displayed arrangement as if the cytoskeleton has rearranged itself (Figs. 5, 6). This can be confirmed as small to enlarged, elongated or spheroid vacuolated spaces created by SER-RER cisterns irradiate outwardly with their long axes as guided by and from the area of the perikaryon through (Figs. 5, 6).

Most of the organelle defects encompass changes of mitochondria, ER components and lysosomes. It is worth noting that, even with TEM, injured mitochondria cannot always be distinguished from lysosomal bodies as they attain an enormous size and can acquire a swollen matrix with huge osmiophilic deposits. Numerous and sometime prominent autophagosomes can be seen adjacent or appearing as an extension of the perinuclear envelope. Diverse stages of autophagocytosis with defects in lysosomal membranes can be observed along with or associated with other injuries that ultimately kill, in this case, human bladder carcinoma RT4 cells similarly to other carcinoma cell types as previously reported [13-22]. During the earliest stages of cell injury growing perinuclear lysosomal complex body complexes can be viewed in the form of huge phagolysosomes as well as diverse cytoplasmic vacuoles, at a later stage. While cell cycle is blocked after this combined treatment, during the further cell demise process, the heterochromatin changes was demonstrated to by of random degradation (Fig. 7). This induced process of cell death is the most frequently observed in vitro and was named autoschizis cell death [13-23]. The process is summarized in Figure 8.



Figures 1 - 4: Representative micrographs of human ovarian carcinoma MDAH 2770 cells following 2-hour vitamin treatments taken from Toluidine blue stained 1 um-thick sections (1A,2A,3A,4A) and SEM views (1B, 2B, 3B, 4B). In A: Sham-treated cells; B: VC treated cells; C: VK3 treated cells; D: VC+VK3 treated cells. Small arrows indicate cell fragments throughout intercellular spaces, arrowheads vacuolar defects and stars mark cell extensions in the process of self-excision exemplifying autoschizis (by permission from Microscopy & Microanalysis, 2001, 7 (Suppl 2): Proceedings, Microscopy Society of America)

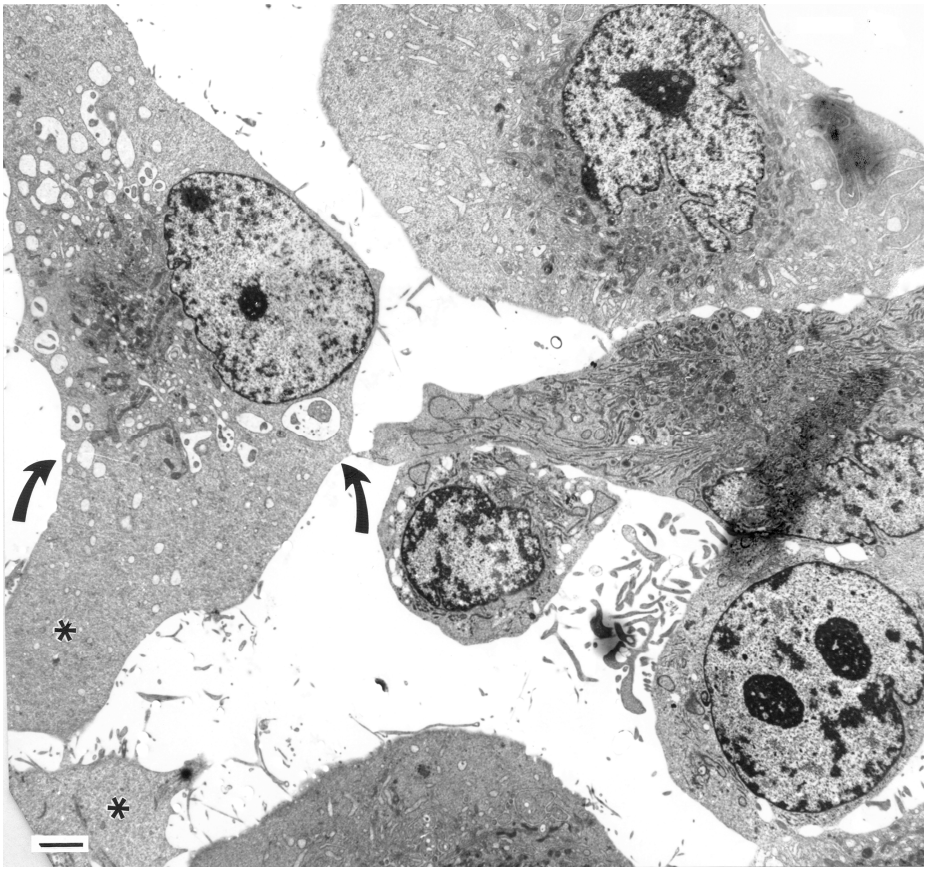


Figure 5: This field of view illustrates several adjacent human bladder carcinoma RT4 cells after 2-hour treatment by VC+VK3 depicting obvious morphologic pleoimorphis and defects: minute to large cells are noted in an ongoing excision (dense curved arrows) and cytoplasm rich in excessive swellings or even vacuolated SER and RER. Often RER appears as a fan-like organisation outwardly from the perikaryon. Organelles are segregated in the perikaryal zones. Nuclei show euchromatic aspects but nucleoli are revealed as round to ovoid condensations as dense ribonuclear masses and denoting cell damage. Scale is 1 um. (by permission from Ultrastructural Pathology [22])

4. Discussion

4.1. Sham-treated RT4 cells

Morphologic features described in our Sham-treated cells fit the fine structure of a carcinoma cell. The nucleus displaying a median indentation and appear as a coffee bean shape with numerous mitochondria clustered in the preikaryon. This large number of mitochondria is likely associated with typical mutations encountered in cancer cells and with an associated, poorly developed aerobic metabolism (Warburg effect). The smooth endoplasmic reticulum (SER) depicts SER aggregations and annulate lamellae with glycogen and even scattered lipid deposits. In addition, tumor cells often contain poor antioxidant detoxification system [27]. Peroxisomes are components of a detoxificating system that can be either either rare or present but show a significant lower catalatic and peroxidatic histochemical activities or biochemical activities than normal cells [e.g. in 27-30]. Similarly than in many other tumours, the expression and activity of DNases and RNases in prostate or other carcinoma cells is inhibited or diminished, as well as many other homeostatic enzymes [31-33]. These intrinsic defects in detoxificating persisting along a main glycolytic metabolism, and others defects, and they could be used as strategy to target tumor cells.

4.2 Cytotoxic actions of Ascorbate (VC) treatment:

In tumour cells, VC exhibits selective cytotoxic action, mutagenic and can kill primary and metastatic cells as well as potentiates chemotherapeutic drugs and also exerts radiosensitizing activity [6- 8, 11, 33]. Among tumour cell defects those that incompass plasma and organelles' membranes resemble those long described in oncotic necrosis: mitochondria, SER, RER, Golgi apparatus, lysosomes, etc. The mitochondrial ones are accompanied by light to abundant electron dense, matrix deposits caused by high levels of ionized calcium impeding ATP production. A decreased of ATP output demonstrated in [31-32] also favours further defects, like the lysosomal membrane leakiness and those increasing intracellular hydrolytic activities, affecting and blocking the cell cycle because damage to the cell

cytoskeleton and others associated with structural and regulatory proteins, especially those involved in the regulation of the cell cycle [34-37] . A cellular acidification can take part in the reactivation of nucleases, such as acid RNAses and DNase II that are usually inhibited in cancer cells [1-6, 45]. In addition, H_2O_2 itself and other reactive oxidative species (ROS) can also alter nucleosides, inhibit DNA synthesis, DNA repairs and induce DNA strand breaks [36,37]. Cytoskeletal damages caused by a remodelling of the superficial and deep framework can affects the redistribution of the organelles as observed throughout the treatments encompassing VC, and more even after both VC+VK3 treatment. Other intrinsic genetic and metabolic defects render RT4 cells unable to pursue cell cycle as VC damage liberate DNase-actin complexes and the freed enzyme contributes to alterations of mitochondrial and nuclear DNAs **[38, 39**]. All of these aforementioned conditions can predispose inexorably those tumor cells into unfavourable survival and instead, toward cell death.

Menadione or vitamin K_3 (VK_3) cytotoxicity

VK3 antitumor activity may be due to the ability of VK_3 to induce cell cycle arrest or delay at both G_1 /S and G_2 /M phases of the cell cycle and pro-oxidant properties [40]. Metabolically, it is also include a significant depletion of glutathione [31,32, 34], exhaustion of the protective NADPH [41], accumulated damages of mitochondria and peroxidations of sulfhydryl groups in many proteins, especially cytoskeleton, and those comprise stress actin, microtubules and cytokeratins [42-44]. Peroxidations of actin and other associated, dendritic macromolecule peroxidations can disrupt the general cell architecture through membrane defects that can disturb the exoplasmic regions (blebs, leakiness, etc) but also the nucleus morphology.

The cytoskeletal defects ultimately contribute to the internal and external phenotypical changes of morphology to favor odd cell shapes, including dwarfing surviving tumor cells caused through self-excisions resulting in some populations of small size, elongated or with lamellar extensions (Fig. 4 A-B) given by the dynamically repairs made by the same damaged cells to preserve the protoplasmic perikarya [13, 15, 18, 22]. In addition to blebbings and self cutting of pieces of cytoplasm and shedding them, forming the autoschizic bodies (pieces of cytoplasm without organelles, contrarily to apoptotic bodies that may contain not only organelles but also pieces of nucleus chromatin compacted without envelope). Again, DNase I reactivation induced by VK3 cause the separation of the G actin-DNase I complex while disturbing the actin network [38,39].

Nuclei, chromatin content and nucleolus alterations viewed after VK3 treatments are morphologically suggestive to resemble that of G1 phase block of the cell cycle (Fig 4 A-C) to that diagrammatically shown in [22,23, 34]. NADPH and glutathione depletion also can induce cell cycle arrest or delay at S/G2 phase [20]. Not only the actin cytoskeleton can be damaged by the treatments, as part of the nucleus as karyoskeleton but also myosin I [40] and other macromolecular oncogenetic factors [43] that affect some of tumour cell replication functions. Within the same realm of injurious events, the energy sources for repair and duplication, synthesis and repairs of DNA are perturbed, inhibited and/or damaged by reactivated DNases and RNAses, stimulated by VK_3 treatment during the first hour or early hours [10). VK3 is also able to induce DNA damages in vitro [7, 33, 43,44] and in vivo, in animal and human tumors [8 -11]. However, the cytotoxicity effects are suggested by the rapid phenotypic alteration of the tumor cells morphology as revealed in several previous: caspase-3 independent reactivation of DNase I within the first hour of treatment [6, 10, 24]. DNA degradation appears as heterochromatic changes, clearing or nucleoplasm while nucleolus sees its components segregated (Fig. 4 A-C). The demonstration of Feulgen densitometry [16, 18] confirms these losses caused by VK3.

Cytotoxicity of the VC: VK_3 combination

The combined vitamin actions have been reviewed several times along with each of the experiments done in vitro [40-44] and in vivo [8-11] and explained in the above paragraphs. The data obtained through several reports have been verified thatboth VC and VK3 interactions foster single electron reduction to produce long-lived semiquinone and ascorbyl radicals with an increase of rate in redox cycling forming H_2O_2 and other ROS [34, 43]. Tumor cells cannot really cope with those concerning our tumour cell killing strategy, McGuire and others [45] recently demonstrated that cathepsin D and L are leaking off those damaged lysosomes and eventually enter the nucleus. Those enzymes can contribute to more intranuclear cytoskeletal and protein injuries an can affect NF-κβ activity and associated mechanisms allowing tumor cells to escape cell death.

Altogether these nuclear injuries can explain some of the changes of nuclear electron density and the DNA degradation o served. Again, histochemical data, using Feulgen stain absorbance densitometry [16,18] demonstrated a significant DNA degradation verified by gel electrophoretic and already verified by cell flow cytometry analysis [21].

Finally, all gel electrophoretgic data support DNA is randomly degraded and certainly not cut internucleosomally as shown in apoptosis (Figure 7). All these DNA gel smears suggest more resemblance of cell death with oncotic necrosis. DNA fragmenting into small pieces can be stimulated by the reactivation of DNase II and reflecting the dysfunctional or ablated RNA polymerase. Again all data confirming the earliest hypothesis developed that a reactivation of nucleases can be related to injuring and eventually killing cancer cells [6].

We have compared the main, three modes of cell deaths earlier [16, 18, 22], but the morphology and data collected in the work done in our laboratories [16, 18, 22, 23] have always and consistently demonstrated that DNA degradation as well as Feulgen staining pattern, with other biochemical data [44] that the events summarized here rand reviewed again confirm that tumour cells can undergo a progressive demise leading to cell death by autoschizis, certainly not apoptosis or necrosis/oncosis as aresult of treatment inducing oxidative stress where lysosomal leaks contribute to this novel cell death mechanism.

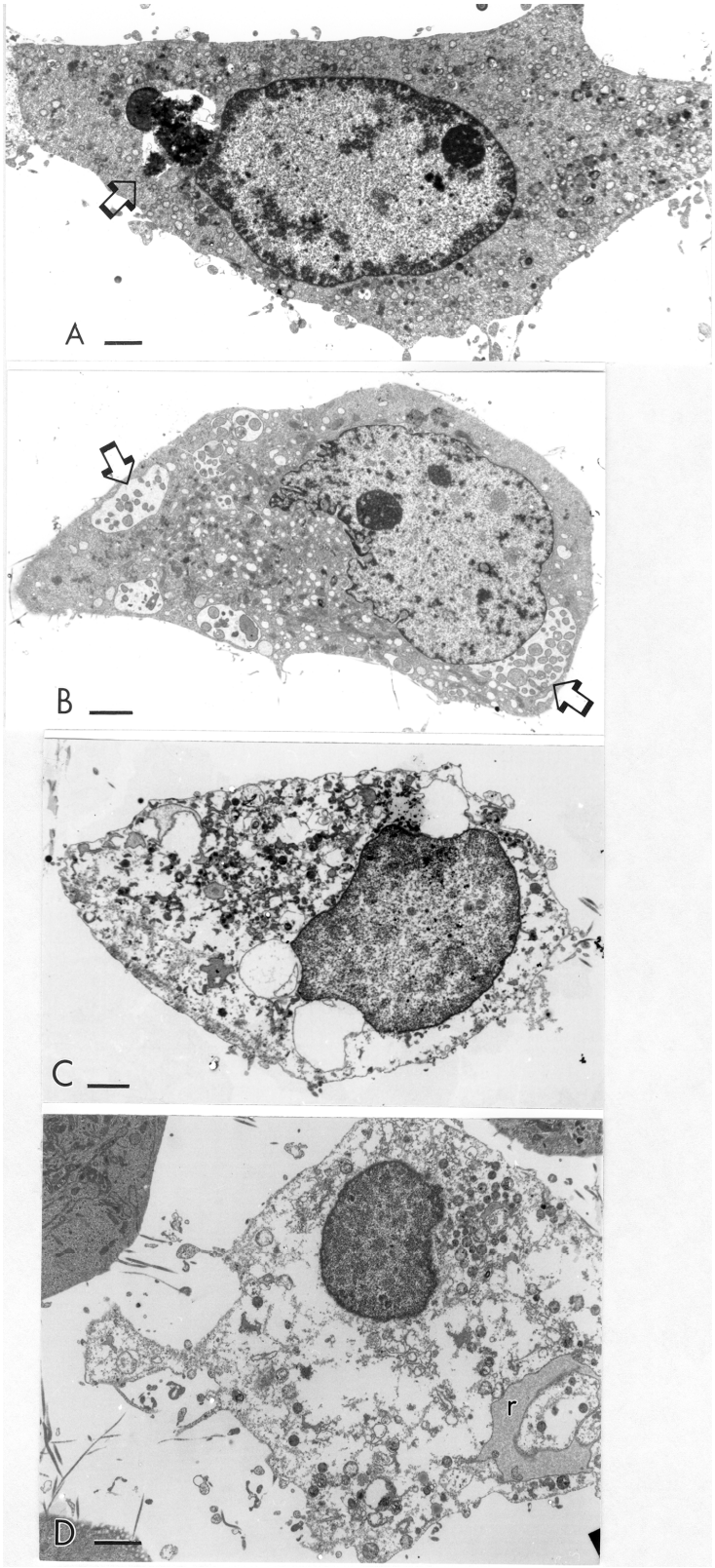


Figure 6: Representative examples and sequence of human bladder carcinoma RT4 cells observed after 2-hour ascorbate (VC) + menadione (VK3) treatment and undergoing cell death by autoschizis, not apoptosis nor necrotic oncosis. Notice from A to D the overall cell shrinkage caused by self-excisions and accompanied by alterations in the nucleus and nucleolus ultrastructure, suggestive of progressive DNA degradation, lack of repairs, transcription and translation defects. Lysosomal defects and cytoplasmic vacuolisations (open arrows) can be viewed along with a progressive degradation of the cells'integrity and demise.Scales in all micrographs is 1 µm (reproduced by permission from Informacare USA, Ultrastructural Pathology 34:140-160, 2010).

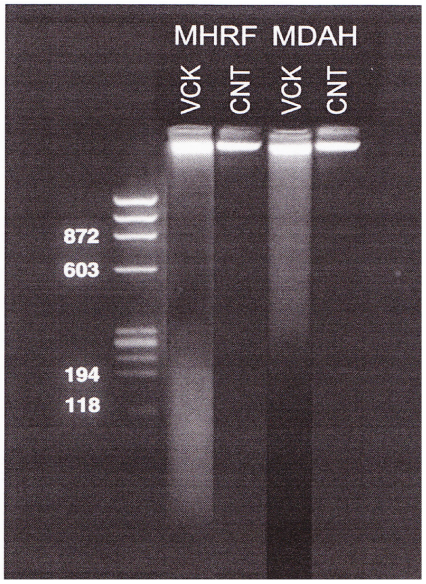


Figure 7: Gel electrophoresis of the total DNA extracted from Sham-treated and VC:VK3- treated human foreskin fibroblasts (MHRF) and MDAH2774 cells. Lane 1 contains molecular weight markers. Lane 2 contains the DNA from VC:VK3-treated MHRFcells (VCK) while lane 3 contains the DNA from Sham-treated MHRFcells (CNT). Lane 4 contains DNA from VC: VK3-treated MDAH cells (VCK) while lane 5 contains DNA from Sham-treated MDAH cells (CNT) (by permission from Elsevier, extracted from Tissue & Cell 2004; 36:197-210)

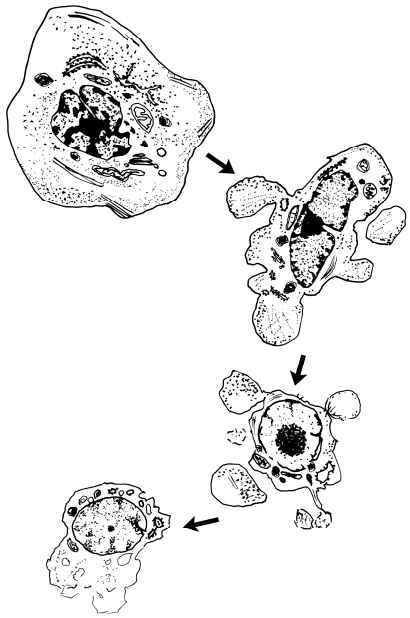


Figure 8: Diagrammatic representation of tumor cells undergoing autoschizic cell death. (reproduced by permission from Informacare USA, Ultrastructural Pathology 34:140-160, 2010).

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