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Biochemical Pharmacology 63 (2002) 1773–1783

Biochemical Pharmacology

Commentary

Autoschizis: a novel cell death

James M. Jamison[a,*], Jacques Gilloteaux[a], Henryk S. Taper[b], Pedro Buc Calderon[b], Jack L. Summers[a]

[a]*Department of Urology, Summa Health System/Northeastern Ohio Universities College of Medicine, Akron, OH, USA*
[b]*Unit of Pharmacokinetics, Metabolism, Nutrition and Toxicology, Universite Catholique de Louvain, Brussels, Belgium*

Abstract

Vitamin C (VC) and vitamin K_3 (VK_3) administered in a VC:VK_3 ratio of 100:1 exhibit synergistic antitumor activity and preferentially kill tumor cells by autoschizis, a novel type of necrosis characterized by exaggerated membrane damage and progressive loss of organelle-free cytoplasm through a series of self-excisions. During this process, the nucleus becomes smaller, cell size decreases one-half to one-third of its original size, and most organelles surround an intact nucleus in a narrow rim of cytoplasm. While the mitochondria are condensed, tumor cell death does not result from ATP depletion. However, vitamin treatment induces a G_1/S block, diminishes DNA synthesis, increases H_2O_2 production, and decreases cellular thiol levels. These effects can be prevented by the addition of catalase to scavenge the H_2O_2. There is a concurrent 8- to 10-fold increase in intracellular Ca^{2+} levels. Electrophoretic analysis of DNA reveals degradation due to the caspase-3-independent reactivation of deoxyribonuclease I and II (DNase I, DNase II). Redox cycling of the vitamins is believed to increase oxidative stress until it surpasses the reducing ability of cellular thiols and induces Ca^{2+} release, which triggers activation of Ca^{2+}-dependent DNase and leads to degradation of DNA. Recent experiments indicate that oral VC:VK_3 increases the life-span of tumor-bearing nude mice and significantly reduces the growth rate of solid tumors without any significant toxicity by reactivating DNase I and II and inducing autoschizis. This report discusses the mechanisms of action employed by these vitamins to induce tumor-specific death by autoschizis.

Keywords: Vitamin C; Vitamin K_3; Menadione; Autoschizis; Cell death

1. Introduction and rationale

1.1. Oxidative stress: the double-edged sword

ROS have been implicated in a variety of biological and pathological processes. For example, excessive production of free radicals can lead to oxidative stress and pathological conditions such as atherosclerosis, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis [1,2]. Moderate levels of oxidative stress can alter other processes including cell signaling, gene activation, cell growth and division, as well as cell death [1]. In fact, it appears that a pro-oxidant or oxidant can stimulate growth, trigger apoptosis, or cause necrosis depending upon its dose and time of exposure [1]. For example, 5 μM VK_3 (Fig. 1) stimulates DNA synthesis and promotes mitogenesis, while 10 μM VK_3 exerts antiproliferative effects, 20 μM VK_3 can induce apoptosis, and 100 μM VK_3 induces necrosis [3,4]. The situation is further complicated by the ability of H_2O_2 to inactivate caspase-3 and delay or inhibit apoptosis, causing tumor cells to die by a necrotic process [5,6]. These observations demonstrate the importance of the cell's ability to regulate the detoxification of ROS. Since many tumors are deficient in catalase, GP, or other detoxifying enzymes (especially manganese-containing SOD), other strategies must be employed to achieve these ends [7–10].

1.2. Antioxidants: the Trojan horse?

One of the strategies to overcome the deficiency of ROS-detoxifying enzymes may be to sequester antioxidants.

* Corresponding author. Present address: Department of Urology, Northeastern Ohio Universities College of Medicine, 4209 St. Rt. 44, P.O. Box 95, Rootstown, OH 44272-0095, USA. Tel.: +1-330-325-6468; fax: +1-330-325-0522.

E-mail address: jmj@neoucom.edu (J.M. Jamison).

Abbreviations: VC, vitamin C, L-threo-hex-2-enoic acid γ-lactone; VK_3, vitamin K_3, 2-methyl-1,4-naphthoquinone, menadione, Q; DNase I, deoxyribonuclease I; DNase II, deoxyribonuclease II; ROS, reactive oxygen species; SOD, superoxide dismutase; GP, glutathione peroxidase; Cdc25, cell division cycle; Cdk1, cyclin-dependent kinase; 2,6-DMBQ, 2,6-dimethoxy-*p*-benzoquinone; SEM, scanning electron micrographs (microscopy); TEM, transmission electron micrographs (microscopy); SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; ER, endoplasmic reticulum.

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PII: S0006-2952(02)00904-8

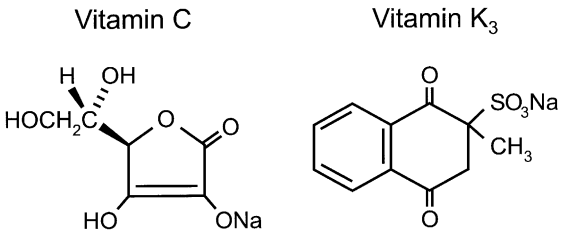


Fig. 1. Structural formulae for vitamin C (VC) and the sodium bisulfite form of vitamin K_3 (VK_3).

VC (Fig. 1) is one of the most abundant antioxidants in the blood. The results of *in vitro* and *in vivo* studies demonstrate that VC preferentially accumulates in malignant melanoma cells [11], human leukemia cells [12], neuroblastoma cells [13], tumor ascites cells [14], and other malignant cells [15]. While VC is traditionally perceived as an antioxidant, it may also act as a pro-oxidant [8]. In the form of ascorbate, VC can be oxidized by either one- or two-electron transfer and can be converted back to ascorbate by NADH-dependent semi-dehydroascorbate reductase or glutathione-dependent dehydroascorbate reductase, respectively [8]. This cycling process generates intracellular H_2O_2 and ROS that deplete cellular thiol levels, initiate membrane lipid peroxidation, and result in tumor cell death.

VK_3 is also known to preferentially accumulate in the tumors of rodents inoculated with Walker rat carcinoma 256 or Ehrlich mouse ascites cells. Furthermore, VK_3 and its derivatives have been employed as radiosensitizers because of their ability to concentrate selectively in malignant cells of certain human tumors and their metastases (including liver, kidney, bladder, prostate, stomach, intestine, and colon cancers) while exhibiting minimal accumulation in bone marrow [16,17]. VK_3 is considered an antioxidant because of its ability to scavenge superoxide ($O_2^{\bullet -}$), but it can also act as a pro-oxidant [18]. VK_3 can be reduced intracellularly *via* one- or two-electron transfer. One-electron reduction of the quinone to the semiquinone can be catalyzed by a number of flavoenzymes including NADPH cytochrome P-450 reductase, NADPH cytochrome b_5 reductase, and NADPH ubiquinone oxidoreductase. Subsequently, the semiquinone reduces O_2 to superoxide and in the process regenerates the quinone. As a result, redox cycling ensues, and large amounts of superoxide are produced. Superoxide may dismutate *via* SOD to form H_2O_2 and O_2 or may take part in metal-catalyzed reactions to form more toxic species of active oxygen, such as the hydroxyl radical and singlet oxygen. Any H_2O_2 that is produced will be reduced by catalase or GP. The GP reaction results in the formation of GSSG, which can be reduced back to GSH by NADPH-linked GSH reductase. If regeneration of NADPH becomes rate-limiting, GSSG accumulates and must be excreted. Therefore, if the rate of redox cycling exceeds the capacity of the detoxifying enzymes, GSH and other cellular thiols become depleted and cytotoxicity occurs [19].

DT-diaphorase is the principal enzyme that catalyzes the two-electron reduction of quinones to hydroquinones, which may form non-toxic conjugates or slowly autoxidize to reform quinones. The autoxidation of the hydroquinone generates the superoxide radical and other ROS (including the hydroxyl radical) and is the rate-limiting step in the redox cycling of the quinone [20]. While two-electron reduction has been considered a mechanism for detoxification, recent evidence suggests that it can also cause redox cycling. In this case, autoxidation of the hydroquinone produces a semiquinone and superoxide, which initiates a free radical chain mechanism that regenerates the quinone [20].

When VC is combined with VK_3, the interaction fosters one-electron reduction to produce the long-lived semiquinone ($Q^{\bullet -}$) and ascorbyl ($Asc^{\bullet -}$) radicals and increases the rate of redox cycling of the quinone [20,21]. According to Jarabak and Jarabak [20], ascorbate generates superoxide by two mechanisms (Fig. 2). In mechanism I, ascorbate ($AscH^-$) reduces VK_3 (Q) by one electron to form the semiquinone and ascorbyl radicals (reaction 1). This reaction is not favored thermodynamically and can occur only because the semiquinone is constantly removed by autoxidation (reaction 2). Ascorbate is oxidized and oxygen is consumed during the redox cycling process (reaction 3). In mechanism II, ascorbate initiates a one-electron reduction of a metal chelate (reaction 4), which is followed by the reoxidation of the metal chelate by oxygen to form superoxide (reaction 5). Alternately, the iron can react with hydrogen peroxide to form hydroxyl radicals ($^{\bullet}OH$) (reaction 6).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (6)$$

The superoxide formed in reactions 2 and 5 will also increase the DT-diaphorase-mediated redox cycling of VK_3 [20].

2. Consequences of exposure to VC, VK_3, and oxidative stress

It is obvious from the preceding discussion that the VC/VK_3 combination exerts its antitumor and antimetastatic activities through a wide array of mechanisms including: blockage of the cell cycle, modulation of signal transduction and potentiation of the immune system, and induction of autoschizic cell death.

2.1. Blockage of the cell cycle

When tumor cells are treated with the VC/VK_3 combination with a VC:VK_3 ratio of 100:1 and are examined using flow cytometry, two populations of cells are seen. The first population (~89%) is growth-arrested in late G_1 or at the G_1/S transition [22,23], while the second population of cells (~11%) undergoes a novel form of non-apoptotic cell death called autoschizis [24–26]. VC and

Mechanism I

$$Q + AscH^- \rightarrow Q^{\cdot -} + Asc^{\cdot -} + H^+ \quad (1)$$

$$Q^{\cdot -} + O_2 \rightarrow Q + O_2^{\cdot -} \quad (2)$$

$$AscH^- + O_2 \rightarrow Asc^{\cdot -} + H^+ + O_2^{\cdot -} \quad (3)$$

Mechanism II

$$Fe^{3+} + AscH^- \rightarrow Fe^{2+} + Asc^{\cdot -} + H^+ \quad (4)$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -} \quad (5)$$

$$AscH^- + O_2 \rightarrow Asc^{\cdot -} + H^+ + O_2^{\cdot -} \quad (3)$$

Fig. 2. Two mechanisms of redox cycling of VC and VK_3.

VK_3 induce a G_1 block in the cell cycle [22,23,27,28]. In the case of VK_3, the mechanism responsible for the block has been well studied. First of all, VK_3 is known to bind to the catalytic domain of Cdc25 phosphatase, which results in the formation of an inactive hyperphosphorylated Cdk1. VK_3 can also inhibit cyclin E expression at the late G_1 phase and cyclin A expression at the G_1/S transition. Together these effects cause cell cycle arrest or can lead to tumor cell death [28].

2.2. Signal transduction and immunopotentiation

When 2,6-DMBQ or other benzoquinones are combined with VC and administered to artificially immunosuppressed mice bearing human tumors, a profound inhibition of metastasis formation is observed in all the tumor models with the VC/benzoquinone combinations (but not VC alone) acting as immunostimulators and the combination treatment being more active than the benzoquinones alone [29]. A second study demonstrated that the synergistic antitumor activity is due to semiquinone radical-induced cytostasis coupled with potentiated CD4+ T-lymphocyte helper activity [30]. Likewise, phylloquinone, VK_3, and vikasol exhibit immunostimulating effects [31,32]. While the mechanism(s) responsible for this immunostimulation remains to be elucidated, the immunostimulatory effects may be attributed to their ability to induce interferon [33]. If interferon is induced, VC is known to increase the kinetics of induction and synthesis of interferon by interferon inducers [34,35]. Once interferon is present, only relatively low titers are necessary to suppress angiogenesis, tumorigenicity, and metastasis of tumor cells by affecting the local balance between positive and negative regulating molecules [36,37]. The immunostimulation may also entail the phosphorylation of the extracellular signal-regulated kinase (ERK2) and the subsequent activation of the Janus-kinase–signal transduction and activators of transcription (JAK–STAT) pathway by VK_3 alone or in conjunction with H_2O_2 generated during redox cycling. This type of activation could result in a coordinate up-regulation of many cytokines [3,38,39].

2.3. Induction of autoschizis

Autoschizis is a novel type of necrosis characterized by exaggerated membrane damage and the progressive loss of cytoplasm through a series of self-excisions. These self-excisions typically continue until the perikaryon consists of an apparently intact, round nucleus surrounded by a thin rim of cytoplasm that contains damaged organelles. During the process of cell death by autoschizis, nucleoplasms initially become more chromatic and then progressively lose chromaticity as their size decreases. Concomitant with this diminution in cell size, the nuclei become smaller and contain large nucleoli, which become round and compact. Therefore, before it dies, the size of the resultant autoschizic cell is much smaller than the tumor cell from which it originated [24,40]. Autoschizis was first observed during our work with prostate cancer cell lines in 1993 [40], but remained a cell death without a name until Gilloteaux coined the term "autoschizis" in 1998 [24] because of the tendency of the cells to cut themselves into small pieces. Subsequently, the ultrastructural characteristics have been described in great detail [25,26]. When sections of solid tumors from nude mice are observed following vitamin treatment, necrosis, apoptosis, and autoschizis can all be seen. However, autoschizis is three times as prevalent as apoptosis [41]. For this reason, it is believed that autoschizis may complement apoptosis in antitumor surveillance.

3. Autoschizis: not typical necrosis

3.1. On the road to autoschizis—where have the nucleases gone?

Roger Daoust was the first investigator who found deficient DNase activity in the cells of more than 60 malignant tumors in humans and experimental animals [42]. These results were extended by Taper and coworkers, who demonstrated that the activities of alkaline DNase (DNase I, EC 3.1.21.1) and acid DNase (DNase II, EC 3.1.22.1) are inhibited in non-necrotic cells of malignant tumors in humans and experimental animals as well as during the early stages of experimental carcinogenesis [43–47]. Indeed, it has been found that VK_3 selectively reactivated alkaline DNase in malignant tumor cells, whereas VC exclusively reactivated acid DNase [48]. Because DNase I and II reactivation occurs in spontaneous- and induced-tumor necroses and regression [41,48–50], compounds capable of reactivating DNase in tumor cells

should offer great potential for therapeutic intervention in cancer.

3.2. Autoschizis in vitro: ultrastructural changes

As was the case for apoptosis, the various stages in autoschizic cell death were first elucidated by light and electron microscopy [22,24–26,40]. Cells treated with VC alone exhibit changes in membrane composition, fluidity, and biochemical integrity. These cells are characterized by flattened perikarya and a loss of intercellular contacts. Prolonged treatment with VC induces more conspicuous membrane defects including blebs, extrusion of large pieces of endoplasm, and the formation of elongated microvilli. Portions of the blebbing membrane exhibit greater fluidity than the neighboring membrane and resemble liquid paraffin. The observed elongation of the microvilli is probably a specific response to membrane defects due to H_2O_2 and to perturbation of intracellular calcium [51]. These observations are consistent with those of Lupulescu [52], who showed that VC treatment of cells from basal and squamous cell carcinomas induced irreversible cytolysis, membrane disruption, mitochondrial alterations, and reduction in nuclear/nucleolar volume [52]. Additional work conducted in our laboratories [22, 40,53,54] has implicated H_2O_2 in the antitumor action of VC.

VK_3-treated cells exhibit cellular changes consistent with disruption of the cell cytoskeleton. VK_3 treatment for 1 hr produces large, round cells with wide, lamellar cytoplasm. The perikarya of these cells are bulging and exhibit damaged membranes. Longer VK_3 treatment produces a dramatic decrease in cell size due to cytoplasmic excisions and doublet formation. Many cells also are enucleated. Mitochondria are generally swollen and damaged and surround an apparently intact nucleus with the other damaged organelles. Ultrastructural studies of cultured mammalian cells [55,56] and human platelets [57] indicate that VK_3 exposure results in a redistribution of plasma membrane proteins and disruption of the normal organization of actin microfilaments, microtubules, and intermediate filaments. These disruptions of the normal organization of the cytoskeletal filaments have been ascribed to superoxide production and the subsequent oxidation and depletion of reduced intracellular glutathione as well as the modification of protein thiols.

Scanning electron micrographs (SEM) of tumor cells grown in titer dishes and treated with the vitamin combination reveal that approximately 25% of the cells are missing from the substrate after 1 hr, and 50% have been lost by the end of the second hour. This decrease in cell number is accompanied by changes in cell morphology as well as a significant decrease in cell size (Fig. 3). The remaining cells are spherical with diameters ranging from 10 to 15 μm. Long cell extensions irradiate from the perikarya and make intercellular contacts through long, tenuous, secondary filopodial extensions. After both 1- and 2-hr vitamin treatment, many cell doublets (cells which appear to be dividing) can be seen. In fact, these doublets represent the commencement of cell morsellation. The smaller portion of the doublet is comprised of a nucleated region or perikaryon surrounded by numerous, delicate filopodia. The anucleate portion of the doublet is biconcave and devoid of filopodia and microvilli. These observations are corroborated by transmission electron micrographs (TEM) in which the same doublet structures and cytoplasmic scissions are observed following a 1-hr exposure to the vitamin combination. This morsellation process can lead to the excision of one or more pieces of cytoplasm from the cell body or the perikaryon. These self-excised, cytoplasmic pieces do not contain organelles. However, at higher magnification, a granular, electron dense material can be seen between a disorganized, fine fibrillar, actin-sized material in the cytoplasm of the excising pieces.

During autoschizis, the portion of the perikaryal cytoplasm that is not excised is filled with many damaged organelles including: smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), mitochondria, membrane whorls, lysosomes, and fatty droplets. Most of the damaged organelles are found clustered in the cytoplasm around the nucleus. However, many small undamaged mitochondria also surround the nucleus. In contrast to the remainder of the cytoplasm which is uniformly electron-dense, the perikaryal region adjacent to the future excision zone shows numerous swollen, hydropic mitochondria with vacuous matrices and a flocculent inner layer which replaces the inner membrane. When this inner membrane is preserved, it appears as interrupted lines or fibers within this deposit. Mitochondria, which appear to be swollen, also display a fuzzy to contrasted precipitate within their matrix. As mentioned previously, a number of condensed organelles are interspersed between mitochondria, including whorls. However, it is difficult to recognize most organelles because of membrane lipid peroxidation and the subsequent distortion of organelle shape due to these peroxidative events.

In tumor cells treated with the vitamin combination, the nucleoli become condensed and fragment early in the process of autoschizic cell death. During the autoschizic process, chromatin becomes progressively less electron-dense. Initially, the chromatin assumes a striped appearance that ultimately appears as a fine granular material with low electron density. There is a continued diminution of the nucleus, which apparently remains intact despite the convolutions and deformations that are observed. While these nuclear changes occur, the tumor cells have reduced their size to average diameters ranging from 6 to 13 μm. Many blisters or small blebs can be observed along the narrow rim of cytoplasm that surrounds the nucleus even after this extreme cell size reduction. RER cisterns are always detected during these events. Additional nuclear and cytoplasmic changes occur through karyorrhexis and

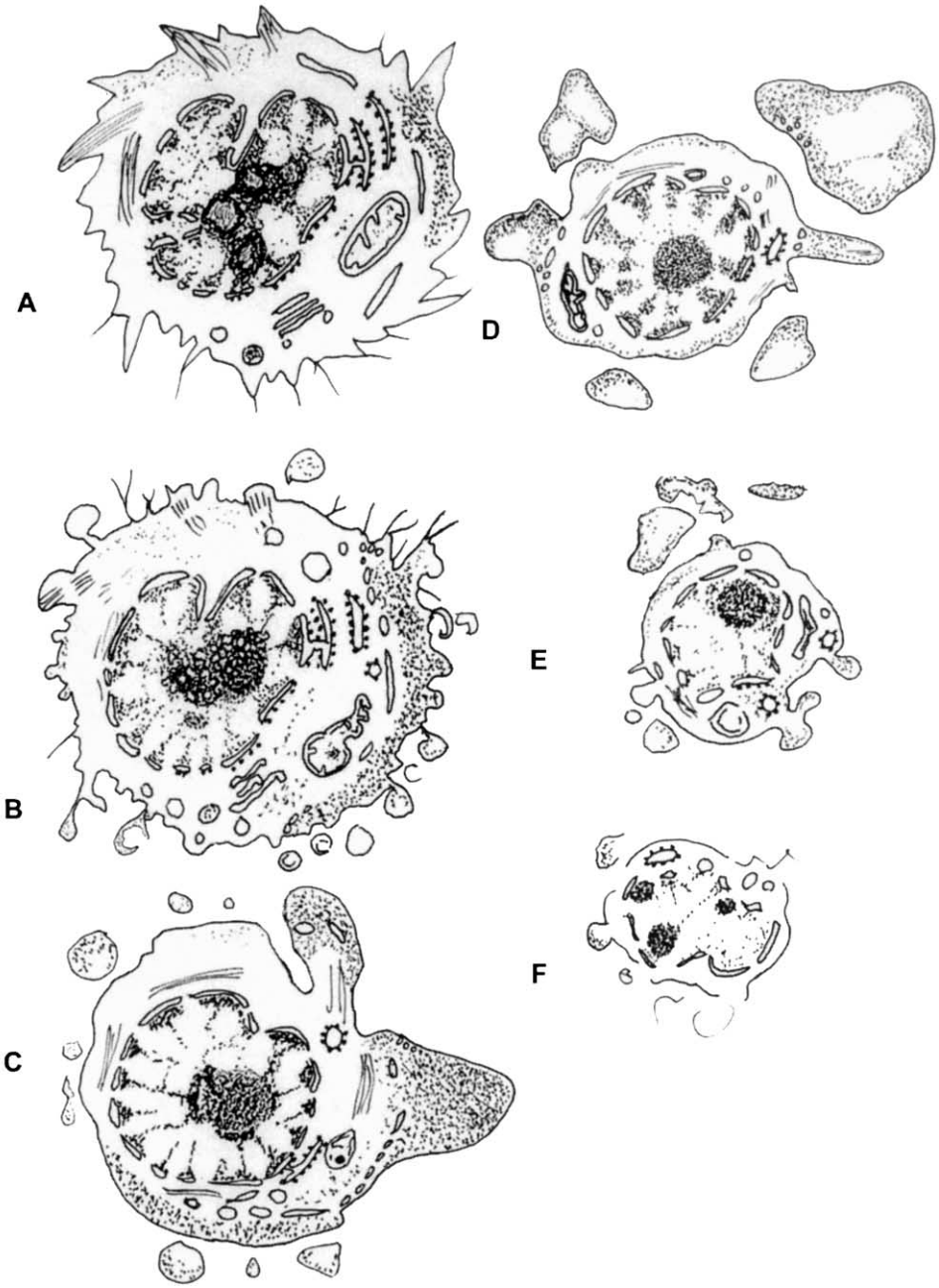


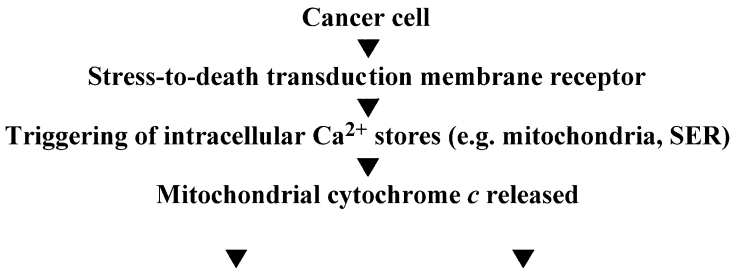
Fig. 3. Diagrammatic representation of the sequential steps in autoschizis. (A) This panel represents an untreated cancer cell. (B) A tumor cell exhibiting the plasma membrane defects, superficial cytoskeleton, and meshed nucleolus characteristic of an early stage of autoschizis. (C) As autoschizis continues, segregation of organelles in the perikaryon, progressive cytoplasmic excisions, rounding of the nucleus, and segregation of the nucleolus can be observed. (D) The nuclear/cytoplasmic ratio is maintained while cell size decreases through further autoschizic processes. Chromatin associated with the nucleolus is extruded leaving a round mass of ribonucleoprotein. (E) The tumor cell is reduced to a perikaryon where the remaining cytoplasm is excised continuously and the nuclear chromatin undergoes further digestion. (F) The tumor cell undergoes karyorrhexis and karyolysis.

karyolysis, which lead to cell death. Finally, the remnants of tumor cell death can be detected throughout the SEM and TEM preparations as cell debris. The ultrastructural changes that occur during autoschizis and apoptosis are compared in Table 1. While the signal that triggers apoptosis may or may not originate at a membrane receptor, the signal for autoschizis probably originates *via* damage-mediated mechanisms that promote intracellular Ca^{2+} increases. Both apoptosis and autoschizis result in the release of cytochrome *c*.

3.3. Autoschizis in vitro: biochemical changes

The antitumor and chemosensitizing effects of the VC and VK_3 combination have been demonstrated by *in vitro* experiments with different human tumor cell lines [15,53].

Table 1
Comparison of apoptosis and autoschizis



1.	Activation of several caspases	*No caspase 3* activation
2.	Caspase-activated DNase(s)	Little condensation of heterochromatin
3.	Little condensation of heterochromatin Nucleolus remains intact	DNA intercalation of VK_3 Segregation and compaction nucleolus DNase I activation (~1 hr post treatment)
4.	Decrease in cell volume from now until death Cell rounding (both *in vivo* and *in vitro*) Nucleus with perinuclear spaces Large masses of chromatin condensed at nuclear periphery (often crescentric)	Decrease in cell volume until death Cell rounding (*in vitro* and *in vivo*) Chromatin margination and peripheral patches; exhibits a peripheral striped patten
5.	Cell blebbing and progressive cytoplasmic self-morsellation *containing organelles and/or* round *nuclear micronuclei* made of hypercondensed chromatin surrounded by nuclear envelope (defined as *apoptotic bodies*)	Cell blebbing, some mitochondrial condensation and progressive cytoplasmic self-morsellation, but excised pieces *do not contain organelles, nor nuclear chromatin (autoschizic bodies)* Cell reduced to perikaryon
6.	Progressive nuclear micronucleation and segregation with excision into intercellular spaces	Progressive nuclear shrinkage while homogenization of nucleoplasmic electron density Nucleolar rounding, chromatin associated with nucleolus extruded from remaining ribonucleoprotein mass
7.	DNA is cleaved at one or multiple internucleosomal subunits, i.e. *ladder pattern in agarose gel*	DNA random cuts by DNase II (induced by VC, 4–16 hr post treatment) i.e. *spread pattern in agarose gel*
8.	Cell disappearance as broken pieces and cell remnants of dying cell are consumed by adjacent cells	Nuclear envelope and RER intact until karyorrhexis, dissolution of nucleolar masses Final cell volume is decreased one-third to one-half original cell size, karyolysis
9.	Minimal decrease of cancer cell population *without* local inflammatory (immune) reaction	Cell death leaving vacuoles and empty spaces leading to significant reduction of tumor volume, *including in vivo* inflammatory response(s)

These *in vitro* studies have been extended to a battery of human urologic tumor cell lines, including DU145, an androgen-independent prostatic carcinoma cell line, and T24, a transitional cell bladder carcinoma cell line [22,40,53,54,58]. Although the individual vitamins exhibit antitumor activity at high concentrations, co-administration of the vitamins in a VC/VK_3 ratio of 100:1 potentiates antitumor activity 4- to 61-fold even when exposure times are as short as 1 hr. These observations suggest that a signal is sent in the first hour of treatment that predisposes the tumor cells to die. For this reason, pulse-treated and continuously-treated tumor cells are run in parallel for all further experiments.

Taper *et al*. [9] have shown that the generation of H_2O_2 is essential for the antitumor activity of the VC/VK_3 combination. Likewise, our results confirm that exposure of tumor cells to VC, VK_3, or the VC/VK_3 combination results in the generation of H_2O_2 and other ROS [53,54]. Because co-administration of exogenous catalase destroys the antitumor activity of the vitamins, H_2O_2 and other ROS are implicated in the antitumor mechanism of these vitamins [53,54]. However, the fact that a greater amount of catalase is required to destroy the antitumor activity of VK_3 than is required to destroy the antitumor activity of the vitamin combination suggests that while H_2O_2 is involved in the mechanism of action of the

vitamins, the enhanced antitumor activity of the vitamin combination is not simply due to the increased production of H_2O_2 [53,54].

Because vitamin administration induces H_2O_2 and other ROS, which are known to induce lipid peroxidation, the amount of lipid peroxidation has been evaluated using the thiobarbituric acid method. While the increase in lipid peroxidation values for VC and vitamin combination treated cells is significantly higher than found in control levels, lipid peroxidation levels rapidly return to near control levels following removal of the vitamins in the 1-hr vitamin exposure experiments. At this time, little damage to the cell membrane is observed. These results suggest that the vitamins must remain in contact with the cells for a longer time period (2–3 hr) before significant levels of lipid peroxidation and damage to the cell membrane occur. However, TEMs demonstrate that mitochondrial architecture is rapidly altered by vitamin treatment and suggest that vitamin-induced lipid peroxidation of mitochondrial membranes occurs rapidly and may be involved in the triggering mechanism of autoschizis [40,53,54].

In light of the mitochondrial damage, intracellular levels of ATP synthesis have been measured to determine if vitamin-induced cell death is related to ''ATP-less'' death. Following a 1-hr pulse treatment with the vitamin combination, cellular ATP levels are transiently depressed and then rebound to a higher than control level. However, the results of Calderon and Taper [59] indicate that prolonged exposure to the vitamin combination significantly depletes ATP production. The mitochondrial damage led us to examine three other factors: DNA synthesis, protein synthesis, and intracellular levels of free calcium.

A tritiated thymidine incorporation assay has been employed to evaluate vitamin-induced changes in DNA synthesis 4 hr after a 1-hr pulse exposure to the vitamins. All the vitamins produce a significant decrease in DNA synthesis [53,54,59]. In addition, flow cytometry reveals that the vitamins induce a block in late G_1 phase that is due, at least in part, to the ability of VK_3 to inhibit cyclin A and E expression [22,28] and also shows the presence of an autoschizic peak [23]. Optical densitometry of Feulgen-stained tumor cells demonstrates a significant decrease in DNA content following vitamin treatment. Staining intensity is related to the length of vitamin exposure with the vitamin combination producing the greatest decrease in staining intensity [60]. A [^{35}S]cysteine incorporation assay employed to follow vitamin-induced changes in protein synthesis 4 hr after a 1-hr pulse exposure to the vitamins reveals a 30% increase in cysteine incorporation [53,54]. These results suggest that, in the case of the vitamin combination, higher ATP levels are consumed during the synthesis of cysteine-containing proteins. As is the case for ATP production, prolonged vitamin exposure to the vitamin combination significantly depresses protein synthesis [59].

The calcium content of the mitochondrial and extramitochondrial compartment has been evaluated using the change in absorbance of arsenazo III at 675 and 685 nm following exposure to the vitamins for 15, 30, 45, or 60 min [61]. There is a concurrent 8- to 10-fold increase in intracellular Ca^{2+} levels due to an equal release of Ca^{2+} from the mitochondria and at least one other subcellular compartment, such as the SER. Both second messenger-mediated and damage-mediated mechanisms can be involved in promoting intracellular Ca^{2+} increases [62]. In the case of autoschizis, VC and VK_3 have been shown to undergo redox cycle and generate substantial amounts of ROS that can damage the Ca^{2+} transport systems of the mitochondria, endoplasmic reticulum (ER), and plasma membrane. This damage allows diffusion of Ca^{2+} down its concentration gradient, disrupts Ca^{2+} homeostasis, and results in sustained Ca^{2+} increases [62,63]. Ca^{2+} dysregulation leads to the exposure of phosphatidylserine (PS) on the outer surface of the plasma membrane and activates a number of enzymes (i.e. phospholipases, proteases, and endonucleases) [61]. Since both apoptotic and necrotic cells display PS at the outer surface of the cell membrane, apoptotic cells must be discriminated from necrotic ones by the simultaneous use of propidium iodide (PI). Co-incubation of tumor cells with an annexin V–FITC/PI mixture following exposure of the cells to the vitamins reveals three populations of cells: the dual-labeled cells are the necrotic cells, the annexin-labeled cells are the apoptotic cells, and the minute, PI-labeled cells are the autoschizic cells. Following treatment with the vitamin combination, the PI-labeled cells are predominant [64].

As mentioned previously, Ca^{2+} dysregulation leads to the activation of DNases during apoptosis. Several candidate deoxyribonuclease molecules have been identified in various cell lines and tissues, including the caspase-activated DNA fragmentation factor caspase-3-activated DNase (DFF40/CAD) nuclease, DNase I, and DNase II. During apoptosis, activation of these endonucleases is usually capase-3-dependent and produces the characteristic laddering pattern on electrophoretic gels [62,65–67]. Conversely, during autoschizis, there is a sequential reactivation of DNase I and DNase II by a caspase-3-independent process, which produces a smearing pattern on electrophoretic gels [41,61]. DNase I has an alkaline pH optimum around 7.5 with enzymatic activity occurring in a pH range of 5.5–9.0 and requires micromolar concentrations of both Ca^{2+} and Mg^{2+} for optimal enzymatic activity. DNase II does not require divalent cations for its activity, and it has an acidic pH optimum at about 5.0 with enzymatic activity occurring in a pH range of 3.0–7.0 [66]. Nuclear DNase II is very stable and is only slightly depleted after 24 hr even in the presence of cycloheximide [67]. As mentioned previously, the activities of DNases I and II are inhibited in malignant tumors in humans [43–47]. In the case of DNase I, monomeric actin has been implicated as the inhibitory molecule [66]. Likewise, a

monomeric, natural protein inhibitor of DNase II has been purified to homogeneity from bovine liver, but has not been characterized. However, small acidic shifts in pH diminish the interaction between the inhibitor and DNase II and lead to recovery of nuclease activity [68]. Sulfhydryl agents, including VK_3, exclusively reactivate DNase I, while reducing agents, including VC, reactivate DNase II [48]. However, the effects (if any) of VC and VK_3 on the DNase–inhibitor complexes are not known.

The redox cycling of VC and VK_3 not only results in the cell insults described in the previous paragraphs, but also causes severe damage to the cytoskeleton. Within the first hour following vitamin treatment, cellular glutathione and thiol levels decrease to less than half of control cells. This loss of protection against ROS is accompanied by the oxidation, arylation, and subsequent disruption of microtubular and other cytoskeletal proteins [55–57]. This cytoskeletal disorganization is reflected by blister and bleb formation as well as by acute distortions in tumor cell shape [26]. Taken together, these results indicate that autoschizis entails the coordinated attack of ROS and other species on the antioxidant thiol buffer system, membranes, cytoskeleton, and DNA of the tumor cells that continues until cell death by self-morsellation (Fig. 4).

3.4. Autoschizis in vivo: chemosensitization and radiosensitization in immunocompetent mice

Combined administration of VC and VK_3 (at a single, intraperitoneal dose of VC = 1 g/kg body weight and VK_3 = 0.01 g/kg) in ascites tumor-bearing mice has been shown to synergistically increase the life-span of the mice by 45%, whereas separate administration of VC and of VK_3 increases life-span by 14.7 and 1.07%, respectively [9]. Combined treatment with VC and VK_3 also potentiates the effects of chemotherapy induced by six different cytotoxic drugs [9] and sensitizes a mouse tumor known to be resistant to vincristine [7]. Furthermore, mice bearing intramuscularly transplanted liver tumors that are pretreated orally and parenterally with single doses of VC and VK_3 and then are locally irradiated with single doses of 20, 30, or 40 Gy of X-rays exhibit a statistically significant potentiation of radiotherapy induced by 20–40 Gy [69]. This potentiated antitumor activity is related to the sequential reactivation of DNase I and II [7]. The weights of the thymus, spleen, testes, and kidneys of the vitamin-treated mice were not significantly different from those of the control mice. In addition, histopathologic examination of the main organs taken from the mice did not demonstrate

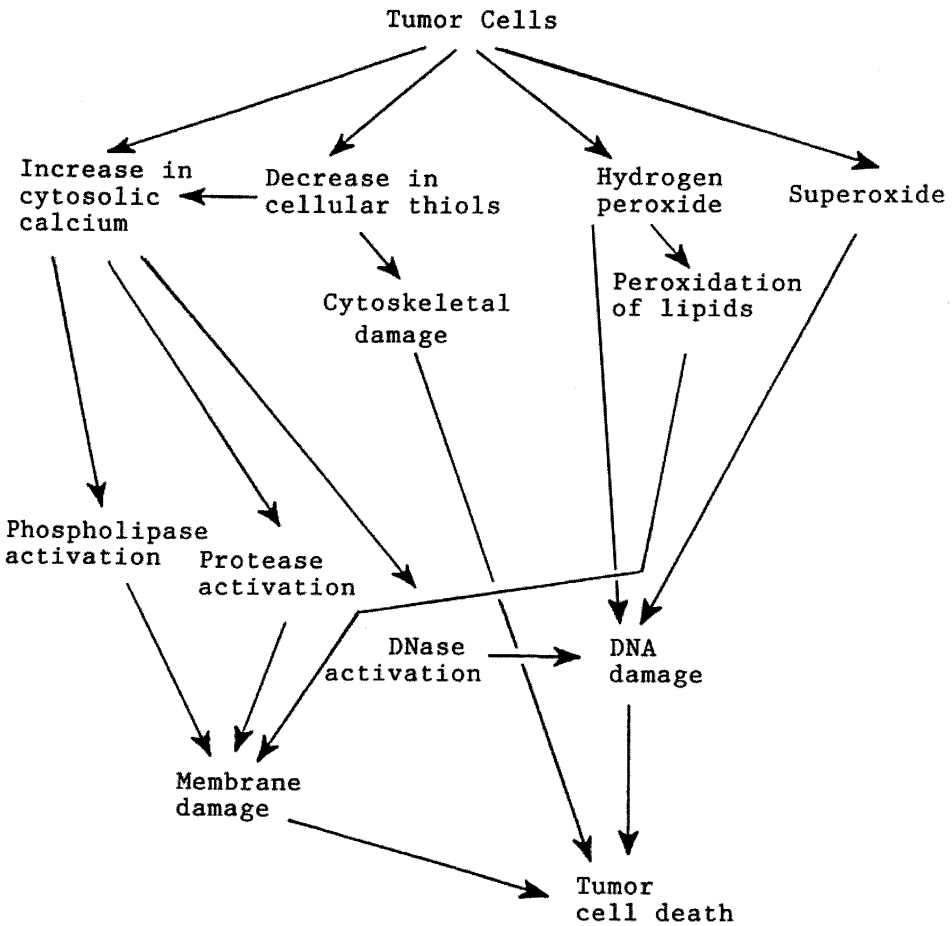


Fig. 4. A schematic of the mechanisms by which VC/VK_3 induce autoschizis.

any necrotic, degenerative, or inflammatory alteration [7,9]. These results suggest that, under these conditions, vitamin pretreatment does not increase the toxicity of the chemotherapeutic agent in the non-tumor tissues of the host.

3.5. Autoschizis in vivo: antitumor activity in nude mice

In vivo studies designed to determine the effect of vitamin administration on the life-span of nude mice demonstrate that mice receiving vitamins both orally and intraperitoneally (at the same doses used by Taper *et al.* [9]) live significantly longer ($P < 0.01$) than control mice. The results of additional *in vivo* studies, designed to determine the effect of vitamin administration on the growth of solid tumors in nude mice, demonstrate that administration of clinically attainable doses of oral vitamins *ad lib.* in drinking water could reduce the growth rate of solid prostate cancer tumors in nude mice significantly ($P < 0.05$). Finally, nude mice receiving the vitamin combination did not exhibit any significant bone marrow toxicity, changes in organ weight, or pathologic changes of these organs [58]. The human prostate cancer cells (DU145) implanted into nude mice are deficient in DNase activity. Following the administration of VC/VK_3, both DNase I and DNase II activities are detected in cryosections using a histochemical lead nitrate technique. DNase I activity appears 1 hr after vitamin administration, decreases slightly until 2 hr, and disappears by 8 hr following treatment. DNase II activity appears 2 hr after vitamin administration, reaches its highest levels between 4 and 8 hr, and maintains its activity 24 hr after treatment. Microscopic examination of 1-μm sections of the tumors indicates that following DNase reactivation autoschizis is the primary form of vitamin-induced tumor cell death [41].

3.6. Autoschizis in vivo: antimetastatic activity in immunocompetent mice

The results of a current study [59] demonstrated that oral administration of VC and VK_3 produces a distinct inhibitory effect on the development of metastases in C3H mice bearing an intramuscularly transplanted mouse liver tumor. Forty-two percent of control mice exhibited lung metastases and 27% possessed metastases in local lymph nodes, whereas 24% of the vitamin-treated mice exhibited lung metastases and 10% possessed local lymph node metastases. Detailed microscopic examination of the main organs as well as the lung and local lymph nodes (other tissues known to harbor metastases from this tumor) revealed a distinct inhibitory effect of oral vitamin treatment on the development of metastases. Microscopic examination of hematoxylin and eosin sections of the metastatic tumors from the vitamin-treated mice revealed the presence of tumor cells undergoing autoschizic cell death. As was the case in previous experiments, oral *ad lib.* administration of clinically attainable doses of VC and VK_3 in drinking water did not produce any adverse symptoms in the mice or macroscopic/microscopic pathologic alterations in the organs and tissues examined during this study.

3.7. Why is autoschizis so selective in vivo?

While various mechanisms have been proposed to account for the antitumor properties of VC–quinone combinations, no evidence has been presented to explain adequately the preferential attack on the primary tumor cells or their metastases, as opposed to normal cells. It has been suggested that the selective toxicity of the vitamin combination in tumor cells may be due to reduced levels of ROS-detoxifying enzymes in these cells, which leads to cellular damage through the accumulation of H_2O_2 and other ROS [7–10]. It has also been suggested that the selectivity of the vitamins for tumor cells may be related to their preferential accumulation in these cells [11–17]. Finally, the selectivity of the antitumor response has been attributed to the immunostimulatory properties of the vitamin combination [29–39]. Additional investigations are necessary to elucidate the importance of each of these mechanisms in the specificity of the antitumor and antimetastatic activities of the vitamin combination.

4. Markers of autoschizis

Autoschizis is a novel type of necrosis characterized by a set of ultrastructural changes, which include exaggerated membrane damage and the progressive loss of cytoplasm through a series of self-excisions that continue until the perikaryon consists of an apparently intact, round nucleus surrounded by a thin rim of cytoplasm which contains damaged organelles. During the process of cell death by autoschizis, nucleoplasms initially become more chromatic and then progressively lose chromaticity as their size decreases. Concomitant with this diminution in cell size, the nuclei become smaller and contain large nucleoli that become round and compact. Therefore, before it dies, the size of the resultant autoschizic cell is much smaller than the tumor cell from which it originated [24,40]. An autoschizic peak can be seen in flow cytometry traces [23]. Co-incubation of tumor cells with an annexin V–FITC/PI mixture following exposure of the cells to the vitamins reveals minute, PI-labeled cells that are the autoschizic cells [64]. Autoschizis is also characterized by the sequential reactivation of DNase I and II with both DNases being active 3–4 hr after administration of the vitamin combination [41]. Other markers are being elucidated even as you read this commentary.

5. VC, VK_3, and autoschizis in cancer therapy

Since the vitamin combination is a chemosensitizer, a radiosensitizer, and perhaps an immunostimulator and seems to induce autoschizis in a relatively tumor-specific fashion, perhaps combined VC and VK_3 administration should be considered as a new, non-toxic, adjuvant cancer therapy, which can be easily introduced into the classical protocols of clinical cancer therapy, without any supplementary risk for patients.

Acknowledgments

This work was supported by a grant from the American Institute for Cancer Research (97B048) and by grants from the Summa Health System Foundation, Akron, OH. We apologize to many colleagues whose work we did not cite due to space restrictions.

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