High Doses of Vitamin C and Leukemia: In Vitro Update

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Additional information is available at the end of the chapter

Abstract

Vitamin C (ascorbic acid) is an essential nutrient with a number of beneficial effects on the human body. Although the majority of mammals can synthesize their own Vitamin C, humans and a few other species, do not produce it and depend on dietary sources for their Vitamin C supply. Among its many effects on cell function and metabolism, Vitamin C has shown, in vitro, a powerful anticancer effect against a number of human tumor cell lines, including myeloid leukemia. There are many different mechanistic explanations for the anticancer/anti-leukemic effects of Vitamin C and the aim of the present review is to illustrate these mechanisms, showing the results of some preliminary in vitro investigations, and outlining their potential clinical relevance.

Keywords: Vitamin C, ascorbate, sodium ascorbate, high doses of ascorbate, intravenous ascorbate, cancer, leukemia, antioxidants, pro-oxidants, free radicals, oxidative stress, redox balance

1. Introduction

Vitamin C is an essential nutrient with a number of beneficial functions, for the organism [1], such as

1. helping the metabolism of tyrosine, folic acid, and tryptophan;

2. increasing the elimination of cholesterol;

3. contributing to the synthesis of catecholamines;

4. helping the body to absorb and breakdown histamine;

5. enhancing the absorption of non-heme iron;

6. promoting the synthesis of collagen (its most widely known physiological function);

7. neutralizing free radicals (it is a reducing agent, "scavenger" of free radicals, and a founder among the natural antioxidants);

8. protecting DNA from damage due to free radicals and mutagens;

9. reducing the risk of premature death;

10. fighting off widespread environmental pollutants; and

11. preventing the development of nitrosamines.

Though ubiquitous, ascorbate is not produced by humans, guinea pigs, some primates, a particular type of fruit eating bat, the majority of fishes and birds [2], who depend on diet for the assumption and use of this fundamental nutrient.

2. Vitamin C and leukemia: historical background

The first mention of the therapeutic potentialities of Vitamin C in leukemia, can be found in the book "*The healing Factor: Vitamin C against disease,*" written by the biochemist Irwin Stone, in 1974 [3]. In his book, Stone refers to a study, performed in 1936 by Stephen and Hawley [4], demonstrating, for the first time, that when the blood is separated into plasma, red blood cells, and white blood cells, there is a 20- to 30-fold concentration of Vitamin C in the white blood cells, as compared to plasma.

Following this report, Barkhan and Howard, by studying a few cases of chronic myelogenous and lymphatic leukemia, added the evidence that leukemic patients have substantially lower than normal plasma levels of Vitamin C [5]. As noted by Stone, although this knowledge could suggest the use of Vitamin C as a therapeutic agent, in leukemia, the first clinical trials showed contrasting results, due to the inappropriately *low doses* administered.

Later on, Vogt, in a literature review [6], confirmed that there are high deficits of Vitamin C in leukemic patients, as also confirmed by the reports of Kyhos and Coll. [7] in 1945, and Waldo and Zipf, in 1955 [8].

According to Stone [3], leukemia reduces the body stores of Vitamin C to very low levels, and any residual Vitamin C circulating in the blood is scavenged and locked in the excessive numbers of leukocytes characterizing this disorder. As a direct consequence, the plasma levels of Vitamin C are reduced to zero or close thereto, and tissues are no longer being supplied with this most important metabolite, since it is accumulated in leukocytes.

Stone [3] defined "*biochemical scurvy*" as the condition of insufficient Vitamin C supply to body tissues, and proposed that its correction required the administration of Vitamin C at a rate of 25 g or more per day.

In 2012, 76 years after the first observations on the "concentration" of Vitamin C in leukocytes, an investigation on 131 patients affected by different types of leukemia, definitively confirmed that leukemic patients have significant lower plasma Vitamin C than normal controls. The reduction of plasma Vitamin C levels in leukemia, as predicted by Stone, is due to an increased uptake and utilization by the actively proliferating leukocytes, leading to tissue biochemical scurvy and consequent increased tendency to bleeding and infections, which are the hallmark of this pathological condition [9, 10]. Interestingly, low plasma levels of Vitamin C, have been, very recently, found in around 30% of cases of Non-Hodgkin Lymphoma (NHL), particularly in patients with high bulk disease [11]. With the above data at hand, it is clear that leukemia can be viewed as a condition of functional Vitamin C deficiency, associated with biochemical scurvy, and therefore, all leukemic patients are suitable candidates for the treatment with this nutrient.

3. How much Vitamin C to treat leukemia? The concept of "mega-doses"

In 1949, Frederik Klenner first reported the successful treatment of bulbar poliomyelitis, with high doses of Vitamin C administered by intramuscular, intravenous, and oral route [12]. Klenner had also established clinical protocols using massive doses of Vitamin C to treat a number of different viral conditions, but only more than two decades later, Stone formally defined the concept and rationale for the use of "*mega-doses*" of Vitamin C. In particular, Stone observed that man and only a few other species do not produce their own Vitamin C, while the great majority of mammals do, according to their physiologic requirements [3]. This observation led the author to hypothesize that due to either insufficient intake or increased consumption of the nutrient, or both, man could easily undergo a condition that he defined "*hypoascorbemia*." Hypoascorbemia is a reduced amount of circulating Vitamin C (also called "ascorbic acid"), due to the lack of the enzyme L-gulonolactone oxidase (GLO), as a consequence of an "inborn error of carbohydrate metabolism" [13–15]. This defect, now very well acknowledged and characterized [16], led Stone to hypothesize that to be in good health, man needs mega-doses of Vitamin C (several grams a day) [17, 18], rather than doses in the order of milligrams, as stated by the Recommended Daily Allowances (RDAs) [19].

The rationale behind the use of mega-doses of Vitamin C was further refined by the chemist and twofold Nobel Prize, Linus Pauling. Pauling soon became an enthusiastic supporter of the use of this nutrient, in high doses, not only to prevent disease [20–23], but also to treat a number of pathologic conditions, ranging from common cold [24, 25] to cancer [26] and AIDS [27].

4. Intravenous Vitamin C and cancer

Studies on dose-concentration relationship in humans, performed by Levine and co-workers [28], revealed that at oral doses exceeding 250 mg/day, the plasma levels of Vitamin C reach a

plateau, and any further increase in the amount administered by mouth, does not determine significant increase in plasma concentration. This is due to multiple "control" mechanisms, including, among others, intestinal absorption, tissue accumulation, renal reabsorption and excretion, and utilization. On the contrary, the intravenous administration of high doses of Vitamin C, bypassing the above control mechanisms, allows plasma concentrations that are 100-fold or higher than maximally tolerated oral doses, and the peak could last for hours within the millimolar (mM) range [29].

More importantly, at plasma concentrations easily achievable by intravenous administration (5–10 mM for 1–2 h), Vitamin C induced death in 75% of 48 cancer cell lines tested in vitro [30], but had no toxic effect on human peripheral white blood cells, fibroblasts, or epithelial cells. This selective cytotoxic effect would be achieved since at high doses, parenteral ascorbate is a *peroxide delivery system* for the generation of sustainable ascorbate radical and H_2O_2. H_2O_2 would be produced in the extracellular space, with consequent oxidative damage to cancer cells [31, 32]. Therefore, Vitamin C in high doses would be cytotoxic to cancer cells because of its *pro-oxidant*, rather than anti-oxidant effect, even though some authors remark that the pro-oxidant activity of Vitamin C, may not be relevant, in vivo [33–35].

More recently, Yun and co-workers [36], by investigating the effects of high doses of Vitamin C on KRAS and BRAF mutants cells derived from colorectal cancer (CRC), have further refined the mechanistic explanation of the anticancer properties of Vitamin C. In particular, according to the authors, the death of KRAS and BRAF cell mutants of CRC is not caused by the Vitamin C itself, but rather, by its oxidized form, dehydroascorbic acid (DHAA). While Vitamin C enter cells though specific receptors, called sodium-Vitamin C co-transporters (SVCTs) [37], DHAA competes with glucose, for intracellular uptake by glucose transporters (GLUT), mainly 1 and 4 subtype receptors [38, 39].

Interestingly, both KRAS and BRAF activating mutations are responsible for the upregulation of GLUT1 expression in different types of cancer, including CRC [40, 41].

However, as reported by Yun and Coll. [36], the upregulation of GLUT-1 expression is not always associated with increased sensitivity of tumor cell lines to the cytotoxic effects of DHAA.

Further investigation into the metabolic makeup of KRAS and BRAF mutations CRC-derived cell lines, showed an accumulation of glycolytic intermediates upstream glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and a contemporary depletion of the metabolites downstream GAPDH. This finding indicates an inhibition or severe reduction of GAPDH activity, which appears to be the key of the cytotoxic effect of DHA.

In summary, the data reported by Yun and Coll. on the effect of DHAA on CRC cell lines, indicate that in glycolysis-addicted KRAS and BRAF mutated cell lines, high amounts of DHAA are transported into the cancer cells, through the overexpressed GLUT-1 receptors. The exceeding amounts of intracellular DHAA are then reduced again to Vitamin C with consequent consumption of glutathione (GSH), redox imbalance, and oxidative stress. Oxidative stress, in turn, causes GAPDH inactivation, with inhibition of glycolysis, and energetic crisis, ultimately leading to cell death [42].

More precisely, beyond being inactivated directly by ROS (including H_2O_2), GAPDH function is also hindered by the depletion of nicotinamide adenine dinucleotide (NAD+), caused by the activation of the DNA repairing enzyme, poly(ADP-ribose) polymerase (PARP), induced by damaged DNA. In fact, the increased production of ROS, in cancer cells, due to the high doses of Vitamin C, produces increased DNA damage and consequent activation of PARP. PARP, in turn, consumes NAD+ with consequent NAD+ depletion, ATP depletion, and cancer cell death [43].

5. High doses of Vitamin C and H_2O_2

The view that Vitamin C in high concentrations, administered by intravenous infusion, acts as a pro-oxidant, leading to the formation of H_2O_2, thus inducing oxidative damage to cancer cells, is not new. In 1969, Benade and co-workers had already demonstrated that Vitamin C could selectively kill cancer cells, without harming normal cells. The authors suggested that the cytotoxic effect of ascorbate could be due ("*in major part*") to the intracellular generation of toxic hydrogen peroxide produced upon oxidation of Vitamin C, by the cells. This view was corroborated by the fact that the toxicity of Vitamin C was greatly enhanced by the con-comitant administration of 3-amino-1, 2, 4-triazole (ATA) that inhibits the enzyme catalase, "*thus decreasing or destroying the ability of the cancer cells to detoxify H_2O_2 effectively*" [44]. Further scientific reports confirmed that human cancer cells have low levels of antioxidant enzymes (including, among others, catalase and glutathione peroxidase), and therefore cannot detoxify hydrogen peroxide [45, 46].

According to the pro-oxidant theory, Vitamin C in high concentrations induces the produc-tion of H_2O_2 through a Fenton-like reaction. This reaction is the oxidation of organic sub-strates by iron and hydrogen peroxide, in which trivalent iron (Fe^{3+}) plays a fundamental role. However, since Fenton-like reactions are usually controlled, in vivo, because of iron seques-tration by metal binding proteins, the pro-oxidant effect of Vitamin C, in vivo, may be scarcely significant [33–35], and other mechanisms should be hypothesized.

Other authors, using two prostate cancer cell lines (LNCaP and PC-3) have shown that iron at physiological concentrations in cell culture medium and human plasma abrogates the anti-cancer/cytotoxic effects of Vitamin C. In particular, at physiological concentrations, iron pro-motes both production and decomposition of H_2O_2, the latter being mediated by a Fenton reaction, which prevents the accumulation of H_2O_2, thus abolishing the cytotoxic effect of Vitamin C. Therefore, for an optimal anticancer effect, Vitamin C should be administered with chelating agents, which remove iron from the medium [47].

On the other hand, Vitamin C readily undergoes pH-dependent autoxidation producing hydrogen peroxide, and catalytic metals only accelerate the oxidation process. Therefore, cata-lytic iron may not be strictly necessary for the production of H_2O_2. This auto oxidation process (oxidation in the absence of catalytic metals) occurs via the ascorbate di-anion ($Asc2-$). In par-ticular, at pH 7.0, 99.9% of ascorbate (Vitamin C) is in the form of mono-anion ($AscH-$). $Asc2-$ increases by a factor ten, with one unit increase in the pH. Therefore, while the production of

H_2O_2 may be scarcely relevant in the absence of catalytic iron (as in the "Fenton chemistry"), it may become considerable when the concentration of ascorbate is in the order of the millimoles, as in the case of the use of Vitamin C as an anticancer compound [48].

Finally, accumulating evidence suggests that cancer cells produce high amounts of hydrogen peroxide [49], and hydrogen peroxide itself is a powerful carcinogen, associated with mutagenic potential [50]. Therefore, the role of Vitamin C as a pro-drug of hydrogen peroxide, to kill cancer cells, is still far from being fully elucidated.

6. Oral vs. intravenous Vitamin C

The pharmacokinetic studies of Levine and Padayatty [28, 29], on Vitamin C, indicate that after oral administration of 200 mg of the nutrient, the maximum plasma concentrations obtained, are not superior to 70–80 μM. This is due to a "tight control," operated by several different mechanisms, including, among others: bioavailability, intestinal absorption, tissue accumulation, renal reabsorption and excretion, and utilization rate as a function of homeostasis. On the contrary, when Vitamin C is administered intravenously, "tight control" is bypassed, until renal excretion restores equilibrium, depending on the dose administered [51].

Therefore, according to these data, the intravenous administration of Vitamin C is the only way to achieve plasma concentrations in the order of millimoles, necessary to kill cancer cells.

However, this view is in disagreement with the following evidences:

a. The results obtained by intravenous administration of Vitamin C, do not show the same large effects reported by Robinson, feeding squamous cell carcinoma implanted mice, with large doses of the nutrient [52];

b. Abram Hoffer [53] used oral high doses of Vitamin C in cancer patients and obtained essentially the same significant results as Cameron and Pauling, Cameron and Campbell [54–58], and Murata [59];

c. Although it is presently believed that only injected Vitamin C delivers the concentrations needed to produce an anti-tumor effect, neither the legendary scientist Linus Pauling nor the consultant surgeon, Ewan Cameron, seemed to know the difference between oral and intravenous administration. In fact, in their clinical trial, the protocol started with a few days of 10 grams of intravenous Vitamin C, followed by 10 grams of oral Vitamin C for the whole life. Interestingly, Cameron and Campbell, who had already reported on the successful treatment of cancer with *oral* Vitamin C, had already observed that "… *with increasing experience, we tend now to believe that the intravenous regime is probably unnecessary as a routine measure, and need only to be employed in situations where vomiting, anorexia, or other complications of malignancy, preclude oral administration*" [58];

d. Plasma concentrations above the 400 μM have been reported, after the administration of a single dose of oral liposomal Vitamin C [60];

e. At times of stress or illnesses (including cancer), the body may absorb extra Vitamin C, as demonstrated by the principle of "bowel tolerance" to the nutrient administered by mouth. According to this principle, when the body is saturated with Vitamin C, slight diarrhea may appear, due to intestinal elimination of the nutrient. However, during stress or disease, the amount of oral Vitamin C a patient can tolerate, before the appearance of diarrhea, increases in proportion with the severity of the condition [61];

f. This means that the "tight control" hypothesized by Levine and Padayatty, over the plasma concentration of Vitamin C, is either inexistent or relative to disease conditions or stress. To achieve the maximum plasma levels, a typical person may need 20 g of oral Vitamin C spread throughout the day (3–4 g every 4 h); but cancer patients may require far more [62]. Such massive intake may result in plasma concentrations that the tumor may absorb, generating hydrogen peroxide that kills cancer cells;

g. More recently, the paradigm according to which antioxidants inhibit tumorigenesis predominantly by decreasing ROS-mediated DNA damage and mutations [63, 64] has been challenged by experimental data. Antioxidants such as N-acetylcysteine (NAC) and Vitamin C exerts their anti-tumorigenic activity by downregulating HIF-1α [65]. Interestingly, these data were obtained not by injecting, but by simply feeding mice with large amounts of NAC or Vitamin C. These findings validate the role of oral administration of Vitamin C (and other antioxidants) in fighting cancer.

## 7. Vitamin C and leukemia: an in vitro update

As we have previously demonstrated, high ("pharmacologic") concentrations of Vitamin C (in the form of the sodium salt of ascorbic acid) are capable of eliciting a clear-cut pro-apoptotic/cytotoxic effect on human promyelocytic leukemia-derived cell lines (HL60), in vitro [66] (**Figures 1** and **2**). This effect is already evident at concentrations of Vitamin C of 1 mM in the culture medium, and it is proportional to the amount of Vitamin C.

Since clinical investigations using high doses of Vitamin C to treat cancer, have reported plasma levels of more than 30 [67], and up to 49 mM [68], it seems reasonable to conclude that using high amounts of Vitamin C, administered by intravenous injection, is not strictly necessary to kill cancer cells in APL.

Further investigations in leukemia, performed by our research group, have shown that a plasma concentration of 3 mM of Vitamin C in the culture medium, is sufficient to kill more than a half of the cells in culture (LC_{50}) in a number of different human myeloid leukemia cell lines [69] (**Figures 3** and **4**) (**Table 1**). It is of interest to consider that according to our protocol, the leukemic cells are exposed to Vitamin C for no more than 2 h, then accurately "washed," re-suspended in fresh culture medium, without Vitamin C, and further incubated for additional 18–24 h, before the evaluation of cell survival and apoptosis. Given the results obtained, it is reasonable to conclude that the Vitamin C added to the culture medium (in the form of sodium ascorbate) is rapidly internalized by the leukemic cells, and
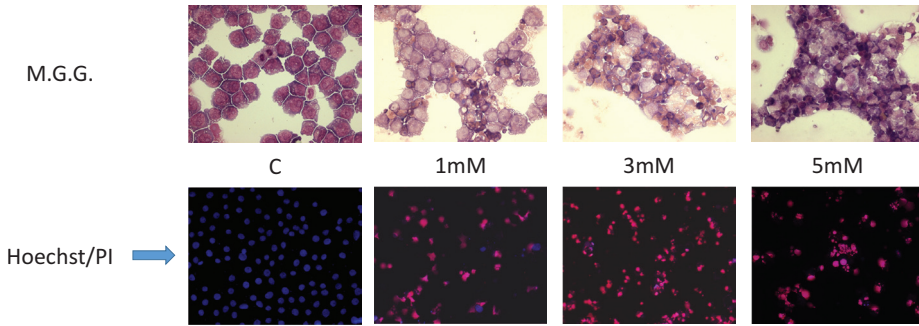
Figure 1. The microphotographs refer to the cytomorphologic modifications of HL60 cell lines (human acute promyelocytic leukemia—APL) exposed for 2 h to increasing concentrations of Vitamin C. It is evident that by increasing the concentration of Vitamin C in the medium (from 1 to 5 mM), APL cells show an increasing degree of morphologic alterations indicating progressive cell death (apoptosis, autophagy, autoschizis). With the Hoechst/PI fluorescent staining, vital cells are colored in blue, while dead/apoptotic cells are stained in red. M.G.G. = May Grunwald Giemsa cell staining; Hoechst33342/Propidium Iodide (PI) = Vital Staining; C = control (untreated) sample; 1 mM, 3 mM, 5 mM = Vitamin C at 1, 3, and 5 mM in the culture medium; original magnification: 400×.
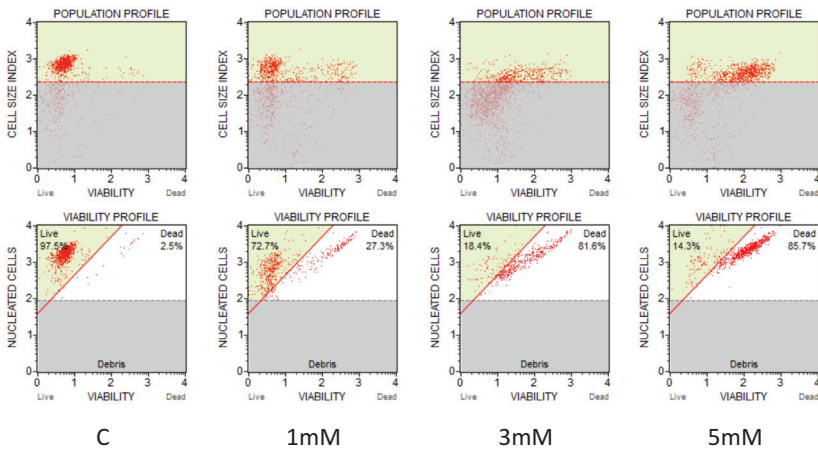

Figure 2. Viability profile of (Human) acute promyelocytic leukemia (APL) cell line (HL60) exposed for 2 h to increasing concentrations of Viability profile of (Human) acute promyelocytic leukemia (APL) cell line (HL60) exposed for 2 h to increasing concentrations of Vitamin C. (Flow Cytometry analysis: see text). The percentage of dead cells in the plates is proportional to the concentration of Vitamin C in the medium. C = control (untreated) sample; 1 mM, 3 mM, 5 mM = Vitamin C at 1, 3, and 5 mM in the culture medium.

its "toxic" effects last for hours (days), even when the nutrient has been removed from the culture medium. This is in agreement with the notion that both normal and leukemic white blood cells tend to concentrate Vitamin C [70–73] to levels that are 10–100 fold higher than plasma [74, 75], and it is in contrast with the view that hydrogen peroxide forms outside the tumor cells [31, 32].
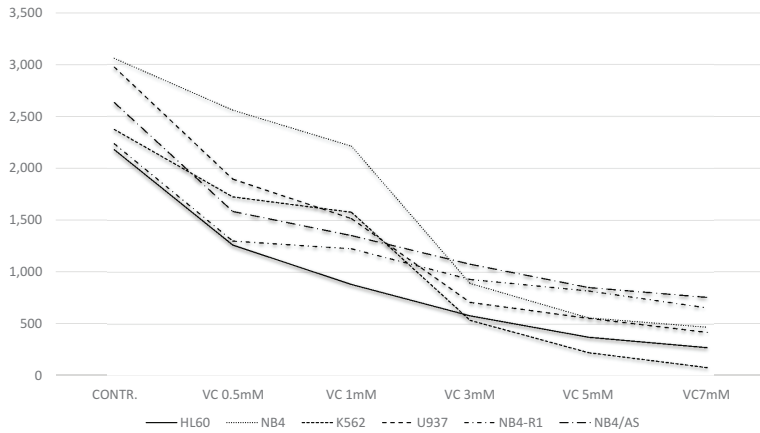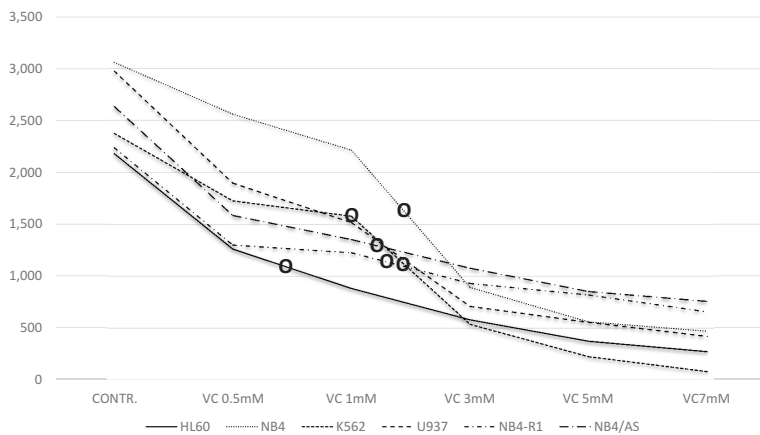
Figure 3. The figure illustrates **Table 1**. The diagram shows the almost uniform decrease in the number of vital leukemic cells in the culture medium, after exposing them to increasing concentrations of Vitamin C, for 2 h.

Figure 4. The figure illustrates **Table 1**. Highlighted with colored circles, the LC50 for each human myeloid leukemia cell line tested.

Neutrophils, in particular, accumulate Vitamin C via the sodium-dependent Vitamin C co-transporter 2 (SVCT2) [76], and have intracellular levels of 1–2 mM [77]. Therefore, while there is agreement on the fact that in solid tumors, Vitamin C, initially oxidized to dehydroascorbic acid (DHAA), is internalized by the cell, via GLUT 1 and 4, and finally reduced

		HL60 (2 h)	NB4 (2 h)	K562 (2 h)	U937 (2 h)	NB4-R1 (2 h)	NB4/As (2 h)
Exp. 1	Contr.	471	912	663	1189	337	819
	VC 0.5	296	680	669	479	82.8	42.7
	VC 1	108	494	628	245	39.7	31.9
	VC 3	22.6	163	226	56.4	47	32.2
	VC 5	15.1	143	82	30.2	35	48.4
	VC 7	6.15	85.4	32.2	10.6	24.6	17.6
Exp. 2	Contr.	869	1020	694	829	936	958
	VC 0.5	423	959	399	704	624	823
	VC 1	349	887	445	585	560	674
	VC 3	217	337	200	232	335	581
	VC 5	143	74.6	111	118	344	402
	VC 7	89.5	147	35.2	93.7	255	329
Exp. 3	Contr.	843	1130	1020	969	967	859
	VC 0.5	545	924	660	716	678	722
	VC 1	438	835	507	689	668	649
	VC 3	343	395	113	480	551	499
	VC 5	218	346	35.2	411	443	453
	VC 7	181	241	17.6	320	380	414

The cell lines used in this experiment are variants of human myeloid leukemia cells, and include: HL60, NB4, K562, U937, NB4-R1, NB4/As. It is evident that the total number of cells in culture decreases by increasing the concentration of Vitamin C in the culture medium. C = control (untreated) sample; VC = Vitamin C; VC 0.5 mM, VC 1 mM, VC 3 mM, VC 5 mM = Vitamin C at 0.5, 1, 3, and 5 mM in the culture medium.

Table 1. The number of vital cells after 2 h of exposure to increasing concentrations of Vitamin C in the culture medium.

again to ascorbic acid, with consumption of GSH; this may not be the case in acute myeloid leukemia.

More importantly, the parallel exposure of normal hematopoietic precursors (CD34+), isolated from cord blood, to Vitamin C, at the concentrations that are cytotoxic for leukemic cells did not affect their survival, or impair their capacity to proliferate and differentiate in response to myeloid growth factors. These data confirm that Vitamin C is harmless for normal hematopoietic precursors and therefore highly selective in its anticancer/antileukemic effect.

8. Hypoxia inducible factor (HIF): the forgotten pathway

Hypoxia and induction of hypoxia-inducible factors (HIF) is a hallmark of many tumors [78, 79].

HIF-1 is a heterodimeric transcription factor discovered in 1991 [80], and is composed of two subunits, α and β. The HIF-1α subunit is oxygen sensitive and it is induced by hypoxic conditions, which are very common in cancer. Direct transcriptional targets of HIF-1 include genes regulating, among others, growth and apoptosis, cell migration, energy metabolism, angiogenesis, vasomotor regulation, matrix and barrier functions, and transport of metal ions and glucose [81].

In normoxic conditions, the HIF-1α unit is downregulated by Vitamin C dependent hydroxylases, while in hypoxic conditions (such as those existing in many different types of cancer), HIF-1α hydroxylation is repressed with consequent increase in HIF-dependent gene transcription, neo-angiogenesis, and tumor growth and progression [82].

More importantly, since Vitamin C stimulates HIF-1α prolyl hydroxylases, low levels of Vitamin C promote tumor growth and progression, by reducing HIF-1α hydroxylation [83], thereby stabilizing HIF1-α. On the contrary, high levels of HIF render cancer cells more sensitive to Vitamin C-induced toxicity. To confirm this view, Kuiper and Coll. [84] have recently found an inverse relationship between intra-tumor levels of Vitamin C and HIF activity in both endometrial cancer [85] and colorectal carcinoma (CRC) [86].

In 1925, Otto Warburg observed that cancer cells manifest increased rates of lactate production under aerobic conditions ("Warburg Effect") or, in other words, they preferentially utilize glycolysis, instead of oxidative phosphorylation, for metabolism even in the presence of oxygen [87, 88].

"Hypoxia" (low oxygen concentration) is a hallmark of solid tumors, usually occurring at the center of the tumor mass, where blood vessels are abnormal or insufficient to supply adequate amounts of oxygen [89].

In response to the reduced oxygen tension, the HIF is activated to mediate the primary transcriptional adaption to hypoxic stress in cancer cells [90, 91].

As previously mentioned, HIFs regulate angiogenesis, cell survival, proliferation, apoptosis, adhesion, and metabolism by transcriptionally activating downstream targets such as vascular endothelial growth factor and erythropoietin. Therefore, HIF (HIF1, in particular) plays a major role in tumor growth, and clinical data suggest that the upregulation of HIF, as determined by the low oxygen tension, is usually associated with increased mortality in a number of different cancers [92–94], and may represent a relevant target for new therapeutic approaches to the disease [95–97].

9. The HIF pathway in leukemia

The role of HIF-1α in leukemia, and in particular in acute myeloid leukemia (AML), has only recently emerged and it is still somewhat controversial. One possible explanation for this delayed interest in the role of hypoxia in leukemia could be the fact that leukemia is not considered a "solid" tumor, and therefore, the role of oxygen, in its pathogenesis, has been

considered inconsequential for long time. This erroneous view, has been recently reviewed, as data have emerged, demonstrating that leukemic cells are sensitive to the oxygen tension, and hypoxia influences leukemic cell proliferation, differentiation, and resistance to chemotherapy [98].

Migliavacca and Coll. have recently demonstrated oncogenic function of HIF-1α, in the M5 Fab subtype of AML [99]. In particular, the authors have demonstrated that in M5 AML, HIF-1α mediates the ability of leukemic cells to migrate and invade extramedullary sites. The same group has demonstrated that PML-RARα and other fusion proteins involved in the pathogenesis of acute promyelocytic leukemia (APL) behave as transcriptional coactivators of HIFs, and both HIFs and PML-RARα enhances the progression of APL, by promoting cell migration, homing to bone marrow, and bone marrow neo-angiogenesis [100, 101].

Further investigations [102] have demonstrated that HIF-1α plays critical and pleiotropic roles in the pathogenesis of chronic lymphocytic leukemia (CLL).

Globally, elevated levels of HIF-1α have been reported in AML [103–106], APL [100], acute lymphoblastic leukemia (ALL) [107], and chronic myelogenous leukemia (CML) [108, 109]. Furthermore, HIF-1α overexpression conditions disease severity and outcome in both AML and myelodysplastic syndrome (MDS) [110–112].

Overall, the available data show that hypoxia and HIF-mediated signaling play a crucial role in leukemia, and targeting HIF with specific drugs or natural inhibitors, such as Vitamin C, represents a potentially useful approach to its treatment [113].

10. Vitamin C as a powerful modulator of TET2 activity

Decreased TET expression and loss of 5hmC have been observed in a wide variety of solid tumors, as well as in many hematological malignancies, including acute myeloid leukemias, myelodysplastic syndromes, and clonal hematopoiesis [114].

Recent experimental studies suggest that pharmacological dose of Vitamin C may represent a potentially important strategy in leukemia therapy through a stimulatory effect on TET2 activation and restoration in leukemic cells. Vitamin C is a co-factor of TET2 enzyme and is capable of interacting with the catalytic domain of TET2, enhancing the enzymatic oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [115]. This epigenetic modulation elicited by Vitamin C is able to improve the generation of pluripotent stem cells [116] and to induce a blastocyst-like state in mouse embryonic stem cells [117].

Two recent studies explored the possible epigenetic effects of Vitamin C on leukemia models, mediated by activation and restoration of TET2 activity in leukemic cells. In the first one, authors used a murine model of IDH1-dependent acute myeloid leukemia [118], and 2-phosphate L-ascorbic acid (Asc 2-P). Asc 2-P, unlike native Vitamin C, remains oxidatively stable under standard cell culture conditions [119], and possesses the same modulatory effects of Vitamin C, but, unlike Vitamin C, it does not induce cytotoxic effects of through stimulation of

H_2O_2 production. Asc 2-P added to the cells in culture is stable and releases Vitamin C by plasma membrane alkaline phosphatase hydrolysis [120]. Therefore, Asc 2-P allows a better characterization of the epigenetic activity of Vitamin C, without the "disturbing" H_2O_2-mediated cytotoxic effects of the native molecule. Asc 2-P treatment of the IDH1 AML-mutant mice induced an increase of 5hmC levels, a reduction of leukemic proliferation and an increase in expression of genes involved in leukocyte differentiation [118]. The stimulatory effect of Vitamin C on myeloid differentiation is mediated though the restoration of a normal expression and function of transcription factors, such as PU.1 and RUNX1, required for normal myeloid differentiation.

A second study provided clear evidence that in various leukemia models, Vitamin C treatment induces the restoration of TET2 function, blocking aberrant self-renewal and leukemia progression. Treatment with Vitamin C, mimics TET2 restoration, driving DNA hypomethylation and, by enhancing 5hmC formation, suppresses leukemic colony formation and leukemic progression of primary human leukemia patient-derived xenografts (PDXs). Interestingly, TET2-mediated DNA oxidation induced by Vitamin C-treated leukemic cells, greatly enhances their sensitivity to PARP inhibition and could provide a safe and effective combination strategy to target TET-deficient leukemic cells. These observations suggest that future clinical trials could incorporate high-dose Vitamin C as an adjuvant to standard chemotherapy/demethylating therapy, particularly in TET2-deficient neoplasms [121].

11. What to do next?

The anticancer properties of Vitamin C are known, since at least six decades, even though its use in clinical practice has only recently re-emerged, after the demonstration that in relatively high concentrations, it can selectively kill a number of different human tumor cells, both in vitro and in vivo.

The proof of the anticancer efficacy of Vitamin C in high doses, administered by mouth, has been reported four decades ago, by Linus Pauling [54–57], and further confirmed, very recently, by experimental in vitro and in vivo data [30–32, 66, 69].

Vitamin C is a natural compound, and it is an antioxidant and a life-saving nutrient with multiple beneficial effects on the human body. Man, some primates, and a few other mammals do no longer produce it. Beyond being a natural and essential nutrient, Vitamin C shows, in vitro, an outstanding efficacy in killing a number of different cancer cells, with an efficiency that no other anticancer drug, presently available on the market, has ever shown.

Vitamin C is extremely selective since it kills only cancer cells, by sparing, at the same time, all the other cells of the organism. As a consequence, it is very well tolerated, and devoid of any significant side effects. In fact, the only (relative) contraindication to its use, is the lack of the enzyme glucose-6-phosphate-dehydrogenase (G6PDH), a rare genetic condition also known as "favism." More importantly, within an expensive and often artificially inflated market, such as that of the anticancer drugs [122, 123], Vitamin C, with its low cost, represents an outstanding opportunity for both the patients and the healthcare system.

Unfortunately, in spite of all the above characteristics, Vitamin C has never been easily or favorably accepted as an anticancer drug, by the western Medicine. This also explains why, although the data on its anticancer efficacy are outstanding and straightforward, many scientists still prefer to consider "controversial" the role of Vitamin C in the treatment of cancer.

As we have seen, the idea that the oral administration of Vitamin C, in high doses, is not effective against cancer is a conceptual artefact, originating from questionable interpretations of pharmacokinetics data, after oral and/or intravenous administration. On the other hand, the idea that Vitamin C, administered in high doses by intravenous infusion, behaves as a pro-drug of H_2O_2 beyond being experimentally questionable, has not led to clinically significant results or outcomes [124–128]. More importantly, encouraging results have emerged from unbiased interpretation of the available data [129]. In particular, as it has been shown up to 110 g/m^2/day are very well tolerated by the majority of patients, and even in the absence of any significant clinical remission, intravenous Vitamin C is almost invariably associated with a clear-cut improvement in patient's quality of life.

As a result, History repeat itself! … and just as Vitamin C was dismissed as ineffective, against cancer, more than 30 years ago, on the ground of questionable clinical trials [130, 131], nowadays, it runs again the risk of being definitively discarded, in spite of the large amount of scientific evidence, demonstrating its extraordinary efficacy in fighting cancer!

It is clear that much remains to be understood about the cytotoxic effects of Vitamin C against cancer, and much more can (and must!) be done, to both improve the intravenous therapy and further investigate the oral administration route of the high doses of the nutrient.

Improving the intravenous treatment can (and should!) be achieved, by considering:

a. The type of pharmaceutical preparation, the sodium salt of the ascorbic acid to be preferred, when administered by the intravenous route [132];

b. The time and schedule of administration (slow infusion to be preferred) [133, 134];

c. The level of tissue oxygenation (cell cultures are better oxygenated than tumor tissues, and this may explain the differences in the outcomes between in vitro and in vivo treatment of cancer) [135]. In clinical settings, an improved tumor tissue oxygenation could be obtained with either ozone or hyperbaric oxygen;

d. The level of blood glucose (glucose may interfere with the uptake of ascorbate by cancer cells) [136, 137], and the possibility of associating an adequate dietetic regimen to the treatment with high doses of oral or intravenous Vitamin C.

12. Latest evidence of the role of Vitamin C in leukemia

A recent study provided clear-cut evidence that Vitamin C is a main regulator of hematopoietic stem cell (HSC) function and leukemogenesis. In fact, Agathocleous and co-workers, using a peculiar strategy for isolation of HSCs and hematopoietic progenitor cells (HPCs) from murine bone marrow, showed that HSCs have unusually high levels of Vitamin C,

which decline with differentiation [138]. Importantly, human HSCs and multipotent progenitor cells (MPPs), such as murine HSCs, display high Vitamin C levels.

Using "GULO" mice (deficient in Vitamin C because of the lack of gulonolactone oxidase, the last enzyme in the synthesis of Vitamin C starting from glucose), Agathocleous and colleagues have shown that Vitamin C deficiency induces an increased number of HSCs. A FLT3-internal tandem duplication (ITD) mutation, found in approximately a quarter of patients with de novo AML, imparts a particularly poor prognosis. Using "GULO" mice (deficient in Vitamin C because of the lack of gulonolactone oxidase), Agathocleous and colleagues have shown that Vitamin C deficiency induces an increased number of HSCs. Therefore Vitamin C deficiency, and TET2 mutations, are likely to cooperates with FLT3-ITD to induce leukemia development in murine models of FLT3-ITD-driven leukemia. [138].

Given the above evidence, it will be worth mentioning, once more, that the biochemist Irwin Stone, in his book "The healing Factor: Vitamin C against disease," published in 1972 (45 years ago!), had already warned the scientific community on the role of Vitamin C as a main factor in the prevention and treatment of leukemia. In his words, "*In a leukemic, the biochemical stresses of the disease process has reduced the body stores of ascorbic acid to very low levels … Any ascorbic acid circulating in the blood has been scavenged and locked in the excessive numbers of white blood cells contained in the blood. The plasmas level of ascorbic acid is usually zero or close thereto. A zero level in the blood plasma means that the tissues of the body are not being supplied with this most important metabolite. The ascorbic acid contained in the leukocytes are unavailable for the tissues. The tissues are in a condition of biochemical scurvy and this explains why these depleted tissues are so susceptible to the characteristic hemorrhaging of leukemia and the infections that kill so many of the leukaemics. A leukemic is not only suffering from leukemia but also from a bad case of biochemical scurvy. To correct this condition, ascorbic acid has to be administered in sufficiently large doses not only to saturate the excess of white blood cells but to provide adequate spill over into the blood plasma and tissues so that the seriously ill leukemic will be given a fighting chance to combat the disease. This may require the administration of ascorbic acid at the rate of 25 or more grams per day, as noted in the following case of leukemia treated with megascorbic levels of ascorbic acid.*" [3].

13. Concluding remarks

The rationale behind the use of high doses of Vitamin C in the treatment of acute leukemia is strong and very well grounded. In summary:

a. Leukemic patients, almost invariably show a severe deficiency of this nutrient;

b. While it is currently supposed to kill cancer cells by inducing pro-oxidant damage, Vitamin C is also very effective as an antioxidant by inhibiting the hypoxia inducible factor (HIF);

c. The mechanistic explanation of the pathogenesis of myeloid leukemia, includes the possibility that a Vitamin deficiency may induce the neoplastic transformation of myeloid precursors, through an upregulation of the HIF, and the consequent cascade of HIF-dependent cancer genes;

d. Although administered by intravenous infusion, in the majority of clinical trials performed so far, Vitamin C appears to be effective, in fighting cancer, even when administered by mouth;

e. Vitamin C is very well tolerated, and has no side or undesired effects;

f. Experimental in vitro data unequivocally show the cytotoxic effect of Vitamin C against leukemia.

g. As shown in our study on leukemic and normal cell lines, Vitamin C can kill almost every type of acute and chronic myeloid leukemia-derived cell, without doing any harm to their normal counterpart CD34+ cells;

h. Vitamin C is a natural compound, and it is very cheap.

Do we really need more information or evidence, to start clinical trials on Vitamin C, in the treatment of acute and chronic myeloid leukemia?

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Conflict of interests

None to declare.

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