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A two-phase strategy for treatment of oxidant-dependent cancers

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Summary In many cancers, a chronic increase in oxidant stress – associated with elevated levels of hydrogen peroxide – contributes to the increased proliferative rate, diminished apoptosis, increased angiogenic and metastatic capacity, and chemoresistance that often characterize advanced malignancies. This oxidant stress often reflects up-regulation of expression and activity of NADPH oxidase, and/or decreased activity of catalase, which functions as suppressor gene in oxidant-dependent cancers. These characteristics of oxidant-dependent cancers suggest a dual strategy for treatment of these cancers. Since ascorbate can react spontaneously with molecular oxygen to generate hydrogen peroxide, high-dose intravenous ascorbate should be selectively toxic to tumors that are low in catalase activity – as suggested by numerous cell culture studies. Measures which concurrently improve the oxygenation of hypoxic tumor regions would be expected to boost the efficacy of such therapy; calcitriol and high-dose selenium might also be useful in this regard. Secondly, during the intervals between sessions of ascorbate therapy, administration of agents which can safely inhibit NADPH oxidase would be expected to slow the proliferation and spread of surviving tumor cells – while providing selection pressure for a further decline in catalase activity. In effect, cancers treated in this way would be whipsawed between lethally excessive and inadequately low oxidant stress. An additional possibility is that ascorbate-induced oxidant stress in tumors might potentiate the cell kill achieved with concurrently administered cytotoxic drugs, inasmuch as oxidant mechanisms appear to play a mediating role in the apoptosis induced by many such drugs, largely via activation of c-Jun NH_2-terminal kinase; cell culture studies would be useful for evaluating this possibility.
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Many cancers are oxidant-dependent

A high proportion of cancers and transformed cell lines have been found to express lower catalase activity than their tissues of origin [1–11]. In some instances, boosting catalase activity of low-catalase cancer cell lines by transfecting the catalase gene or adding catalase to cell cultures has been shown to suppress proliferation, survival and/or angiogenic capacity of these cells [10,12]. Conversely, increased production of oxidants via NADPH oxidase activity has been demonstrated in many cancer cell lines, and transfection with Nox1 has been shown to transform certain non-malignant cell lines [13–24]. In these cells, inhibi-

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41 tors of NADPH oxidase often reduce proliferation or 42 induce apoptosis.

43 In aggregate, these findings indicate that, in 44 moderate concentrations, hydrogen peroxide often 45 promotes proliferation, survival, and malignant 46 behavior in cancer cells. Hydrogen peroxide ap- 47 pears to achieve these effects by reversibly inhibit- 48 ing tyrosine phosphatase activities that target 49 growth factor receptors [12,25], by promoting the 50 transactivation of certain growth factor receptors 51 (via c-Src or related tyrosine kinases whose activity 52 is redox regulated) [26–30], and by boosting the 53 activity of the NF-kappaB and AP-1 transcription 54 factors [31,32].

55 Ascorbate selectively kills low-catalase 56 cancers

57 Low catalase activity may imply greater suscepti- 58 bility to the cytotoxic activity of hydrogen perox- 59 ide. Indeed, there are numerous reports that 60 cancer cell lines are selectively killed by exposure 61 to low millimolar concentrations of ascorbate 62 [33–36]; exposure times as short as 1 h are suffi- 63 cient to achieve substantial cell kill in many cancer 64 cell lines [36]. This cytotoxicity has been traced to 65 the hydrogen peroxide generated by the spontane- 66 ous reduction of molecular oxygen by ascorbate; 67 thus, co-administration of catalase (or of blood 68 containing erythrocyte catalase activity) abolishes 69 the toxicity of ascorbate to these cells [36–38]. 70 Catalysis by free transition metal ions is not re- 71 quired for this cytotoxic effect, as it is not inhib- 72 ited by intra- or extracellular chelators [36].

73 Intriguingly, as long ago as 1969, Benade, How- 74 ard, and Burk demonstrated that Ehrlich ascites 75 cells, which express low catalase activity, were 76 selectively susceptible to the toxicity of ascorbate 77 [39]. Hypoxia alleviated this toxicity – presumably 78 because less hydrogen peroxide was generated – 79 whereas the catalase inhibitor 3-amino-1,2,4-tria- 80 zole amplified it.

81 Much more recently, Riordan, Levine and col- 82 leagues [36,40–43] have re-examined the mega- 83 dose vitamin C therapy for cancer originally 84 espoused by Cameron, Campbell, and Pauling 85 [44–48]. They note that the original protocol advo- 86 cated by these researchers incorporated intermit- 87 tent i.v. administration of high-dose ascorbate 88 (10 g per session) as a complement to daily high- 89 dose administration of vitamin C. Subsequent 90 double-blind trials that evaluated the utility of 91 high-dose ascorbate as a cancer treatment [49,50] 92 – and which failed to confirm the promising results reported by Cameron and Pauling – omitted the i.v. 93 component of the protocol. It is now known the i.v. 94 administration of high-dose ascorbate can achieve 95 plasma ascorbate concentrations in the low milli- 96 molar range; such concentrations can be main- 97 tained for several hours with the right infusion 98 protocol [43,51]. In contrast, oral administration 99 of vitamin C cannot achieve plasma concentrations 100 much in excess of 200 μM – an insufficient concen- 101 tration to kill most cancer cell lines in vitro. Hence, 102 Riordan and Levine are now engaged in an effort to 103 evaluate high-dose i.v. ascorbate as a cancer ther- 104 apy. Intriguing case reports suggest that such a 105 strategy can be genuinely useful in selected cases 106 [52]. Levine insightfully characterizes ascorbate 107 as ''a pro-drug to deliver hydrogen peroxide to tis- 108 sues'' [36]. 109

Stromal cells can contribute to the total cata- 110 lase activity of a tumor. It can be presumed that 111 tumors high in stromal elements expressing normal 112 activities of catalase will be less susceptible to 113 high-dose ascorbate therapy. 114

Adjuvant measures may boost ascorbate 115 cytotoxicity 116

Since oxygen is required for production of hydrogen 117 peroxide, it can be anticipated that hypoxic tumor 118 regions will be relatively resistant to the cytotoxic- 119 ity of ascorbate, regardless of their catalase sta- 120 tus. Thus, concurrent measures which boost 121 tumor oxygenation – such as those developed as 122 adjuvants to radiotherapy – may amplify the 123 responsiveness of many tumors to high-dose ascor- 124 bate. Such measures may include ozone autohemo- 125 therapy [53–56] (which right-shifts the oxygen 126 dissociation curve of hemoglobin, decreases blood 127 viscosity, increases distensibility of leukocytes 128 and erythrocytes, and promotes endothelial nitric 129 oxide production), administration of perfluoro- 130 chemical oxygen carriers [57–59], niacinamide 131 [60] (which prevents episodic closure of tumor cap- 132 illaries), and inhalation of carbogen (95% O_2/ 133 5%CO_2) [57,58,61,62]. 134

Koren and colleagues have demonstrated that 135 pretreatment with activated vitamin D (calcitriol) 136 enhances the cytotoxic impact of hydrogen perox- 137 ide on MCF-7 breast cancer cells; this effect did 138 not reflect any impact on hydrogen peroxide catab- 139 olism or on generation of hydroxyl radical [63]. On 140 the other hand, their previous work has shown that 141 calcitriol has a pro-oxidant effect on this cell line, 142 as indicated by an increase in the ratio of digluta- 143 thione to reduced glutathione [64]. Such an effect 144

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could be expected to amplify the physiological impact of hydrogen peroxide, including its ability to activate and up-regulate the JNK pathway. Moreover, this might rationalize the ability of calcitriol to potentiate the cytotoxic impact of various chemotherapeutic agents on vitamin D-responsive cancers [63,65]. Whether these findings can be generalized to other cancers remains to be seen; calcitriol exerts an anti-apoptotic effect on stressed keratinocytes [66].

Methylseleninic acid (MSeA) — a metabolite of the naturally occurring selenoamino acids methylselenocysteine and selenomethionine — potentiates the apoptotic impact of various cytotoxic agents on androgen-independent cancer cell lines. This effect was abolished in the context of JNK inhibition — although MSeA exposure did not enhance JNK activation [67]. These findings thus suggest that MSeA potentiates the interaction of JNK with a downstream target that promotes apoptosis. Conceivably, MSeA could influence the apoptotic impact of hydrogen peroxide via a similar mechanism — in which case concurrent administration of methylselenocysteine or selenomethionine might amplify the response of cancers to intravenous ascorbate.

Protective impact of G6PD activity

Reduced glutathione protects against the toxicity of hydrogen peroxide both by providing substrate for glutathione peroxidase, and by promoting the reversal of oxidations catalyzed by peroxides [68]. Reduction of the cellular glutathione pool is contingent on glucose oxidation via the pentose phosphate pathway, and there is evidence that the sensitivity of cancer cells to the toxicity of hydrogen peroxide correlates inversely with glucose-6-phosphate dehydrogenase (G6PD) expression [68]. The fact that high-dose ascorbate can induce hemolysis in individuals with genetic G6PD deficiency is well known [69]; indeed, such deficiency is an absolute contraindication to ascorbate therapy [70]. Conversely, Burk and colleagues reported that glucose diminished the toxicity of hydrogen peroxide to Ehrlich ascites cells in vitro [39]. Oxidative stress can induce increased expression of G6PD at the transcriptional level [71] — a mechanism which some cancers might employ to evade ascorbate toxicity.

Glucose levels tend to be low in many tumors owing to the phenomenon of aerobic glycolysis, often compounded by inefficient perfusion [72,73]; thus, induced hypoglycemia might differentially impact the pentose phosphate pathway activity of such tumors. Burt has suggested that infusion of glycerol might render hypoglycemia more tolerable when induced hypoglycemia is employed in cancer therapy [74,75]. Whether such strategies might be feasible for potentiating the anti-cancer efficacy of intravenous ascorbate could be studied in rodent models.

A clinical protocol

The data of Chen et al. are of particular interest, as these researchers determined the concentrations of ascorbate that achieve 50% cell kill after 1 h exposure for 9 cancer cell lines (mouse and human) in vitro [36]. This concentration was less than 5 mM for 5 of the 9 cell lines — whereas the non-transformed cell lines tested were not harmed by 20 mM. It should be borne in mind, however, that relative hypoxia and the presence of catalase in stromal cells will likely render tumors less sensitive in vivo than their constituent cells would be in vitro. It should be feasible to maintain plasma concentrations of 5 mM or more for several hours with a simple infusion regimen. For example, data and extrapolations in Padayatty et al. [43] suggest that intravenous infusion of 30 g sodium ascorbate i.v. (1 g/min), followed 2 h later by a 30 g booster dose, would maintain a plasma ascorbate concentration in excess of 5 mM for over 4 h.

Inhibition of NADPH oxidase may slow the spread of oxidant-dependent cancers

As noted, various isoforms of NADPH oxidase are often responsible for the increased oxidant stress that drives malignant behavior and promotes survival in many cancers. Thus, drugs or phytonutrients which can inhibit this enzyme complex in clinically feasible doses may have utility for slowing the growth and spread of cancers that are oxidant-dependent. Indeed, it may prove feasible to treat such cancers with a two-part strategy. Intermittent application of high-dose i.v. ascorbate may achieve significant cell kill in low-catalase tumors. In the phases between courses of ascorbate therapy, daily administration of NADPH oxidase inhibitors would be expected to slow the proliferation of surviving cancer cells.

On the other hand, some researchers suggest that the oxidant stress in some cancers is counterproductively high, such that a moderate reduction of this stress might have a favorable impact on tu-

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mor growth [76]. In this regard, an agent which blocks heme oxygenase-1 induction selectively in tumors was shown to slow the growth of Sarcoma 180 in mice [77]; heme oxygenase activity would be expected to decrease oxidant production via NADPH oxidase [78], so agents which block its induction should have a pro-oxidant effect. Thus, the impact of NADPH oxidase inhibitors on growth of oxidant-dependent tumors might be expected to vary depending on the intensity of baseline oxidant production in the tumor and the degree of NADPH oxidase inhibition achieved.

Interactions with concurrent cytotoxic chemotherapy

A high proportion of cancer chemotherapeutic agents boost oxidant stress in targeted cells; activation of NAPDH oxidase appears to mediate this excess oxidant stress in at least some cases [76,79,80]. Moreover, there is reason to believe that this oxidant stress is often an important mediator of chemotherapy-induced apoptosis. Thus, measures which suppress oxidant production or reverse sulphydryl oxidation (such as glutathione or *N*-acetylcysteine administration) tend to reduce the cellular lethality of chemotherapeutic drugs and radiotherapy, whereas pro-oxidant measures can potentiate their cytotoxicity [76,79–89]. These considerations suggest that ascorbate-catalyzed hydrogen peroxide production might potentiate the cell kill achieved by pre-administered cytotoxic drugs — whereas inhibitors of NADPH oxidase would be expected to have the opposite impact.

Hydrogen peroxide can up-regulate apoptotic mechanisms by activating c-Jun NH_2-terminal kinase (JNK) via a signaling pathway involving protein kinase D and ASK-1 [90,91]. JNK activation can promote apoptosis via a range of complementary mechanisms, and its role in mediating response to cytotoxic therapy appears to be of particular prominence in cancers that lack effective p53 activity [92,93]. When cellular stress is low, phosphorylated forms of many pro-apoptotic proteins (including Bad, FOXO3a, and c-Abl) are bound by 14-3-3 proteins; this sequestration eliminates their pro-apoptotic activity. Activated JNK, by phosphorylating 14-3-3 proteins in a key region, induces release of these pro-apoptotic proteins, thereby up-regulating apoptosis [94,95]. This phosphorylation also liberates unphosphorylated Bax from association with 14-3-3, allowing it to associate with mitochondrial membranes [96]. Phosphorylation of the BH_3-only pro-apoptotic proteins Bim and Bml by JNK releases them from sequestration by dynein motor complexes, enabling them to antagonize the anti-apoptotic activity of Bcl-2 and Bcl-XL [97]. JNK can also phosphorylate anti-apoptotic Bcl-2 and Bcl-xL; this promotes apoptosis in some, but not all [98], contexts [99,100]. Another target of JNK is p53, its phosphorylation inhibits its association with Mdm2, thereby enhancing its half-life.[101] Finally, sustained activation of JNK and its target c-Jun, in conjunction with p38 MAP kinase activatrion, can promote extrinsic apoptosis by boosting transcription, via AP-1, of Fas ligand [102–104]. While hydrogen peroxide can induce JNK activation, it can also up-regulate cytotoxin-mediated JNK activation by alleviating the inhibitory impact of glutathione S-transferase p on JNK [105].

However, the impact of ascorbate on response to cytotoxins is complicated by the fact that ascorbate can donate an electron to quench free radicals; such an effect might antagonize the activity of cytotoxic agents that produce primary lesions by radical-dependent mechanisms. The magnitude of this effect may hinge on the extent to which a marked elevation of extracellular ascorbate can increase ascorbate levels within cancer cells; in endothelial cells, capacity for facilitated ascorbate uptake is maximized at high physiological plasma concentrations [106]. In any case, cell culture studies are needed to evaluate the impact of concurrent exposure to millimolar concentrations of ascorbate to the cell kill achieved by various cytotoxic agents. Selective elevation of hydrogen peroxide in catalase-deficient tumors might have the potential to potentiate the efficacy of concurrent chemotherapy.

Indeed, exposure of various cancer cells to superoxide dismutase mimics — which boost the hydrogen peroxide content of superoxide-producing cells — was shown to markedly increase the cell kill achieved with oxaliplatin, paclitaxel, and 5-FU [76]. The authors note that ''the simultaneous exposure to ROS-generating agents and to cytotoxic drugs dramatically increases the rate of cell death''. Perhaps high concentrations of ascorbate would exert a similar potentiating effect.

It should be noted that, in high concentrations, hydrogen peroxide has the potential to suppress apoptosis by blocking the active sites of caspases or diminishing ATP availability [107,108]. This may not be a great disadvantage if these high levels of hydrogen peroxide induce pyknotic/necrotic cell death, but in one type of Burkitt's lymphoma cell hydrogen peroxide decreases the net cell kill achieved with cytotoxic agents [109]. Also, hydrogen peroxide has the potential to inhibit apoptosis

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by inhibiting tyrosine phosphatase activities and thereby up-regulating Akt activation; Akt works to counter apoptosis in various complementary ways [110–113]. Thus, the net impact of hydrogen peroxide generation on apoptotic mechanisms can be complex, and may vary as a function of the concentration of hydrogen peroxide achieved and molecular biology of a given tumor.

The tumor oxygenating strategies recommended as adjuvants during ascorbate therapy could also be expected to increase the response of hypoxic tumor regions to concurrent chemotherapy, as hypoxia promotes chemoresistance in a variety of ways [114–119]. Thus, simultaneous administration of high-dose ascorbate, cytotoxic chemotherapy, and tumor oxygenating agents may have intriguing therapeutic potential. Other potential adjuvants discussed above — selenium and calcitriol — might likewise be expected to potentiate response to concurrent chemotherapy [63,65,67, 120,121].

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