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Effect of Vitamin C on Prostate Cancer Cells In Vitro: Effect on Cell Number, Viability, and DNA Synthesis

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BACKGROUND. Many studies describe the protective role of vitamin C (ascorbic acid) against cancer development and in treatment of established cancer. The present study investigated whether ascorbic acid demonstrates a therapeutic benefit for prostate cancer.

METHODS. Androgen-independent (DU145) and androgen-dependent (LNCaP) human prostate cancer cell lines were both treated in vitro with vitamin C (0–10 mM). Cell counts, cell viability, and thymidine incorporation into DNA were determined.

RESULTS. Treatment of DU145 and LNCaP cells with vitamin C resulted in a dose- and time-dependent decrease in cell viability and thymidine incorporation into DNA. Vitamin C induced these changes through the production of hydrogen peroxide; addition of catalase (100–300 units/ml), an enzyme that degrades hydrogen peroxide, inhibited the effects of ascorbic acid. Superoxide dismutase, an enzyme that dismutates superoxide and generates hydrogen peroxide, did not prevent decreases in cell number and DNA synthesis, suggesting further the involvement of hydrogen peroxide in vitamin C-induced changes. These results clearly indicate that reactive oxygen species (ROS) are involved in vitamin C-induced cell damage. However, that singlet oxygen scavengers such as sodium azide and hydroquinone and hydroxyl radical scavengers such as D-mannitol and DL-α-tocopherol did not counteract the effects of ascorbic acid on thymidine incorporation suggests that vitamin C-induced changes do not occur through the generation of these ROS.

CONCLUSIONS. Vitamin C inhibits cell division and growth through the production of hydrogen peroxide, which damages the cells probably through an as yet unidentified free radical(s) generation/mechanism. Our results also suggest that ascorbic acid is a potent anticancer agent for prostate cancer cells. Prostate 32:188–195, 1997. © 1997 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; vitamin C; cell death; free radical mechanisms

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer among men and is the second leading cause of death among U.S. men [1]. Although a variety of treatment modalities, chemotherapy, and radiation therapy have been employed in an effort to manage prostate cancer, none of these regimens has proved to be efficacious. These observations point to the need for the development of an effective regimen for treating prostate cancer.

Vitamins and combinations of vitamins have been evaluated for their anticancer activity because of their low systemic toxicity. Vitamin C (vit.C) especially has been suggested to be a protective agent against cancer development and a therapeutic agent against established cancer [2]. More specifically, studies have indicated the therapeutic value of vit.C in the treatment of estrogen-induced renal cancer [3], mammary tumors, and ultraviolet light-induced skin tumors [4] and in inhibition of neoplastic transformation induced by methyl-chloranthrene in C3H/101T/2 cells [5].

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Protective effects of vit.C appear to require high doses and long-term treatment [6–8]. Not surprisingly, epidemiological studies in which the population ingested much lower doses (60–100 mg/day) of vit.C have not shown a reduction in the incidence of prostate cancer [9]. Randomized control studies have shown that dietary vit.C supplementation does not delay progression or enhance survival in patients with advanced colon cancer [10]; however, such studies have not been performed with prostate cancer. Increased utilization of vit.C by tumor tissues has been suggested as a major cause for the lower levels of vit.C in patients with malignancy [11–13]. A deficiency of ascorbic acid has been reported in association with dissolution of the intercellular matrix, which might facilitate local infiltration and dissemination of neoplastic cells [14]. Human beings cannot synthesize vit.C and therefore depend on dietary sources of vit.C. These facts clearly emphasize the need for vit.C supplementation at doses higher than FDA-recommended levels of 60–100 mg/day in the prevention and treatment of cancer. Therefore, we tested the effect of vit.C on prostate cancer cell lines in vitro. Our results indicate that vit.C exhibits anticancer effects both on androgen-dependent and on androgen-independent prostate cancer cells. The present investigation indicates that the anticancer effects of vit.C might be due to hydrogen peroxide-mediated free radical generation.

MATERIALS AND METHODS

Chemicals

RPMI 1640 medium, penicillin/streptomycin, and calf serum were obtained from GIBCO BRL (Grand Island, NY). 3H-thymidine was obtained from New England Nuclear Corporation (Cambridge, MA). Sodium ascorbate, catalase, superoxide dismutase, and other chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture of Cells

Human prostate cancer cell lines, derived from lymph node carcinoma of the prostate (LNCaP) and prostatic adenocarcinoma metastatic to brain (DU145) were obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI + 10% fetal bovine serum (FBS) containing penicillin and streptomycin (100 μg/ml) and 1 mM glutamine at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. Testosterone (10⁻⁸ M) was added to LNCaP cultures [15].

Cell Number and Trypan Blue Exclusion Technique

To obtain cell counts, cells were plated in 24 well plates at a density of 0.5–1 × 10⁴ cells/well and grown for 24 hours. The cells then received fresh medium containing 10% FBS and sodium ascorbate (0–10 mM) and were grown at 37°C, 5% CO₂ and 95% O₂. Depending on the experimental condition, the medium was not changed or was changed on alternate days. At the end of incubation, the medium was removed and the cells were treated with trypsin-ethylene diamine tetraacetic acid (EDTA; 0.125 M) at 37°C for 5–7 min. Control cultures had medium without vit.C. Trypsin action was stopped by adding FBS (200 μl/ml) before the samples were spun down at 1,500 rpm for 15 min, washed once with PBS, and suspended in 70% ethanol until counted. The cells were counted using a hemocytometer.

To determine cell viability, the trypan blue exclusion technique was carried out. About 100 μl of cell suspension in PBS was mixed with 100 μl of trypan blue (Sigma Chemical Co.), and the numbers of stained and unstained cells were counted using a hemocytometer [16]. Dead cells take up the stain, whereas living cells do not.

3H-Thymidine Incorporation

The cells were plated at a density of 20–50 × 10³ cells/well, and, 24–48 hr after plating, the medium was replaced with fresh medium containing 10% FBS plus different concentrations of sodium ascorbate (0–10 mM), and the cells were grown at 37°C for various lengths of time. Control cultures received medium without vit.C. In the time course study, the medium was changed on alternate days.

The cells were incubated with 1–2 mCi of ³H-methyl thymidine (specific activity 5–18 Ci/mmol) in 100 μl of 100 μM unlabeled thymidine and incubated for 5–6 hr at 37°C to incorporate the radiolabeled thymidine into DNA. After incubation, the medium was removed and the cells were washed twice with PBS to remove the residual radioactive material from the plate and precipitated with 10% trichloroacetic acid (TCA). Radioactivity in the TCA-precipitable material was counted after solubilization with 0.3 N NaOH and 1% sodium dodecyl sulfate (SDS) [17].

Mechanism of Vit.C Inhibition of Cancer Cell Proliferation

To understand the mechanism by which ascorbic acid effects prostate cancer cells, cells were incubated in the presence of 2.5 mM ascorbic acid with and with-
out various free radical scavengers such as hydroquinone (10,100 μM), sodium azide (500 μM), mannitol (5 mM), and α-tocopherol (500 μM) and enzymes such as catalase (100–300 U/ml) and superoxide dismutase (10–600 U/ml); thymidine incorporation and cell counts were determined.

**Statistical Analysis**

The data were analyzed by one-way analysis of variance and Neuman-Keul’s multiple test at the 5% level of significance.

**RESULTS**

**Effects of Vit.C on Cell Proliferation**

Dose-dependent effects on cell counts. Experiments were carried out to determine the dose-dependent effects of ascorbate on LNCaP and DU145 prostate cancer cells by incubating them with 0–10 mM vit.C for 5 days. Vit.C treatment decreased the cell number. The cell counts decreased to 12% and 50% with 1 mM vit.C for LNCaP and DU145, respectively ($P < 0.05$). Further increase in vit.C concentration resulted in greater decrease in cell number for these two cell lines ($P < 0.05$; Fig. 1A).

Trypan blue exclusion studies to determine cell viability. The results from trypan blue exclusion studies indicated that <5% and 15% of LNCaP and DU145 cells were stained, respectively, when they were incubated in medium containing only 10% FBS. In contrast, in the presence of vit.C (2.5 mM), the percentage of cells that were stained increased to approximately 50% for both these cell lines, suggesting that ascorbic acid induces cell death in prostate cancer cells (Fig. 1B).

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gers such as hydroquinone, sodium azide, mannitol, and \( \alpha \)-tocopherol and enzymes such as catalase and superoxide dismutase, and thymidine incorporation and cell counts were determined. The results are shown in Figures 2–4. Cells received any one of these scavengers followed by the addition of ascorbic acid and were then incubated for 3 days.

As shown in Figure 1, vit.C by itself inhibited thymidine incorporation into DNA, decreased the cell count, and increased the percentage of dead cells. Addition of catalase (100–300 units/ml) along with vit.C increased thymidine incorporation, maintained the cell number similar to 10% serum control, and decreased the percentage of dead cells. Heat-inactivated catalase did not counteract the vit.C-induced decrease in thymidine incorporation (Fig. 2). These results suggest that catalase, an enzyme that facilitates the breakdown of hydrogen peroxide, prevents the effects of vit.C on prostate cancer cells.

Superoxide dismutase, an enzyme that breaks down superoxide and generates hydrogen peroxide did not counteract the effects of vit.C (2.5 mM) on thymidine incorporation and cell number (Fig. 3). Incubation of prostate cancer cell lines either with singlet oxygen free radical scavengers such as hydroquinone and sodium azide or with hydroxyl radical scavengers such as D-mannitol and DL-\( \alpha \)-tocopherol did not prevent the decrease in thymidine incorporation induced by 2.5 mM vit.C (Fig. 4).

**DISCUSSION**

Ascorbic acid and its oxidized form, dehydroascorbic acid, are postulated to play an important role in inhibitory control over the division and growth of a number of cancer cells such as malignant melanoma [18], human leukemia cells [19], neuroblastoma cells [20], and ascites cells [21]. The present study indicates that ascorbic acid also inhibits the division and growth of prostate cancer cells. A 50–90% inhibition of cell growth was seen in the range of 1–10 mM vit.C concentration (Fig. 1A).

The current study utilized both androgen-dependent and -independent human prostate cancer cell lines. DU145 and LNCaP are androgen-independent [22] and androgen-dependent [23] human cell lines, respectively. The results clearly demonstrate that ascorbic acid inhibits proliferation and survival of these tumor cells in vitro. The effects are dose and time dependent. The sensitivity of these two cell lines to ascorbic acid differed; LNCaP, the androgen-dependent cell line, was more sensitive to the presence of ascorbic acid than was DU145.

We also noted in our study that DNA synthesis
varied between 5–25% (LNCaP) and 25–60% (DU145) of control levels after vit.C treatment (Figs. 1–4). This variation could be due to the initial plating density and the time of vit.C addition. In some of our experiments, vit.C was added 24 hr after plating; in others, it was added 48 hr after plating. When vit.C was added 24 hr after plating, the inhibitory effect on DNA synthesis was greater than when vit.C was added after 48 hr of plating. It is not known whether longer preincubation reduces the sensitivity of the cells to vit.C. In most of our experiments, cells were plated at an initial density of 20,000 cells/well, whereas in some they were plated at a density of 50,000 cells/well. As a result, the number of cells that have already entered S phase of the cell cycle would be greater. Our preliminary experiments (data not shown) to determine the phase of cell cycle sensitive to vit.C indicate that cells in G0/G1 phase are more sensitive to vit.C and that they are not able to overcome the cytotoxic effects of vit.C in order to enter S phase. However, once they have crossed the G1/S interphase, addition of vit.C does not affect DNA synthesis as significantly as when it is added at the G0/G1 interphase. Therefore, if more cells are in S phase, DNA synthesis is also going to be relatively greater in spite of the fact that vit.C significantly inhibits DNA synthesis.

Our experiments (manuscript in preparation) on ascorbic acid transport indicate that there is a rapid and greater uptake of only oxidized vit.C by prostate cancer cells. Similar results have been reported for HL-60 myeloid leukemia cells, which transport dehydroascorbic acid and accumulate reduced ascorbic acid within the cells [24]. Therefore, when we added vit.C to culture medium, the medium was either not changed if the cells were incubated for 2 days or changed only on alternate days, thereby allowing the conversion of reduced ascorbic acid into dehydroascorbic acid. We also used ascorbic acid in the higher physiological range (1–10 mM) to obtain the maximal inhibitory effect. Studies indicate that cells such as human neutrophils or B cells are capable of accumulating vit.C in millimolar concentrations, although the physiological concentration of vit.C in the blood may be 100–250 μM in ascorbic acid-supplemented individuals [25,26]. Therefore, the concentration we have used in our experiments is well within the physiological range that the cells accumulate.

The mechanism by which vit.C induced damage in

Fig. 3. Effect of superoxide dismutase (SOD) on vitamin C-induced changes in prostate cancer cells. A: Cell number (5,000 cells/well). B: Thymidine incorporation. Cells (20,000 cells/well) received vitamin C 48 hr after plating in RPMI + 10% FBS. The values are mean ± SE from two different experiments. SOD, superoxide dismutase.
cell survival is not clearly understood at present. However, generation of free radicals (hydroxyl and superoxide) has been proposed as one of the mechanisms of vit.C-induced cell death. The generation of hydroxyl radicals at physiological pH is thought to be mediated by a vit.C-driven Fenton reaction (reaction 1) in which ascorbyl anion (AH) or ascorbyl radical (AH−) reduces metal ions, ferric or cupric ions [27–29]. The reduced metal ions then participate in the Fenton reaction with hydrogen peroxide to produce the reactive hydroxyl radical (OH*; reaction 2) [30]:

\[
AH^- + M^{n+} + H_2O_2 \rightarrow A^+ + M^{(n-1)+} + H^+ + \text{OH}^-
\]  

Alternatively, AH− or AH may react with O2 to give superoxide (O2−*), which is capable of reducing Fe3+ and thereby initiating a classical Haber-Weiss cycle with resultant formation of OH* [31]. Vitamin C may form H2O2 either by reacting with O2−* itself or by superoxide dismutase catalysis. Thus, it provides both substrates needed for the Fenton reaction (2).

That catalase, an enzyme that degrades hydrogen peroxide into H2O and O2, increased thymidine incorporation in the presence of ascorbic acid and maintained cell viability at 10% serum control level suggests that hydrogen peroxide is involved in vit.C-induced cell death. Furthermore, superoxide dismutase [32], an enzyme that breaks down superoxide and generates hydrogen peroxide did not counteract the effects of vit.C on cell number and thymidine incorporation, thus supporting the involvement of hydrogen peroxide in ascorbic acid-induced cell damage. That higher concentrations of hydrogen peroxide can have deleterious effects on mammalian cells and can lead to cell death resulting from generation of free radicals has been demonstrated [33]. The efficacy of extracellular superoxide dismutase and catalase in hydrogen peroxide generation and degradation is consistent with a significant extracellular concentration of oxygen radical species, insofar as the prostate cancer cell membrane is not permeable to these large-molecular-weight scavenging enzymes. Superoxide and hydrogen peroxide species thus produced in the extracellular space might then traverse the cell membrane to react with macromolecules and start cellular damage. It has been suggested in this regard that the superoxide anion can traverse the erythrocyte cell membrane via anion channels [34,35].

Studies also indicate that hydrogen peroxide forms bonds with monomeric and polymeric compounds, including nucleic acid bases, nucleosides, amino acids, peptides, and proteins. Histidine is known to be one of the amino acids that strongly bonds with H2O2. Hydrogen peroxide-histidine adduct inhibits growth and promotes the cytotoxicity of hydrogen peroxide in hamster lung fibroblasts and Chinese hamster fibroblasts as well as the number of sister chromatid exchanges and the frequency of micronuclei induced by hydrogen peroxide [36]. These adducts as such may react with metals or enzymes or with Cl− in the presence of myeloperoxidase to generate tissue-destructive HOCl. Further studies will be required to delineate the pathway of cellular damage induced by ascorbic acid.

The results of the present study indicate no improvement in the vit.C-induced decrease in cell proliferation in terms of thymidine incorporation in the presence of singlet oxygen free radical scavengers or hydroxyl free radical scavengers. These data suggest that the vit.C-induced cell damage might not occur through the generation of hydroxyl free radical or sin-
glet oxygen radicals. Other, as yet unidentified free radicals such as hydroperoxyl (HO$_2^*$) and peroxynitrite radicals (ONOO$^-$) might have caused cell death. Alternatively, the inability of these radical scavengers to improve thymidine incorporation could be due to the compartmentalization of the scavengers separated from effective scavenging agents. Further experiments will be required to determine whether other hydroxyl/oxygen radical scavengers could prevent the free radical induced cellular damage.

In conclusion, our results clearly indicate that vit.C at the concentrations we have used inhibits proliferation and survival of prostate cancer cells in vitro. These effects of vit.C are brought about by the generation of hydrogen peroxide. Our preliminary results from the in vivo study (data not shown) indicate that athymic nude mice bearing PC3 tumors respond to vit.C treatment.

Further studies are in progress to investigate vit.C transport as well as to measure hydrogen peroxide generated by vit.C directly at different temperatures and time courses of generation and with other components present in the medium. Studies are also in progress to determine the signaling pathway of ascorbic acid-induced cell death.

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