Inhibition of the development of metastases by dietary vitamin C:K₃ combination

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Abstract

The tumor growth-inhibiting and chemo-potentiating effects of vitamin C and K₃ combinations have been demonstrated both in vitro and in vivo. The purpose of this study was to investigate the influence of orally administered vitamin C and K₃ on the metastasis of mouse liver tumor (T.L.T.) cells implanted in C3H mice. Adult male C3H mice were given water containing vitamin C and K₃ (15 g/0.15 g dissolved in 1000 ml) beginning 2 weeks before tumor transplantation until the end of the experiment. T.L.T. cells (10⁶) were implanted intramuscularly in the right thigh of mice. All mice were sacrificed 42 days after tumor transplantation. Primary tumor, lungs, lymph nodes and other organs or tissues suspected of harboring metastases were macroscopically examined. Samples of primary tumors, their local lymph nodes, lungs and main organs such as liver, kidneys, spleen were taken for histological examination. Forty-two percent of control mice exhibited lung metastases and 27% possessed metastases in local lymph nodes whereas 24% of vitamin-treated mice exhibited lung metastases and 10% possessed local lymph nodes metastases. The total number of lung metastases was 19 in control group and 10 in vitamin C and K₃-treated mice. Histopathological examination of the metastatic tumors from the vitamin-treated mice revealed the presence of many tumor cells undergoing autoschizic cell death. These results demonstrate that oral vitamin C and K₃ significantly inhibited the metastases of T.L.T. tumors in C3H mice. At least a portion of this inhibition was due to tumor cell death by autoschizis.

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Keywords: Metastases; Vitamin CK₃; Ascorbate; Menadione; Lung; Lymph node
Introduction

The influence of dietary components on tumor growth and development has recently become a subject of major interest (Williams and Dickerson, 1990; Roberfroid, 1991; Milner, 1994). Amongst different alimentary factors, vitamin C and vitamin K3 were investigated as possible antitumor agents (Park et al., 1980; Prasad et al., 1981; Gold, 1986). These vitamins were of particular interest because vitamin C (ascorbic acid or sodium ascorbate) was shown to exclusively reactivate acid DNase (DNase II) in malignant tumor cells, while vitamin K3 (2-methyl-1-4-napthoquinone) selectively reactivated alkaline DNase (DNase) (Taper, 1980; Taper et al., 2001).

These observations were of great interest because previously published studies demonstrated that the activity of alkaline and acid DNases was inhibited in non-necrotic cells of malignant tumors in human and experimental animals as well as during early stages of experimental carcinogenesis (Taper, 1967; Taper et al., 1971a,b; Fort et al., 1974; Taper and Bannasch, 1976). Furthermore, reactivation of alkaline and acid DNase-induced tumor cell death and tumor regression (Taper, 1980; Taper et al., 1981). In fact, the tumor growth-inhibiting and chemotherapy-potentiating effects of vitamin C and K3 combinations were evaluated using a variety of human tumor cell lines (Noto et al., 1989; De Loecker et al., 1993). These in vitro studies were extended to a battery of human urologic tumor cell lines, including DU145, an androgen-independent prostate carcinoma cell line (Gilloteaux et al., 1995, 1999; Venugopal et al., 1996a,b; Jamison et al., 1996, 1997, 2001). In these studies, autoschizis, a new type of cell death which differs from necrosis and apoptosis, was described (Gilloteaux et al., 1998, 1999, 2001a,b; Jamison et al., 2001).

In vivo administration of the vitamin C and K3 combination to ascites tumor-bearing mice (at a single, intraperitoneal dose of vitamin C = 1g/Kg body weight and vitamin K3 = 0.01 g/Kg) synergistically increased the life span of mice by 45% when compared with sham treated control mice. Individual administration of vitamin C and vitamin K3 increased life span by 14% and 1.07%, respectively (Taper et al., 1987). Combined vitamin C and K3 administration also potentiated the chemotherapeutic effects of six different cytotoxic drugs commonly utilized in the classical protocols of human cancer treatment (Taper et al., 1987). The same vitamin C and K3 combination also sensitized a mouse tumor that was resistant to vincristine and potentiated the therapeutic effects of radiotherapy (Taper and Roberfroid, 1992; Taper et al., 1996). More recently, vitamin C administration with a benzoquinone was shown to inhibit the metastasis of several colon cancer cell lines that had been implanted into immunocompetent mice (Hidvégi et al., 1998). The structural similarity of vitamin K3 (a napthoquinone) to the benzoquinone employed in these studies suggested that the vitamin C and K3 combination may decrease tumor metastasis as well as inhibiting the growth of the primary tumor.

Metastases are one of the greatest problems in cancer patients. They appear frequently and are the primary cause of mortality in cancer patients (Fidler, 1999). Different mechanisms are involved in the so-called metastatic cascade, including angiogenesis, cellular adhesion, local proteolysis and tumor cell migration (Kohn, 1993; Fidler, 1999). Development of chemotherapeutic agents which target and intervene in one or more processes in the metastatic cascade should lead to a favorable outcome for a large number of cancer patients.

The current study evaluates the ability of orally administered vitamin C and K3 to inhibit the development of metastases of mouse liver tumor (TLT) cells that have been implanted into the thigh of C3H mice.
The TLT cell line is a primary liver tumor of spontaneous origin that was first observed in a 2 month old female Swiss-Webster mouse. The TLT tumor was derived from in vivo passage in mice and characterized by Taper and his colleagues at Sloan Kettering Institute for Cancer Research (Taper et al., 1966). Tumor growth is rapid, invasive and not strain-specific. TLT cells were employed because these tumor cells provide an excellent model for metastasis. Unlike many putative metastatic models in which tumor cells are injected into the tail vein of immunosuppressed rodents and “metastasize” to the lungs, a solid primary tumor is formed in the TLT model. Subsequently, cells from the primary tumor recapitulate all the stages of metastasis.

Materials and methods

Animals and diets

Young adult male C3H mice weighing 25–30 g at the beginning of experiments were purchased from IFFA Credo, Domaine des Oncins, France and were fed basal diet for experimental animals AO4, supplied by U.A.R., Villemoisson-sur-Orge, France. Vitamin C (sodium ascorbate) and vitamin K3 (2-methyl-1,4-naphthoquinone) were purchased from Sigma, St. Louis, MO, USA. Control mice received water ad libitum. The mice in the experimental group received vitamin C and K3 (15 g/0.15 g) dissolved in 1000 ml of water. This dose and route of administration of the vitamin C and K3 mixture was shown to produce potent antitumor activity in mice in previous experiments (Taper et al., 1987; Taper and Roberfroid, 1992; Taper et al., 1996). This mixture of vitamins was prepared each second day and given ad libitum in drinking water beginning 2 weeks before tumor transplantation until the end of the experiment.

Tumor transplantation and metastases counting

Viable neoplastic cells (10^6) of a transplantable mouse liver tumor T.L.T. were implanted intramuscularly in the right thigh of C3H mice (Cappucino et al., 1966; Taper et al., 1966). All mice were sacrificed by general anaesthesia when spontaneous mortality appeared (i.e. 42 days after tumor transplantation). After the sacrifice of animals, a detailed general autopsy of each mouse was performed in order to identify the metastases. Primary tumors and all organs or tissues suspected of harboring metastases were macroscopically examined. Samples of primary tumors, their local lymph nodes, lungs and main organs such as liver, kidneys, spleen were taken for detailed histological examination. Special sections up to 10 for each tumor and organ were microscopically examined. Mainly one, the most suspected macroscopically local lymph node per each primary tumor was taken for histological

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice with lung metastases per total mice</th>
<th>% mice with lung metastases</th>
<th>Total number of lung metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14/33</td>
<td>42%</td>
<td>19</td>
</tr>
<tr>
<td>Vitamin CK₃</td>
<td>7/29</td>
<td>24%</td>
<td>10</td>
</tr>
</tbody>
</table>
examination. These samples were fixed in 10% neutral formalin, embedded in paraffin cut into 5–7 μm thick sections with microtome and stained with hematoxylin and eosin. Detailed identification of metastases was performed by a microscale equipped microscope and the number and mean diameter were calculated in all lobes of lungs for each mouse. Identification and counting of possible metastases in other organs was also macro- and microscopically performed. A one tailed z distribution (Tables 1 and 3) and Wilcoxon non-parametric test (Table 2) were utilized for statistical analysis of the results.

Results

At early stages after tumor transplantation, vitamin C and K₃ treatment produced a distinct inhibition of solid and ascitic TLT tumor growth without producing any distinct difference in morphology of treated and control TLT tumors (Taper et al., 1987; Taper and Roberfroid, 1992; Taper et al., 1996). However, the progression of intramuscularly transplanted T.L.T. tumors in C3H mice rapidly became lethal and obliged the sacrifice of all mice 42 days after tumor transplantation. This mortality was accelerated by tumor necrosis, ulcerations and infections. Before the sacrifice, 1 mouse was dead in CK3-treated and 2 in control group. All these spontaneously dead mice were without macroscopically detectable metastases. Due to the advanced post-mortem decomposition, histological examinations were not performed. Between experimental and control groups of mice there were not any difference in water consumption. These primary tumors were voluminous and often exhibited large ulcerations and subsequent dissemination of their contents. Few differences were observed between the tumors in the control and experimental groups at this terminal stage of their evolution. It appeared, that there was not any difference in the number of mitosis in primary tumors and metastases between the control and experimental groups of mice, but they were not counted in this preliminary experiment. However macroscopic and microscopic examination of serial lung sections revealed a distinct difference in the number of mice bearing lung metastases between the control group and the vitamin-treated group. In the experimental group, 7 of 29 mice (24%) exhibited lung metastases. Conversely, in the control group, 15 of 33 mice (42%) possessed lung metastases (Table 1). Similarly, when mice did possess lung metastases, there was a distinct reduction of the total number of lung metastases in the vitamin-treated group as compared with the control group. The total number of lung metastases was 19 in control group and 10 in the vitamin-treated group (Table 1).

In the majority of mice with lung metastases, there was a single lung metastasis per mouse. Mice with two or more lung metastases were more frequent in control group (5) than in the vitamin-treated group (2) (Table 2). Microscopic evaluation of the diameter of lung metastases demonstrated that the
majority of the lung metastases (7 of 10) in the vitamin-treated group exhibited diameters less than 100 μm, while the majority of diameters of the metastases in the control group were greater than 100 μm (range: 100 to 1000 μm). Specifically, 9 of 19 (45%) mice had lung metastases with diameters greater than 100 μm in control group compared to only 3 of 10 (30%) of mice in the vitamin-treated group.

Besides the lungs, metastases were found only in local lymph nodes (in the proximity of the implanted primary tumors and in the right retroperitoneal area). Once again, control mice exhibited more metastases than vitamin treated mice (Table 3). In the control group, local lymph node metastases were observed in 9 of the 33 examined mice (27%), whereas in the vitamin-treated group, lymph nodes metastases were found in 3 of 29 examined mice (10.2%). Statistical analysis of the differences in the incidence of metastases between the control and vitamin-treated mice has given

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of metastases in local lymph nodes per total number of examined mice</th>
<th>% of mice with lymph nodes metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9/33</td>
<td>27.2%</td>
</tr>
<tr>
<td>Vitamin CK₃</td>
<td>3/29</td>
<td>10.2%</td>
</tr>
</tbody>
</table>

Table 3

Effect of combined vitamin C and K₃ given in drinking water on the Incidence of metastases in local lymph nodes

Fig. 1. A–D: Histological aspects of small (A–B, i.e. <100 μm diam) and large (C–D) metastatic TLT tumors in mouse lung photographed at low (A and C) and high magnification (B and D). Notice that the small metastasis is circumscribed by a vascular endothelium and shows clusters of tumor cells in its lumen co-aggregated with some rare leukocytes. The large tumor contains a large central necrotic zone where numerous autoschizis and apoptosis are the principle types of cell death are observed. An apoptotic cell displaying nuclear breakdown is indicated by short arrows, while autoschizic nuclei are noted with curved arrows.
statistically significant results concerning the data presented in Tables 2 and 3 (p<0.05) and closely significant (Table 1, p = 0.06).

Detailed autopsies and microscopic examinations did not demonstrate metastases in any other organs besides the lungs and local lymph nodes. In addition, no pathological symptoms or microscopic alterations in tissue or cellular structure was visible in any of the vitamin-treated mice. This apparent lack of toxicity is consistent with the results of previously published studies (Noto et al., 1989; De Loecker et al., 1993; Gilloteaux et al., 1998; Jamison et al., 2001; Buc Calderon et al., 2002; Verrax et al., 2003).

Histopathological examination of hematoxylin and eosin stained sections of the lungs of control (Fig. 1) vitamin-treated (Fig. 2) mice reveals several small to large solid tumors. Fig. 1 shows a small (A–B)
and a large (C–D) metastatic TLT tumor in mouse lung photographed at low (A and C) and high magnification (B and D). The small metastasis is circumscribed by a vascular endothelium and shows clusters of tumor cells in its lumen which are co-aggregated with some rare leukocytes. While the large tumor contains a large central necrotic zone, autoschizis and apoptosis are the principle types of cell death observed outside this zone.

Fig. 3. This figure shows a local lymph node taken from an untreated mouse (A–D) and a local lymph node taken from a VC:VK3 treated mouse (E–I). A: A general view of the lymph node shows the collections of metastatic cells in the subcapsular sinus and mantle zone of the perilymphoid space which exhibit a glassy pink-orange contrast after H&E staining. B–D: A higher magnification of the same lymph node depicts a few metastatic cells including some that are undergoing mitosis. In E: A general view of a region of a vitamin-treated lymph node which is equivalent to the region shown in Fig. A reveals many open spaces (open arrows) due to the dramatic reduction in the size of the metastatic cells. F–I: Higher magnification of the same lymph node demonstrates the presence of many apoptotic cells (small arrows) and autoschizic cells (curved arrows). The open arrows indicate large pieces of excised cytoplasm among smaller nuclei which are greatly reduced in size and are indicative of autoschizis. The scales are 10 μm for all micrographs in this figure.
Upon initial examination, the peripheral zone of the tumors display the pleomorphism that is characteristic of tumor cells (Fig. 3). However, at higher magnifications, one can see carcinoma cells with large nuclear/cytoplasmic ratios, indented nuclei as well as several small apoptotic cells, apoptotic bodies and autoschizic cells (Fig. 1B–D). Autoschizis is a novel type of cell death which is 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 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 which become round and compact (Fig. 2F). Therefore, before it dies, the size of the resultant autoschizic cell is much smaller than the tumor cell from which it originated (Gilloteaux et al., 1998, 1999, 2001a,b). Cells in both the central and peripheral zones of the tumors display morphologic characteristics representative of various stages of autoschizic cell death (Fig. 2B–F). First, there is a decrease in nuclear indentation and a concomitant development of compact spherical nuclei. Second, the nuclei display round to oblong compacted nucleoli which occupy a large volume of the nucleoplasm. Third, the degraded chromatin gives the nucleoplasm a reticulated or striped pattern (Fig. 2C and D). Fourth, the perikarya decrease their size through self-excisions of cytoplasmic pieces (Fig. 2E). Fifth, the size of the cells is greatly reduced while the nucleoli appear compact to fragmented in a nucleoplasm with greatly decreased contrast (Fig. 2A, E and F).

Histopathological examination of hematoxylin and eosin stained sections of local lymph nodes taken from control and vitamin-treated C3H mice reveal an interesting phenomenon. The subcapsular sinus of the control lymph node is clogged with many metastatic TLT cells. These cells can be distinguished from the lymphocytes by their larger size and by the pink-orange color of their cytoplasm. As is the case for the tumor cells in vitamin-treated lung metastases, the tumor cells in the vitamin-treated lymph node appear to be undergoing both apoptosis and autoschizis with autoschizis being the most prominent form of tumor cell death. The incidence of autoschizis seems to be more prevalent in the lymph nodes than in the lung metastases.

Discussion

The results of the current study demonstrated that oral administration of vitamin C and K3 produced a distinct inhibitory effect on the development of metastases in C3H mice bearing an intramuscularly transplanted mouse liver tumor. Detailed microscopic examination of the main organs as well as the lungs 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. As was the case in previous experiments, oral ad libitum administration of clinically attainable doses of vitamin C and K3 in drinking water did not produce any adverse symptoms in the mice or macroscopic/microscopic pathological alterations in the organs and tissues examined during this study (Taper et al., 1987, 1996, 2001; Taper and Roberfroid, 1992).

Both direct antitumor and immunomodulatory effects are among the different mechanisms that may be involved in the therapeutic and antimetastatic effects of the vitamin combination. The antitumor effects of vitamin C are related to the ability of the VC to induce a G1 block in the cell cycle as well as the ability of the VC to redox cycle (Saitoh et al., 1997; Vojdani et al., 2000). This cycling process
generates intracellular H$_2$O$_2$ and other reactive oxygen species (ROS) that deplete cellular thiol levels, initiate membrane lipid peroxidation and result in tumor cell death. A concomitant reactivation of DNase II occurs during the redox cycling process and leads to the degradation of tumor cell DNA (Taper, 1980; Taper et al., 2001). In addition, enrichment of intracellular ascorbate content of tumor cells following the addition of exogenous vitamin C has been shown to inhibit tumor metastasis and invasion by inducing a marked decrease in metalloproteases (MMP) 2 and 9 via a post-transcriptional inhibition of MMP proenzyme production (Nagao et al., 2000).

As was the case for VC, the antitumor effects of vitamin K$_3$ are a function of the vitamins ability to block the cell cycle, to stimulate redox cycle and to reactivate DNase I. VK$_3$binds to the catalytic domain of Cdc25 phosphatase which results in the formation of an inactive hyperphosphorylated Cdk1. VK$_3$ also inhibits cyclin E expression at late G$_1$ phase and cyclin A expression at the G$_1$/S transition. Together these effects cause cell cycle arrest and can lead to tumor cell death (Wu and Sun, 1999). Administration of VK$_3$ is also known to induce a variety of effects on cells including: reduction of nicotinamide adenine dinucleotide phosphate and adenosine triphosphate pools; depletion of glutathione; induction of single-stranded DNA breaks; and oxidation of sulfhydryl groups in cytoskeletal proteins (Gant et al., 1988; Mirabelli et al., 1989). A concomitant reactivation of DNase I occurs during the redox cycling process and leads to the degradation of tumor cell DNA (Taper, 1980; Taper et al., 2001).

When VC and VK$_3$ are combined in a VC: VK$_3$ ratio of 100:1, the combination exhibits tumor specific antitumor activity. The potentiation and specificity of the cytotoxic antitumor activity has been attributed to the ability of intracellular redox cycling of the VC and VK$_3$ to cogenerate the long-lived ascorbyl and semiquinone free radicals as well as other ROS; to generate peroxides which induce membrane lipid alteration, and to reactivate both DNase I and DNase II which destroy DNA (Noto et al., 1989; Taper and Roberfroid, 1992; De Laurenzi et al., 1995). The importance of sequential DNase I and II reactivation to the in vivo antitumor activity of the vitamin combination has been demonstrated histochemically in both the TLT model and in a system in which human androgen-independent prostate cancer cells were implanted in nude mice. In these studies, loss of tumor cell DNA content and the incidence of tumor cell death by autopschesis were well correlated with the reactivation of tumor cell DNases (Taper, 1980; Taper et al., 2001).

While various mechanisms have been proposed to account for the antitumor properties of vitamin C-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 (Morgan et al., 1998). It has been suggested that the selective toxicity of the vitamin combination in tumor cells may be due to reduced levels of catalase, superoxide dismutase and/or glutathione peroxidase as well as other ROS detoxifying enzymes in these cells which leads to cellular damage through the accumulation of hydrogen peroxide and other ROS (Noto et al., 1989; Benade et al., 1969; Josephy et al., 1978). Supression of the antitumor activity of the vitamin combination by simultaneous administration of catalase supports this hypothesis (Noto et al., 1989; Taper and Roberfroid, 1992). It has also been suggested that the selectivity of the vitamins to tumor cells may be related to their preferential accumulation in these cells. The results of in vitro and in vivo studies demonstrate that VC exhibits selective toxicity towards malignant melanoma cells, human leukemia cells, neuroblastoma cells, tumor ascites cells and other malignant cell lines that has been ascribed, at least in part, to their preferential accumulation in tumor cells (Prasad et al., 1979; Bram et al., 1980; Park et al., 1980; Liotti et al., 1984). Likewise, VK$_3$ and its derivatives have been shown 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 radio-
sensitizers 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 (Marrian et al., 1969; Halsall et al., 1973). Finally, the selectivity of the antitumor response has been attributed to the immunostimulatory properties of the vitamin combination. A variety of mechanisms have been proposed for this immunostimulation including: modulation of CD4+ T-lymphocyte helper activity, induction and increased production of interferon, and activation of the JAK-STAT pathway (Dahl and Degre, 1976; Askarkhodzhaev et al., 1979; Terekhova et al., 1991; Rybnikov et al., 1997; Hidvégi et al., 1998; Markovits et al., 1998; Morgan et al., 1998; Simon et al., 1998; Dong et al., 1999; Slaton et al., 1999; Bystrova et al., 2000).

It is obvious from the preceding discussion that the VC/VK3 combination exerts its antitumor and antimetastatic activities through a wide array of mechanisms including: blockage of the cell cycle, stimulation of redox cycling, induction of autophagic cell death, reactivation of DNases, decreasing MMP activity and potentiating the immune system. Additional investigations are necessary to elucidate the importance of each of these mechanisms in the antitumor and antimetastatic activities of the vitamin C/K3 combination.

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