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4. Enhanced survival of B16-F10 melanoma tumour-bearing C57BL6/N mice treated with a mixture of antioxidants

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Abstract. There is compelling evidence that habitual intake of adequate quantities of micronutrients with antioxidative properties helps reduce the risk of developing tumors in the general population, and a similar effect may exist for other diseases as well. For the purposes of the present work we focused our attention on a commercial available mixture of micronutrients (Citozym), testing antioxidant supplements, with the aim of collecting evidence linking suboptimal micronutrient status and tumor-bearing animals survival. A highly sensitive computerized image analysis method, performed on histological lung sections of mice injected with B16-F10 melanoma cells, was used to quantify the efficacy of the treatments. This study demonstrated that the treatment of tumor-inoculated mice with antioxidant supplements at low or high doses, was unable to reduce the invasive potential of melanoma cancer cell, meanwhile significantly improved the survival of tumor-bearing animals.

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Introduction

About one third of the world's population suffer from micronutrient deficiencies and hundreds of millions suffer from chronic diseases of lifestyle. Cardiovascular diseases, and cancer as one of the most important causes of mortality and morbidity globally, will continue to be first and second leading causes of death in the world. Most developing countries, currently are in the process of transition and experiencing the double burden of both communicable and non-communicable diseases in which chronic diseases of lifestyle have emerged while the battle against infectious diseases has not been won. Food supplements are concentrated sources of nutrients or other substances with a nutritional or physiological effect whose purpose is to supplement the normal diet. A micronutrient is defined as a substance needed only in small amounts for normal body function. Micronutrient deficiencies are an important cause of malnutrition and associated ill health in the developing world. Adding single or multiple nutrients to the food chain has been shown to be of great benefit in terms of combating clinical deficiency (1). Vitamins are essential nutrients for human metabolism, playing an important role as coenzymes or enzymes in many vital processes for the normal functioning of the body. In recent years, it has become apparent that vitamins are crucial in health and human disease, due to several investigations that studied this relationship. Currently, it is known that vitamins can have an important role in the prevention and treatment of cancer, but until now no conclusive results were obtained (2). For example, vitamin A supplementation and fortification of table salt with iodine, have both shown enormous health impact in several populations (3), while a recent trial in Tanzania suggests that a multivitamin supplement (thiamin, riboflavin, pyrodoxine, niacin, cobalamin, folate and vitamins C and E) may delay the progression of HIV disease (4). Clinical deficiency of micronutrients is uncommon in the developed world, but interest has increasingly focused on non-clinical deficiencies, or suboptimal status of micronutrients and the effect such deficiencies may have on risk of chronic disease. Suboptimal status of micronutrients such as vitamins C, B and E and folate has been proposed to play a role in the development of cancer, at various sites, chronic renal failure and age-related macular degeneration (5). Vitamin D deficiency is epidemic affecting some 1 billion people worldwide and is mainly caused by chronically inadequate sun exposure. This deficiency is associated with harmful effects on almost all tissues including a predisposition to cancer. In cancer patients vitamin D deficiency is associated with a worsening of the prognosis. While supplements of vitamin D improve musculoskeletal symptoms, proof is still

lacking that these doses convey a protection from cancer. Interventional studies that administer vitamin D versus placebo in patients with cancer should be a high priority because of the hypothesized benefits and the low risk of supplementation with vitamin D (6). Correcting micronutrient deficiencies can be approached in a number of ways. Programmes can be implemented that are designed to encourage individuals to consume more micronutrient-rich foods, commonly-eaten foods can be fortified with micronutrients or the use of micronutrient supplements can be encouraged (1). Cancer cachexia, a progressive wasting syndrome experienced by approximately 80% of patients, is characterized by loss of adipose tissue and lean body mass. This complex metabolic process reflects both reduced nutrient availability and increased nutritional demand. Though cachexia is most commonly associated with particular tumors, no patient or tumor are excluded (7). High-dose multiple micronutrients, including antioxidants, as an adjunct to standard or experimental therapy, may improve the quality of life of cancer patients by increasing tumor response to therapy and decreasing toxicity. Several *in vitro* studies and some *in vivo* investigations support this hypothesis. This assumption is based on the concept that antioxidants will destroy free radicals that are generated during therapy, thereby protecting normal cells against death (8). For the purposes of the present work we focused our attention on a commercial available mixture of micronutrients (Citozym), testing antioxidant supplements, with the aim of collecting evidence linking suboptimal micronutrient status and tumor-bearing animals survival.

Material and methods

Materials

D-MEM, glutamine, penicillin (10,000UI/ml) and streptomycin (10 µg/ml) were from Eurobio Laboratoires (Le Ulis Cedex, France). Fetal Calf Serum (FCS) was from Gibco (Grand Island, NY). [^{14}C]-methylamine (46.6mCi/mmol) was purchased from Amersham International (Bucks, UK). Citozym (CYZ) were from CITOZEATEC, S.r.l. (Peschiera Borromeo, Milano). EDTA, FCS, and HPLC solvents were purchased from Sigma (St. Louis, MO).

Cell culture

Highly metastatic murine B16-F10 melanoma cell line was purchased from the Division of Cancer Treatment, Tumor Repository NIH (Frederick, MD) and propagated under standard culture conditions (9). Cells were

cultured in D-MEM with 10% FCS, supplemented with 200 mM glutamine, 100 U/ml penicillin/streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were harvested twice a week with EDTA, re-fed every other day and used at about 80% confluence.

Cell proliferation

Melanoma cells were plated and grown in 35 mm dishes in D-MEM supplemented as above reported, and treated with two solutions (1:2v/v and 1:4v/v) of CYZ in distilled water for 24, 48 and 72 h. Contamination was excluded by visual control under light microscope. Cells were detached with EDTA and viability assessed after Trypan Blue staining. Cells were counted using a Neubauer modified chamber.

Animals

Six- to 8-week-old male C57BL6/N mice were purchased from IFFA Credo (L'Abreole, France) and were housed throughout the experiments in air conditioned animal room. 15 animals were used each experimental group. Treated mice were fed *ad libitum.* All experimental protocols were carried out following the Guidelines for the Welfare of Animals in Experimental Neoplasia and the ECC Council Directive 86=609, OJL 358, 1st December 1987.

Primary tumours and experimental lung metastases

The B16-F10 murine melanoma cell line is the commonly used model to test *in vivo* formation of tumours and metastases in C57BL6/N mice. This syngeneic cell line easily developed tumor metastases in the lungs when injected into the caudal vein, due to their high invasiveness (10). For antineoplastic study at day 20, the animals were sacrificed, the lungs explanted, measured and fixed for subsequent histological examination. For the experimental lung metastases formation, B16-F10 cells, cultivated *in vitro* were used for the assay when in log phase. In order to evaluate survival animals-bearing tumor were kept under control and sacrificed at the first sign of uncomfortable pain. We treated 15 mice for each group of experiments (15 mice for group 1 control not treated; 15 mice for group 2 treated daily with intraperitoneal (i.p.) administration of CYZ at a concentration of 1:4 in distilled water; 15 mice for group 3 treated daily with i.p. administration of CYZ at a concentration of 1:2 in distilled water, with a total number of 45 mice, by injecting 2×10^5 viable B16-F10 murine melanoma cells, suspended in 0.1 ml sterile PBS, into the caudal vein of the animal. The

plasma concentration of CYZ was determined daily by a HPLC assay of pantothenic acid and calciferol (Dabre et al., 2011) as markers of the mixture, in all experimental groups. The analysis of metastasis formation rate was randomly conducted on 2 animals for each group at 10, 20, 30, 40 and 50 days post-injection. The mice were sacrificed by cervical dislocation and the lungs removed, washed in PBS, fixed in formalin and then metastasis number was assessed by counting under a dissection microscope (11). Survived animals-bearing melanoma tumor, were kept under control and sacrificed at the first sign of uncomfortable pain.

Histology

Invaded lung samples were fixed in 10% formalin for 48 h, dehydrated in ethanol and embedded in paraffin. Tissue 10 μm-sized serial sections performed every 100 μm were obtained with an ultramicrotome Leitz 1512 (Germany) and were stained with haematoxylin and eosin.

Statistical analysis

All experiments were repeated three times, and the results are expressed as the mean ± SD of three different determinations. Data were analyzed by the t Student’s test.

Results

Effect of CYZ treatments on metastases formation

The effect of treatment with CYZ on number and size of metastases formation in mice inoculated intravenously (i.v.) with B16-F10 melanoma cells was investigated. Animals were treated by i.p. administration of CYZ as described in Materials and Methods. The plasma concentration of CYZ was determined daily by a HPLC assay of markers (see Material and methods) as components of the mixture (see Material and methods) , in all experimental groups, and found stable between the overall time of treatments (data not shown). As shown in Table I, the number of lung metastases detected by visual control under light microscope were not reduced, compared to untreated animals. The rate of proliferation of melanoma cell into the target organ (lung), expressed as Growth Index and the frequency of foci expressed as Invasion Index (10) was unchanged with both CYZ concentrations (Table I). Higher magnification of the histological sections of the mouse lungs is shown in Figure 1. In the control experiments (CTR), the

Table 1. Effect of CYZ treatments of mice injected with B16-F10 melanoma cells on lung metastasis analyzed by a morphometric parameters obtained by computer-assisted evaluation performed on histological sections of B16-F10 invaded C57BL6/N mice lungs.

Treatment	Number of lung metastases	Growth* Index	Invasion* Index
None	483 ± 37	210± 25	400± 32
CYZ 1:4	448 ± 12	220± 30	385± 42
CYZ 1:2	410 ± 28	180± 37	420± 38

*(Lentini et al., 2000)

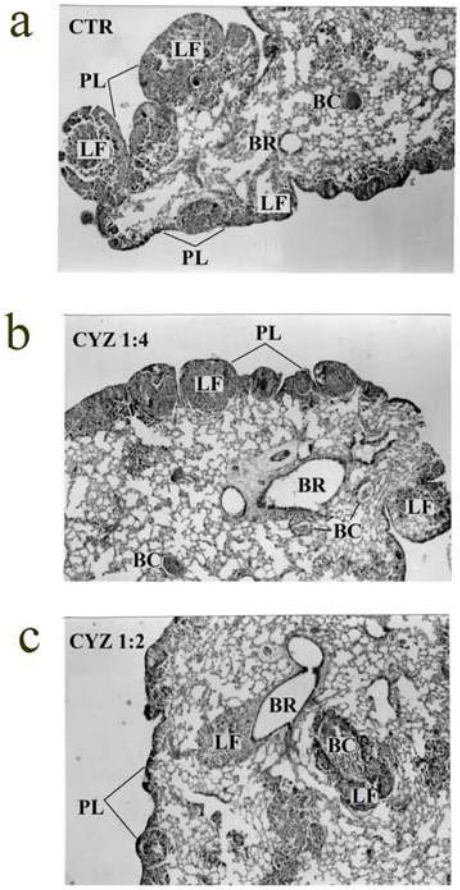

Figure 1. Histological sections of lung of C57BL6/N mice i.v. injected with B16-F10 melanoma cells **a**: untreated animals (CTR); **b**: treated animals with CYZ 1:4 (CYZ 1:4); c: treated animals with CYZ 1:2 (CYZ 1:2). **LF**: lung foci; **PL**: pleura; **BC**: blood capillary; **BR**: bronchus.(x200).

lung foci (LF) appeared larger and mainly in contact with pleura (PL), of the organ (Figure 1a). In animals treated with 1:4 CYZ (CYZ 1:4) negligible variations in tumor colonization were observed (Figure 1b), whereas in the lung sections from mice treated with 1:2 CYZ (CYZ 1:2), lung foci appeared slightly reduced in contact with pleura, and much larger around bronchus (BR) and blood capillary (BC) with respect to the control (Figure 1c).

Effect of CYZ treatments on mice survival

The effect of treatment with CYZ on the survival of mice inoculated i.v. with B16-F10 melanoma cells was investigated. Animals were treated by i.p. administration of CYZ as described in Materials and Methods. The plasma concentration of CYZ was determined daily by a HPLC assay of markers as components of the mixture (see Material and methods), in all the experimental groups, and found stable between the overall time of treatments (data not shown). As shown in Figure 2, CYZ treatment caused a long-term delay in mortality; 50% of the 1:2 CYZ-treated animals died by 38± 3.1 days from the intravenous inoculation of B16-F10 melanoma cells, whereas 1:4 CYZ-treated mice died by 27±2.3 days after melanoma cells i.v. inoculation (control group: 16±1.5 days; $p<0.001$). Therefore, i.p. administration of 1:4

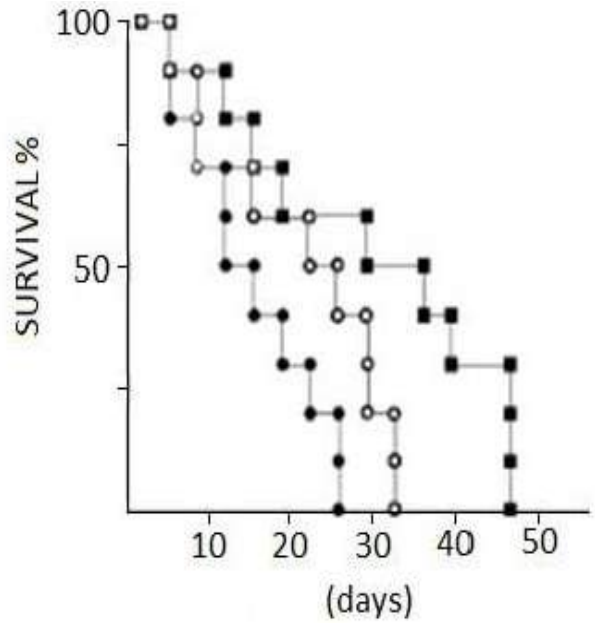

Figure 2. Effect of CYZ administration on the survival of B16-F10 pulmonary metastasis-bearing C57BL6/N mice. Kaplan Meier survival curves for untreated mice (, n=10), CYZ 1:4-treated mice (, n=10) and CYZ 1:2-treated mice (, n=10).

solution of CYZ (group 2), increased the survival of C57BL6/N i.v. injected B16-F10 melanoma cells mice, of about 69 % and with a solution 1:2 of CYZ (group 3) of about 137 % respect with the control group (group 1).

Discussion

While research generally supports the potential of natural antioxidants for reducing cancer risk, only a few have been widely studied. Some standouts include polyphenols, particularly EGCG (epigallocatechin-gallate), found in green tea; genistein, found in soybeans and some other legumes; quercetin, found in apples and onions; PCOs (procyanidolic oligomers, also known as proanthocyanidins), found in abundance in pine bark and grape seed extract, as well as in red wine; citrus flavonoids, including naringenin found in oranges, grapefruits, tangerines and other citrus fruits. Researchers feel that dietary antioxidants may be the most effective cancer-preventing compound discovered to date (12).

Searching for the molecular mechanisms by which antioxidants exert antiproliferative effects on tumor cells, several experimental evidences have been described in the literature (13). Most of authors agree with the antioxidants-induced impairment of the signal transduction pathway mediated by tyrosine kinase and or protein kinase C (14). Some authors have observed the inhibition of the insulin-induced glucose uptake in NCF-7 breast cancer cells as a possible mechanism for the antiproliferative role of naringenin (15), and others demonstrate a possible involvement of the anti-estrogenic activity of naringenin in two cancer cell lines (16). In addition, the anti-invasive role of numerous antioxidants has been well documented, as a result of inhibition of metalloproteinase activity or antiangiogenic action (17).

The aim of the present work was to extend our knowledge about the mechanisms involved in antioxidant mediated antineoplastic activity, investigating the possible induction of cancer cells differentiation in B16-F10 melanoma cells treated with a commercial mixture of antioxidants (CYZ).

The choice of this commercial available mixture of antioxidants was planned because CYZ is reach of vitamin B5, B9, C, D, and amylase and lactase together several carbohydrates. Previously published reports suggest that natural antioxidants, like retinoids or methylxanthines (18), may be considered effective activators of transglutaminases (TGase), a class of enzymes involved in cell differentiation (19). It is well known that intracellular activation of soluble TGase may give rise to cross-linked proteins leading to the formation of envelopes in apoptotic cells (20), whereas extracellular activation contributes to stabilization of the

extracellular matrix (ECM) and promotes cell-substrate interactions (21,22). Therefore it is possible to believe that antioxidants exert their antiproliferative property by the induction of cancer cell terminal differentiation and their antimetastatic activity by the enhancement of cell-cell and cell-ECM adhesion.

The reduction of polyamine synthesis is one of the earliest biochemical events associated with the reduction of cell proliferation (23). In the light of the observed increased activity of TGase, the reduction of SPD and SPM levels in NG- and HP-treated cancer cells may give a crucial contribution to the antiproliferative capability of these flavonoids. A full evaluation of the *in vivo* activity of potential antineoplastic molecules would require that the bioactive concentration of the agents is maintained long enough in the target organs to produce pharmacodynamic effects. Such *in vivo* evaluation necessarily needs knowledge concerning the formulation, administration, and dose-limiting host toxicity as a function of formulation, route of administration and dose scheduling. Although oral administration of CYZ, may appear to be an effective route for the potential antineoplastic treatment of C57BL6/N mice, it was obviously difficult to monitor the amount of product consumed by mice with the food intake. To overcome this difficulty, CYZ was administered by an i.p. route and the plasma levels of two marker components were determined daily. CYZ-markers reached a peak plasma concentration at about the fourth day of treatment, and this value was found to be quite stable during the experimental time. Furthermore, CYZ treatment did not exert any effect on mice liver or lung weights. We have shown that CYZ treatment of B16-F10 melanoma-bearing mice was unable to reduce tumor implantation and proliferation, but surprisingly, significantly increased the animals' lifespan. The wider distribution and the abundant presence of antioxidants in the CYZ formulation, together with the present results, may suggest that these mixture of antioxidants may have the potentiality of a possible contribute to the improvement of the quality of life of cancer patients without any effects on cancer progression. However, the effects of CYZ on cancer, merits further epidemiological investigation and at the moment our preliminary results can therefore not to be extrapolated directly to humans.

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