Reactive oxygen species, antioxidant mechanisms and serum cytokine levels in cancer patients: impact of an antioxidant treatment

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Abstract

Objective. So far, it is not well established whether oxidative stress found in cancer patients results from an increased production of oxidants in the body or from a failure of physiological antioxidant systems. To further investigate this question we have assessed the blood levels of reactive oxygen species as a marker of free radicals producing oxidative stress and the most relevant of the physiological body enzymes counteracting reactive oxygen species, namely glutathione peroxidase and superoxide dismutase. Serum levels of proinflammatory cytokines and IL-2 were also investigated. All these parameters were studied in relation to the clinically most important index of disease progression, namely Performance Status (ECOG PS). We also tested the reducing ability of different antioxidant agents on reactive oxygen species levels by measuring the increase in glutathione peroxidase activity, and the reduction of serum levels of IL-6 and TNF. *Design, setting and subjects*. We carried out an open non randomized study on 28 advanced stage cancer patients (stage III, 10.7 %, and stage IV, 89.3%) with tumours at different (8) sites: all were hospitalized in the Medical Oncology Dept, University of Cagliari Interventions. The patients were divided into 5 groups and a different antioxidant treatment was administered to each group. The selected antioxidants were: alpha lipoic acid 200 mg/day orally, N-acetylcysteine 1800 mg/day i.v. or carboxycysteine-lysine salt 2.7 g/day orally, amifostine 375 mg/day i.v., reduced glutathione 600 mg/day i.v., vitamin A 30000 IU/day orally plus vitamin E 70 mg/day orally plus Vitamin C 500 mg/day orally. The antioxidant treatment was administered for 10 consecutive days. *Results*. Our results show that all but one of the antioxidants tested were effective in reducing reactive oxygen species levels and 2 of them (cysteine-containing compounds and amifostine) had the additional effect of increasing glutathione peroxidase activity. Comprehensively, the "antioxidant treatment" was found to have an effect both on reactive oxygen species levels and glutathione peroxidase activity. The antioxidant treatment also reduced serum levels of IL-6 and TNF . Patients in both ECOG PS 0-1 and ECOG PS 2-3 responded to antioxidant treatment.

Keywords: antioxidant agents • reactive oxygen species • glutathione peroxidase • cytokines • disease progression • cancer patients

Introduction

Normal human cells produce small amounts of reactive oxygen species (ROS), which are reduced by antioxidant enzymes and low molecular weight radical scavengers [1-4].

It is widely accepted that ROS play both positive and negative roles in vivo. Positive are those related to ROS involvement in energy production, phagocytosis, regulation of cell growth and intercellular signalling, and synthesis of biologically

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Moreover, we tested the ability of different antioxidant agents to reduce ROS levels, to increase the antioxidant enzyme activity and to reduce the serum levels of proinflammatory cytokines IL-6 and TNFα. These parameters were then associated with the most relevant of the clinical indexes of patient status, the Performance Status. The main distressing symptoms experienced by cancer patients, which include fatigue, asthenia, anorexia, loss of appetite and nausea, may be related to OS and to an overproduction of selective cytokines such as IL-6 and TNFα. Overall, these symptoms may account for the patients' poor Performance Status (ECOG PS). This is the reason why the serum levels of proinflammatory cytokines (IL-6 and TNFα) and IL-2 were measured in addition to ROS and GPx.

Patients and methods

Patients

The protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects, last amendment of which was adopted by the 52nd WMA General Assembly, Edinburgh, October 2000). The study was approved by the Ethical Committee of the Policlinico Universitario, University of Cagliari Medical School. Written informed consent was obtained from all patients. Twenty-eight advanced stage cancer patients with tumours at different (8) sites were included in the study (mean age 58.6 years, range: 34-75; M/F ratio: 10/18; mean weight 63.6 kg, range 44-96). They were stage III (10.7 %) and stage IV (89.3%) according to the International Union Against Cancer [18]. Approximately 50% of the patients were habitual smokers and some were also heavy alcohol drinkers. Being almost all habitual smokers and heavy alcohol drinkers, the patients affected by head and neck cancer and lung cancer they were distributed homogenously into five treatments as it appears from the Table 2. During the antioxidant treatment (10 days) these patients gave up alcohol and smoking. The rationale for having selected this great variability of tumor sites was to get information on as large as possible a number of tumors in a relatively small sample of patients. All patients were referred to the Medical Oncology Department, Policlinico Universitario, University of Cagliari Medical School, Cagliari. Their clinical characteristics are reported in Table 1. Twenty age-sex-weight/height matched healthy individuals were used as controls (mean age 51.4 years, range: 35-65; M/F ratio: 10/10; mean weight 63.5 kg, range 44-95); among the control group, only one person was a smoker. The mean value of ROS was within the normal range of our laboratory for controls (standard values). Performance status was quantified using the WHO-approved Eastern Cooperative Oncology Group (ECOG) performance status (PS) scale [19]. This scale evaluates three dimensions of health status simultaneously (activity, work, self-care), and scores range from 0=fully active to 5=dead. All but two patients included in the study were chemotherapy-naive: two patients were studied during a chemotherapy regimen, but the antioxidant agents were administered at least two weeks after chemotherapy.

The patients were divided into five groups and a single different antioxidant agent was administered to the patients of each group (Table 2). The selected antioxidant agents were: ALA capsules (Tiobec, Laborest, Nerviano, Milan, Italy) 200 mg/day orally (arm 1), N-acetylcysteine vials 1800 mg/day i.v. or carboxycysteine-lysine salt sachets (Fluifort, Dompè, Milan, Italy) 2.7 g/day orally (arm 2), amifostine vials (Ethyol, Schering Plough, Milan, Italy) 375 mg/day i.v. (arm 3), GSH vials 600 mg/day i.v. (arm 4), vitamin A tablets 30000 IU/day orally plus vitamin E tablets 70 mg/day orally plus vitamin C tablets 500 mg/day orally (arm 5). The antioxidant treatment was administered for 10 consecutive days. The patients were studied at baseline and after antioxidant treatment. Clearly, this was only a preliminary study which did not aim at selecting the most effective antioxidant treatment.

Assessment of blood levels of reactive oxygen species and antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD)

In order to measure the OS in the patients we assessed the two most important parameters able to define the OS in a clinical setting, namely the quantitative assay of both ROS and the two most important of the body antioxidant enzyme systems, GPx and SOD.

Table 1. Patient characteristics

	NO. OF PATIENTS	%
PATIENTS	28	
AGE (years)		
Mean (Range)	58.6 (34-75)	
SEX		
Male	10	35.7
Female	18	64.3
PERFORMANCE STATUS		
0	4	14.2
1	15	53.6
2	8	28.6
3	1	3.6
STAGE		
III	3	10.7
IV	25	89.3
CANCER SITE		
Head and neck	11	39.4
Lung	3	10.7
Breast	5	17.9
Colorectal	2	7.1
Ovary	2	7.1
Endometrium	2	7.1
Melanoma	2	7.1
Myeloma	1	3.6
WEIGHT (kg)		
Mean (range)	63.6 (44-96)	
HEIGHT (meters)		
Mean (range)	1.62 (1.51-1.74)	

Table 2. Characteristics of patients included in the different arms of the study

	NO. OF PATIENTS				
	ARM 1	ARM 2	ARM 3	ARM 4	ARM 5
PATIENTS 28	6	5	7	6	4
SEX					
Male	3	2	3	2	-
Female	3	3	4	4	4
PERFORMANCE STATUS (ECOG)					
0	1	2	1	-	-
1	3	3	2	3	4
2	2	-	4	2	-
3	-	-	-	1	-
STAGE					
II	-	-	-	-	-
III	-	1	-	1	1
IV	6	4	7	5	3
CANCER SITE					
Head and neck	3	3	4	1	-
Lung	-	-	-	2	1
Breast	2	1	-	1	1
Colorectal	1	1	-	-	-
Ovary	-	-	2	-	-
Endometrium	-	-	1	1	-
Melanoma	-	-	-	1	1
Myeloma	-	-	-	-	1

Arm 1: Alpha lipoic acid (ALA) 200 mg/day orally
Arm 2: N-acetylcysteine 1800 mg/day i.v or carboxy-cysteine-lysine salt sachets 2.7 g/day orally
Arm 3: Amifostine 375 mg/day i.v
Arm 4: Reduced glutathione (GSH) 600 mg/day i.v
Arm 5: Vitamin A 30000 IU + Vitamin E 70 mg + Vitamin C 500 mg once day orally
All treatments were administered during 10 days continuously

The ROS levels, GPx and SOD activity were measured on fresh blood samples. Blood samples (5 ml) were drawn from patients by venipuncture after overnight fasting, before the administration and 24 h after the last administration of each antioxidant agent.

The ROS were determined using the D-Roms test (Callegari, Parma, Italy). The test is based on the concept that the amount of organic hydroperoxides present in the blood is related to the free radicals from which they are formed. When the blood sample is dissolved in an acidic buffer, the hydroperoxides react with the transition metal

important compounds [5]. Their negative effects may be very damaging, since they firstly may attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates, and DNA, and secondly induce oxidations, causing membrane damage, protein modification including enzymes, and DNA damage. This oxidative damage is considered to play a causative role in aging, in several degenerative diseases, such as heart diseases, cataracts, cognitive dysfunction, and in cancer [6]. Humans have evolved with antioxidant systems as a protection against free radicals. These systems include some endogenous antioxidants which are produced in the body, and other exogenous systems, which are supplied through the diet. Endogenous antioxidants include both enzymatic and non-enzymatic defences. Enzymatic defences are Se-glutathione peroxidase, catalase, and superoxide dismutase. Non-enzymatic defences are glutathione, histidine-peptides, the iron-binding proteins transferrin and ferritin, lipoic acid, reduced CoQ10, melatonin, urate, and plasma protein thiols. The last two account for the major contribution to the radical-trapping capacity of plasma.

Several mechanisms may lead to oxidative stress (OS) in cancer patients. By preventing a normal nutrition, and thereby a normal supply of nutrients, anorexia/cachexia may contribute to OS by causing an altered energy and nutrient metabolism together with proinflammatory processes, which eventually lead to increased levels of ROS [7]. Indeed, a nonspecific chronic activation of the immune system accompanied by an excessive production of proinflammatory cytokines may in turn increase the ROS production [8-10]. The use of antineoplastic drugs such as alkylating agents and cisplatin may also result in an excess of ROS and may therefore lead to OS [11]. Thus, we can hypothesize that the body redox systems, which include antioxidant enzymes and low molecular weight antioxidants, may be downregulated in cancer patients as a function of the administration of antineoplastic drugs just as it may be a result of disease progression.

In order to counteract ROS and OS several approaches have been tried both in experimental systems and in humans. Among the most used antioxidant agents are alpha lipoic acid (ALA), cysteine-containing compounds, amifostine, reduced glutathione (GSH) and vitamins. ALA is present in a bound lipoillysine form in the human cell mitochondrial proteins, which play a central role in oxidative metabolism. ALA has recently gained considerable attention as an antioxidant [12] for its capacity of inducing a substantial increase in cellular reduced glutathione and thereby restoring severely glutathione deficient cells [13].

Among the cysteine-containing compounds, carboxycysteine-lysine salt appears to be one of the most interesting. Cysteine is a known precursor of glutathione synthesis, which has been shown to act on redox balance and to be capable of significantly improving the antioxidant potential by increasing reduced glutathione levels [14]. Carboxycysteine-lysine salt protects alpha 1 antitripsin from inactivation by hypochlorous acid. In fact, having a chemical structure similar to methionine, it competes with the latter against the oxidative activity of ROS.

Amifostine, an analogue of cysteamine, is a phosphorilated aminothiol prodrug which is dephosphorilated at the tissue site by membrane-bound alkaline phosphatase to its active metabolite, the free thiol, WR-1065. WR-1065, being the form of the drug which is rapidly taken up into cells, is the major cytoprotective metabolite.

GSH is a key molecule in redox body homeostasis. OS induces the transformation of GSH into oxidized glutathione (GSSG) by the action of glutathione peroxidase: GSSG may in turn be transformed into glutathione protein mixed disulfide or reduced back to GSH by glutathione reductase. During cancer growth, the glutathione redox status (GSH/GSSG) decreases in the blood of both tumor-bearing animals and humans. This effect is mainly due to an increase in GSSG levels. The blood GSH/GSSG ratio also decreases in patients with breast or colon cancers, and this change is associated with higher GSSG levels, especially in advanced stages of cancer progression [15].

Antioxidant vitamins, which include vitamin A, vitamin C, and vitamin E, are hypothesized to prevent cancer progression by trapping organic free radicals and/or deactivating reactive oxygen molecules [16-17].

The purpose of the present study was to find out if the blood levels of ROS in advanced stage cancer patients are pathologically high and if the antioxidant enzyme activity, especially GPx, is low.

ions liberated from the proteins in the acidic medium and are converted to alkoxy and peroxy radicals. These newly formed radicals are able to oxidize an additive (N,N-diethyl-para-phenylendiamine) to the corresponding radical cation. The concentration of this persistent species can be easily determined at 505 nm using a spectrophotometer (Form CR 2000, Callegari, Parma, Italy). Results are expressed in CARR U (Carratelli Units), where 1 CARR U corresponds to 0.8 mg/l of hydrogen peroxide [20-21]. The method is considered specific and sensitive: within-run variations were less than 2.6 % and between-run variations less than 4.6 % [21].

Erythrocyte GPx activity was measured using a commercially available kit (Ransel; Randox Lab, Crumlin, U.K.). Heparinized whole blood samples were diluted with a diluting agent to convert the glutathione peroxidase to the reduced form. The sample was incubated for 5 min and then diluted with Drabkin's reagent to avoid falsely elevated results due to the presence of peroxidases in human blood. The diluted sample was mixed with reagent (constituted by glutathione, glutathione reductase and NADPH) and Cumene Hydroperoxide. GPx catalyses the oxidation of reduced Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidized Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance after 1 and 2 min at 340 nm was measured. The result obtained was expressed in units/litre of haemolysate and was multiplied by the appropriate dilution factor (41) to obtain the result in U/l of whole blood. Erythrocyte SOD activity was measured using a commercially available kit (Ransod; Randox Lab, Crumlin, U.K.). The role of SOD is to accelerate the dismutation produced during oxidative energy processes of superoxide radicals (O^{2-}) to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase in order to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. The assay is carried out on washed red blood cells, by diluting the samples to give between 30 and 60% inhibition. Together with the kit, a standard is supplied, which is diluted to provide a range of standards and a calibration curve. All standard and diluted sample rates must be converted into percentages of the blank rate and subtracted from 100% to give the inhi-

bition. A standard curve is produced by plotting % inhibition for each standard against Log 10. The result was multiplied by the appropriate dilution factor (100) and expressed in units/litre (U/L) of whole blood.

Serum levels of proinflammatory cytokines and IL-2

Proinflammatory cytokines (IL-6 and TNFα) and IL-2 were detected by a "sandwich" ELISA test (Biosource Europe SA, Belgium for IL-6 and TNFα; Immunotech SA, Marseille, France for IL-2) using monoclonal antibodies (mAbs) for 2 different epitopes of the cytokine molecule.
The absorbance of the sample at 450 nm for IL-6 and TNFα, and at 405 nm for IL-2 was measured with a spectrophotometer (Sirio, Seac, Florence, Italy). A standard curve was prepared by plotting the absorbance value of the standards versus corresponding concentrations. The concentration of the cytokine in the sample was determined by extrapolating from the standard curve. Ranges of assay results were 2-1,500 pg/ml for IL-6; 10-1,500 pg/ml for TNFα; 5-1,000 pg/ml for IL-2. Intra-assay variations were 3% for IL-6, 6% for TNFα and 3% for IL-2. Inter-assay variations were 7% for TNFα, 8% for IL-6 and 7% for IL-2.
The results were expressed in pg/ml. More details of the techniques used are described in our previous report [23-24].

Statistical analysis

The results were expressed as mean ± SD, and the significance of the difference between the mean values of cancer patients and controls was determined by the Student's t-test.
The level of significance was corrected by multiplying the p-value by the number of comparisons performed (n) according to Bonferroni's correction. The paired Student's t-test was used to compare the values of the parameters before and after antioxidant treatment. Significance was determined at the 5%, 1% and 0.1% level, two-sided. Confidence intervals at 95% were also calculated.
For data analysis ANOVA test was performed by comparing the different arms of treatment for the single variables (ROS, GPx, etc.).

Table 3. Assessment of blood levels of ROS, antioxidant enzymes, serum proinflammatory cytokines and IL-2 of 28 cancer patients and 20 controls.

	CONTROLS	PATIENTS	95% CI	p*
ROS (Carr U)	172 ± 32.2	414.4 ± 86	-283.10; -201.70	**0.006**
GPx (U/l)	10813 ± 2134.7	6793 ± 2310.5	2700.19 ; 5339.81	**0.006**
SOD (U/ml)	84 ± 43.2	58.4 ± 28.6	4.76; 46.44	0.102
IL-6 (pg/ml)	1 ± 2.5	29.8 ± 18.6	-37.25; -20.35	**0.006**
TNFα (pg/ml)	19 ± 6.7	42.9 ± 24	-35.03; -12.77	**0.006**
IL-2 (pg/ml)	37.2 ± 23	21.5 ± 14.2	4.88; 26.52	**0.036**

Results are expressed as mean ± standard deviation. Significance was calculated by Student's t-test.
*The level of significance was corrected multiplying the p-value by the number of comparisons performed (n) according to Bonferroni's correction.
PS, ECOG PS; ROS, reactive oxygen species; GPx, glutathione peroxidase; SOD, superoxide dismutase

Results

Assessment of blood levels of reactive oxygen species and antioxidant enzymes

The blood levels of ROS at baseline were significantly higher in cancer patients (414.4 ± 86.0 CARR U) than in controls (172.0 ± 32.2 CARR U, p=0.006). Conversely, GPx activity at baseline was significantly lower in cancer patients (6793.0 ± 2310.5 U/l) than in controls (10813.0 ± 2134.7 U/l, p = 0.006) (Table 3). The association between the levels of ROS and GPx with patient ECOG PS showed that ROS were lower and GPx was higher in patients with PS 0-1 than in patients with PS 2-3 but not at the statistically significant level (Table 4).

Assessment of serum levels of proinflammatory cytokines and IL-2

The values of serum proinflammatory cytokines IL-6 and TNFα were significantly higher in cancer patients than in controls (p = 0.006). The values of serum IL-2 were lower in cancer patients as compared to controls (p=0.036) (Table 3).

Assessment of blood levels of reactive oxygen species, antioxidant enzyme activity, serum proinflammatory cytokines and IL-2 at baseline and after antioxidant treatment

The comparison of the blood levels of ROS and GPx activity at baseline and after antioxidant treatment of all patients (taking into consideration all arms) showed a significant decrease of blood levels of ROS after antioxidant treatment compared to baseline (p = 0.006) and a significant increase of GPx activity (p = 0.000) (Table 5).
The comparison of the serum values of proinflammatory cytokines at baseline and after antioxidant treatment of all patients showed a significant decrease of IL-6 (p=0.048) and TNFα (p = 0.012) (Table 5).
Considering the effect of the single antioxidant treatments, blood levels of ROS decreased signifi-

Table 4. Association of blood levels of ROS, antioxidant enzymes, serum proinflammatory cytokines and IL-2 with ECOG PS of patients

	CONTROLS	PATIENTS PS 0-1	PATIENTS PS 0-1 VS. CONTROLS		PATIENTS PS 2-3	PATIENTS PS 2-3 VS. CONTROLS		PATIENTS PS 0-1 VS. PS 2-3	
			95% CI	p*		95% CI	p*	95% CI	p*
ROS (Carr U)	172±32.2	386.6±73.5	-251.28; -117.92	**0.006**	464.5±87.5	-337.13; -247.87	**0.006**	-141.65; -14.15	0.114
GPx (U/l)	10813±2134.7	7150.7±2634.9	2091.44; 5233.16	**0.006**	6149.1±1478.2	3118.49; 6209.31	**0.006**	-864.06; 2867.26	1.680
SOD (U/ml)	84±43.2	57.3±26.2	2.86; 50.54	0.174	60.2±34	-8.31; +55.91	0.840	-26.52; 20.72	4.818
IL-6 (pg/ml)	1±2.5	22.5±9.8	-26.10; -16.90	**0.006**	42.9±23.7	-52.68; -3.1.12	**0.006**	-33.40; -7.40	0.066
TNFα (pg/ml)	19±6.7	40.6±20.1	-31.25; -11.95	**0.006**	46.9±30.5	-42.30; -13.50	**0.006**	-25.93; 13.33	3.09
IL-2 (pg/ml)	37.2±23	24.5±15.6	-0.38; 25.78	0.342	16.1±9.5	5.47; 36.73	0.060	-2.79; 19.59	0.810

Results are expressed as mean ± standard deviation. Significance was calculated by Student's t-test.
*The level of significance was corrected multiplying the p-value by the number of comparisons performed (n) according to Bonferroni's correction.
PS, ECOG PS; ROS, reactive oxygen species; GPx, glutathione peroxidase; SOD, superoxide dismutase

Table 5. Assessment of blood levels of ROS, antioxidant enzyme activity, serum proinflammatory cytokines and IL-2 of 28 cancer patients at baseline and after antioxidant treatment.

	ALL PATIENTS				
	BASELINE	AFTER	95% CI	P VALUE	Δ%
ROS (Carr U)	414.4 ± 86.0	352.1 ± 80.1	40.53; 84.11	0.006	-15
GPx (U/L)	6793.0 ± 2310.5	9032.0 ± 2289.0	-2953.05; 1525.09	0.000	+33
SOD (U/mL)	58.4 ± 28.6	70.1 ± 34.2	-27.64; 9.42	0.388	+20
IL-6 levels	29.8 ± 18.6	20.9 ± 14.0	4.03; 13.86	0.048	-30
TNFα levels	42.9 ± 24.0	30.0 ± 15.9	1.36; 24.22	0.012	-30
IL-2 levels	21.5 ± 14.2	21.1 ± 9.8	-5.70; 6.58	0.786	-2

Δ %, percentage of variation.
Significance between values at baseline and after antioxidant treatment was calculated by paired Student's t-test.
ROS, reactive oxygen species; GPx, glutathione peroxidase; SOD, superoxide dismutase.

Table 6. Assessment of blood levels of ROS and GPx activity in the single arms of antioxidant treatment in 28 cancer patients.

	No. of Patients	ROS				GPx			
		Baseline	After	P value	Δ%	Baseline	After	P value	Δ%
Arm 1	6	445.2±99.8	347.3±98.3	0.051	-22	6033.8±1040.9	8146.8±2741.1	0.082	+35
		95% CI -5.77; 201.44				**95% CI -4610.79; 384.79**			
Arm 2	5	330.8±83.4	257.8±46.2	**0.018**	-22	6412.4±1340.7	9823.6±2363.9	**0.005**	+53
		95% CI 20.51; 125.49				**95% CI -5108; -1713.81**			
Arm 3	7	440.6±87.0	396.3±68.0	**0.007**	-10	6625.0±1797.2	9160.6±1277.6	**0.006**	+38
		95% CI 17.19; 71.38				**95% CI -4026.78; -1044.36**			
Arm 4	6	434.8±64.4	382.4±57.7	**0.050**	-12	6765.0±2288.7	8511.6±2295.6	0.052	+26
		95% CI 1.84; 88.83				**95% CI -2993.80; -204.20**			
Arm 5	4	401±63.4	348.3±56.0	**0.047**	-13	9009.8±4689.2	10414±3034.2	0.340	+16
		95% CI 1.38; 104.12				**95% CI -5353; 2545.28**			
ANOVA test*				0.433				0.460	

* ANOVA test was performed by comparing the different arms of treatment for the single variables. Significance between values at baseline and after antioxidant treatment was calculated by paired Student's t-test. %, percentage of variation.
Arm 1: Alpha lipoic acid (ALA) 200 mg/day orally; Arm 2: N-acetilcysteine 1800 mg/day i.v or carboxycysteine-lysine salt oral solution 2.7 g/day; Arm 3: Amifostine 375 mg/day i.v.; Arm 4: Reduced glutathione (GSH) 600 mg/day i.v; Arm 5: Vitamin A 30000 IU + Vitamin E 70 mg + Vitamin C 500 mg once day orally

cantly compared to baseline in all but 1 arms and with different levels of significance. The GPx activity increased significantly in two arms compared to baseline (Table 6).

The comparison by ANOVA test of the different arms of treatment for the single variables studied (ROS, GPx, etc.) showed no significant difference between the treatment arms (Table 6 for ROS and GPx, not reported for remaining variables).

Association of ECOG PS with different biological parameters (ROS, GPx activity, cytokines)

The blood levels of ROS decreased significantly after antioxidant treatment compared to baseline both in patients with ECOG PS 0-1 and in those with PS 2-3. Conversely, the GPx activity increased significantly after antioxidant treatment in both groups. IL-6 decreased significantly

Table 7. ROS, GPx activity and Cytokines at baseline and after antioxidant treatment in the 2 different groups of patients: ECOG PS 0-1 and ECOG PS 2-3.

	PATIENTS PS 0-1			PATIENTS PS 2-3		
	BASELINE	AFTER	P VALUE	BASELINE	AFTER	P VALUE
ROS (Carr U)	386.6±73.5	315.7±68.6	0.000	464.5±87.5	417.5±54.3	0.008
	95% CI 40.19 ; 101.48			**95% CI 15.85 ; 78.15**		
GPx (U/L)	7150.7±2634.9	9656±2463.2	0.000	6149.1±1478.2	7908.9±1441.2	0.004
	95% CI -3507.97 ; -1502.69			**95% CI -2797.10 ; -736.50**		
SOD (U/mL)	57.3±26.2	77.4±32.6	0.088	60.2±34.0	57.0±34.7	0.784
	95% CI -43.52; 3.30			**95% CI -22.47; 28.87**		
IL-6	22.5±9.8	14.4±4.0	0.000	42.9±23.7	32.5±17.9	0.117
	95% CI 4.21; 12.05			**95% CI -3.16; 24.00**		
TNFα	45.1±27.1	32.8±17.1	0.265	46.9±30.5	25.1±13.0	0.063
	95% CI -6.13; 21.86			**95% CI -0.34; 44.08**		
IL-2	24.5±15.6	23.3±10.6	0.916	16.1±9.5	17.1±7.0	0.726
	95% CI -9.83; 8.88			**95% CI -7.4; 5.36**		

Significance between values at baseline and after antioxidant treatment was calculated by paired Student's t-test.

after treatment only in patients with ECOG PS 0-1 (Table 7).

Safety

The administration of antioxidant agents has been proven to be safe: no adverse events were recorded except in one patient who, after amifostine administration, had a short episode of orthostatic hypotension, which cleared up spontaneously in a few minutes. The compliance to the antioxidant treatment was very high, and no patient was withdrawn from or refused to continue treatment.

Discussion

The ROS cause extensive damage to DNA, protein and lipid and it is argued that this damage is a major contributor to aging and to degenerative diseases of aging such as cancer [25-26]. Despite our increasing understanding of the possible mechanisms through which OS exerts a regulatory role in tumor growth and progression, including genomic instability [27], oncogene activation [28] and angiogenesis [29], several important questions remain unanswered. It is unclear whether OS in tumor results from an increased oxidant production or from a failure of antioxidant systems [30]. While important changes in cellular redox homeostasis

during tumor growth have been documented in experimental models [31, 15], such variations have not been demonstrated in humans. Most of the difficulties encountered in these studies are related to the complexity of the biochemical pathways which regulate the cellular redox balance. A wide variety of oxidizing molecules such as ROS and/or depleting agents can alter the glutathione redox state, a key compound in the regulation of body redox homeostasis. The glutathione redox state is normally maintained by the activity of GSH-depleting (GPx) and -replenishing enzymes (GR). The importance of glutathione and related enzymes and their variation in tumors has so far been poorly investigated [15, 32].

In the present study we have demonstrated that the ROS production is pathologically high (significantly higher than that of normal individuals) in advanced stage cancer patients, and that it is somehow associated with the general patient status, *i.e.* PS: indeed, the highest values were found in patients with ECOG PS 2-3. The GPx activity (being one of the physiologically most important antioxidant defence systems) shows an inverse trend just as cancer patients exhibit significantly lower values than controls [33-36]. The same behaviour is evident in patient PS values: indeed, the lowest GPx values are seen in patients with PS 2-3. Considering the results of ROS and GPx together, cancer patients show a typical pattern of overt OS, in which the reduced antioxidant defence systems are associated with the increased oxidant production [37].

The main goal of the study was to verify if the administration of different antioxidant agents, given either orally or i.v. to cancer patients, is feasible and effective, *i.e.* if it reduces the blood levels of ROS and increases antioxidant enzymes.

Our interest was also focused on the ability of antioxidant agents to downregulate the serum levels of proinflammatory cytokines IL-6 and TNFα, ??? which are well known to be involved in the onset of cancer cachexia [8-10]. Indeed, OS can mediate upregulation of the production of proinflammatory cytokines, which in turn are central to the induction of cachexia [7-8].

The antioxidants we have selected for the present study included ALA and cysteine-containing compounds, which had already shown, alongside their antioxidant efficacy, to be able to restore important immunological functional defects in peripheral blood

mononuclear cells isolated from cancer patients [38]. The other antioxidants, *i.e.* vitamins A, C, E, amifostine and GSH were tested for the first time. The reasons for this choice of antioxidants were based on different considerations. Firstly, all may be administered both orally and i.v. Thereby the different personal preferences and/or patient compliance may be addressed. Secondly, they have been shown to be effective in our hands [39] and in several of the papers below cited. Thirdly, they have different mechanisms of action. Indeed, numerous recent data demonstrated that antioxidant agents are effective in reducing the OS and they even have an impact on cancer progression. In fact, supplementation with vitamin C or an antioxidant mixture containing vitamin C, ALA and vitamin E increases plasma F(2)-isoprostane levels, an index of oxidative stress in humans with high body mass index [40]. A recent paper provides evidence that N-acetylcysteine has a strong antiangiogenic potential that could be exploited for preventing cancer progression [41].

The present study shows that all but one of the antioxidants tested were effective in reducing ROS levels and two of them, namely cysteine-containing compounds and amifostine, were also effective in increasing GPx activity. Indeed, comprehensively, the "antioxidant treatment" was found to be effective both on ROS levels and GPx activity. Considering the results, it is to be taken into account that the duration of treatment was short (10 days) and perhaps not all its potential benefit was exploited. Obviously, in clinical use this treatment must be planned over a much longer period of time. Which is to be considered the best antioxidant treatment has not yet been established. Several factors must be taken into consideration before making the decision: effectiveness, safety, patient compliance, treatment feasibility and the costs or cost/effectiveness of the treatment. From the results of the present study, we would suggest the combination of ALA and carboxycysteine for outpatient at home self administration as the best treatment. For inpatients a treatment with GSH + ALA + carboxycysteine may be a better choice considering both the patient preferences and its effectiveness. Amifostine, proven to be one of the most effective of the antioxidants, may not be the drug of choice considering firstly the high costs involved (the drug itself and its exclusively i.v. administration)

and secondly its potential adverse effects, although their incidence is rare and not severe (hypotension).

In the present study we confirm, as reported in several our previous papers [8-10], that the levels of proinflammatory cytokines, and particularly IL-6 and TNFα, were higher in cancer patients as compared to controls, and that antioxidant treatment reduced serum levels of IL-6 and TNFα in all patients. In agreement with our results, antioxidants agents such as GSH [42], the precursor of the synthesis of GSH N-acetylcysteine together with other glutathione prodrugs were found to decrease the production of TNFα, IL-6 and IL-8 [43]. Moreover, both patients with ECOG PS 0-1 and those with ECOG PS 2-3 responded positively to antioxidant treatment with respect to ROS and GPx, while only patients with ECOG PS 0-1 responded with respect to IL-6. The short duration of the antioxidant treatment must also be taken into account when evaluating its effect on the proinflammatory cytokine levels.

Summing up, our results warrant further investigation with an adequate clinical trial to test the hypothesis that the supplementation of antioxidant agents may prevent/protect cancer patients from oxidative stress, occurring either spontaneously or enhanced by treatment with cisplatin or other oxidative damage-inducing drugs [44, 25].

A phase III clinical trial based on the reported results is soon to be activated in our Institution.

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