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Plasma Peroxyl Radical Trapping Capacity in Lung Cancer Patients: A Case-Control Study

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Increasing evidence suggests that cancer patients express oxidative disturbances. The main objective of this cross-sectional case-control study (n = 57 + 76) was to explore whether lung cancer patients, when compared to healthy controls, have alterations in their plasma peroxyl radical trapping capacity (TRAP). Group matching was used with respect to age, sex and smoking history. A secondary objective was to observe the effects of life-long cigarette consumption on plasma TRAP and its components. Mean TRAP values were significantly lower in the cancer patients than in the control group (1143 vs 1273 umol/l, p = 0.0002). Moreover, all the components of TRAP (except uric acid) were significantly lower in the cancer group: protein SH-groups 442 vs 571 umol/l, ascorbic acid 34.0 vs 46.5 umol/l and vitamin E 25.0 vs 33.8 umol/l. The as yet unidentified antioxidant compounds in plasma contributed 26.5% of plasma TRAP in the cancer group and 30.2% in the control group. There was no correlation between cigarette consumption in pack-years and plasma TRAP; however, plasma concentrations of uric acid and ascorbic acid were negatively correlated with cigarette consumption.

Keywords: Lung cancer, oxidative stress, antioxidants, vitamin E, vitamin C, uric acid, smoking

INTRODUCTION

Persistent oxidative stress—expressed as an imbalance between the pro-oxidant and antioxidant defense systems in favor of the former[1]—has been suggested to partly explain those characteristics of cancer such as activated proto-oncogenes and transcription factors, genomic instability, chemotherapy resistance, invasion and metastasis.[2] Metabolic consequences of reactive oxygen species and reactive nitrogen species have been linked both with the initiation and progression of cancer[3] and with changes in cell proliferation with chronic inflammation.[4] It has been reported that several human tumor cell lines produce large amounts of reactive oxygen intermediates.[5] Hence it can be hypothesized that not only carcinogenesis-related factors are relevant when viewing oxidative pathways in human cancer, but also the metabolic consequences of the cancer itself, possibly leading to a

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further imbalance between pro-oxidant and antioxidant defense systems.

The synergistic actions of various plasma antioxidant compounds[10] are of great importance in controlling complex intra- and extracellular oxidant pathways. Evaluating the responses of all major plasma antioxidant compounds to the hypothesized oxidative stress persistent in cancer is thus vital. However, there is no consensus concerning the preventive or therapeutic effects of vitamin C or vitamin E in lung cancer.[6–8] A major ATBC study[7] raised the possibility that beta-carotene could even have harmful effects on male smokers. This finding, emphasized by similar observations in the CARET study,[9] led Finnish health care authorities to place antioxidant preparations containing beta-carotene under doctors' prescription.

Very few attempts have been made to explain the status of overall oxidative stress in relation to human cancer. The problem with clinical studies has been an inadequacy of methodology in explaining the status of oxidative stress. The most recent methods have been the measurement of lipid peroxidation products,[11] the total peroxyl radical trapping antioxidant capacity of plasma [TRAP],[12–14] alkanes in exhaled air,[15] and assessing the oxidatively modified DNA bases in urine, e.g. 8-hydroxydeoxyguanine.[2,16] Furthermore the total peroxyl radical trapping ability of a biological fluid can be evaluated by determining its total peroxyl radical trapping antioxidative parameter. Based on the original method of Wayner,[17] we have developed a chemiluminescene method of this measurement. Our method,[18] while incapable of explaining tissue specific situations, nevertheless provides a good view of *in vivo* situations and has previously been applied in clinical research[12–14] work.

To the best of our knowledge, there are no previous studies which measure the plasma total peroxyl trapping capacity and the individual components of plasma in lung cancer and control patients. We conducted a cross-sectional case control study of 57 *untreated* lung cancer patients and 76 controls having no lung or other cancer in their history. The present study was undertaken primarily to explore the effects of lung cancer on TRAP and its components (uric acid, protein SH-groups, vitamin C and vitamin E). Secondarily, since smokers are known to be prone to concomitant oxidative burden,[19] we were interested in exploring the effects of long-term heavy smoking on plasma TRAP. If oxidative stress is present in lung cancer per se, it is logical to hypothesize that the plasma TRAP of cancer patients should be lower than that of control patients.

MATERIAL AND METHODS

Patients

The study was conducted at two Finnish institutions within the same county (Tampere University Hospital and Lahti Central Hospital) between June 1992 and March 1996 among new, *untreated* patients who had histologically defined lung cancer. The Ethical Committee of both hospitals approved the study, and an informed consent form was signed by all patients and controls. The criteria included all new, *previously untreated* lung cancer patients with histologic verification of the cancer and without: 1) regular allopurinol treatment 2) acute serious infection 3) unstable angina pectoris 4) regular use of antioxidant vitamin preparations during the three months prior to the study 5) ingestion of any antioxidant vitamin supplement during the 7 days preceding blood sampling for TRAP and 6) any other previous cancer.

A total of 57 patients participated in the study, consisting of 47 men and 10 women. Cigarette life-time consumption expressed in pack years (i.e. packs smoked per day × years smoked) was 27.7 in the cancer patients and 29.2 years in the control group. Histologic classification revealed 43 nonsmall cell- and 14 small cell carcinomas (SCLC). Staging of the cancers resulted in 14

stage II, 12 stage IIIa, 12 stage III b and 17 stage IV lung cancers. Histologic classification and staging were performed according to the criteria of ICD-O[20] and UICC.[21]

The Karnofsky performance status describing the capacity of patients' daily activities placed them accordingly in different categories: Performance Status 100 (No symptoms or signs of cancer) = 7 patients; 90 (Very few symptoms) = 27 patients; 80 (Some symptoms) = 9 patients; 70 (Symptoms, incapable of working, capable of living at home) = 12 patients, 60 (Some help needed, mostly capable of taking care of personal needs) = 1 patient; 40 (Hospitalized patient, rapidly progressing disease, unable to take care of oneself) = 1 patient. At the time of sampling 5 patients were hospitalized; the remaining were polyclinic patients. All patients were ambulatory and had received no previous treatment for any cancer. The possible effects of impaired nutrition reflected as weight loss on plasma TRAP were evaluated by using a standardized questionnaire formula which was first filled by all the patients and controls and later reinterviewed by the first author. The formula was similar to the one used in the ATBC[7] study. Questions concerning loss of weight during 6 months prior to the blood samping were analyzed.

Controls

The control persons were smoking volunteers without lung or other cancer in their history and were from the same county. The protocol included voluntary smokers or ex-smokers aged over 40 years. Exclusion criteria were the same as for the patient group. Twelve of the controls resided in the local War Veterans Home; none were hospitalized. The control persons were recruited to the study within the above-mentioned study time; thus no historical controls were used. All control persons were interviewed by an oncologist and a medical status was performed. None of them were scheduled to further examinations due to suspected cancer. Furthermore all the patients came for a follow-up visit four months after the blood sampling. At that time-point none of them had been diagnosed to have cancer, nor were they scheduled for any examinations due to suspected cancer. Two patients were excluded from the study due to taking antioxidant vitamin preparations during the last week before blood sampling. One person was excluded after the sampling due to previously undiagnosed gout.

Blood samples for TRAP

Plasma samples for evaluating TRAP were collected at the time of diagnosis before the patients were hospitalized to receive any treatment (except the five hospitalized patients). The control persons provided their blood samples at the hospital laboratory (64 persons) and at the War Veterans Home (12 persons). All samples were collected after 8 hours of fasting, mainly from 7 am to 11 am. Twenty ml of venous blood was drawn from the antecubital vein into Vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). The samples were immediately protected from light and transported in ice to the laboratory, where plasma was separated by a centrifuge. For ascorbic acid determination, a special tube containing meta-phosphoric acid (5% final concentration) was prepared. All samples were stored at –80°C in a CO_2 atmosphere for analysis within three months.

Chemicals and instruments

For the measurement of TRAP, ABAP (2,2-azo-bis 2-amidinopropane hydrochloride) was purchased from Polysciences (Warrington, PA, USA) and luminol (5-amino-2,3-dihydro-1,4-phatalazideedione) from Sigma (St. Louis, MO, USA); TROLOX (&-hydroxy-5,7,8-tetramethyl-chroman-2-carboxylic acid) was a gift from F. Hoffman-La Roche Ltd. (Basel, Switzerland). A Pharmacia LKB Wallac Luminometer 1251 was used in measuring chemiluminescence. The lumi-

nometer was controlled and processed by a PC computer, whose program was purchased from TriStar Enterprises Oy (Tampere, Finland).

Determination of TRAP

The main principles of TRAP determination are described in greater detail elsewhere.[17,18] Briefly, constant production of peroxyl radicals by ABAP is followed by luminol-enhanced chemiluminescence. The time in which the added test sample extinguishes the reaction is directly proportional to the peroxyl radical-trapping antioxidant capacity of the sample. TRAP is thus expressed as micromoles of peroxyl radicals trapped by one litre of the sample. TROLOX was used as a standard.

In addition to the direct measurement of TRAP, calculated TRAP ($TRAP_{calc}$) was also derived from concentrations of individual peroxyl radical-trapping antioxidants in plasma. In order to determine the contributions of individual peroxyl radical trapping antioxidants to TRAP, the stoichiometric factor of each antioxidants is required. These factors indicate the molar amount of peroxyl radicals trapped by each mole of antioxidants (n). The stoichiometric factor for TROLOX is known to be 2.0.[17] Thus $TRAP_{calc}$ = 2.0[tocopherol concentration] + 2.0[uric acid concentration] + 0.7[ascorbic acid concentration] + 0.4[protein SH-groups concentration]. The percentage contribution of individual antioxidants was calculated from the total TRAP by using the stoichiometric factor of each antioxidant component. The difference between total measured TRAP and calculated TRAP (TRAP-$TRAP_{calc}$) is composed of the antioxidative capacity of yet as unidentified antioxidants (i.e. UNID).

Other Chemical Determinations

The concentrations of plasma ascorbic acid and urate were measured by high performance liquid chromatography (HPLC) with an electrochemical detector, according to the method of Frei et al.[22] Vitamin E was determined by the modified HPLC method.[23] Protein SH-groups were determined according to Ellman.[24]

Statistics

Sample size calculations were based on plasma concentrations of TRAP and on existing lung cancer data (n = 60). It was calculated that at least 50 healthy controls were required to ensure an 80% power of detecting a difference of 100 umol/l between the groups with a significance level of 5%.

Pearson correlations were calculated to examine relationships between age, smoking history and TRAP or its components. An unpaired t-test was used to compare the two groups with respect to age, smoking history, plasma cholesterol and the plasma concentrations of TRAP and its components. When appropriate, an analysis of variance (ANOVA) was performed; where smoking or cholesterol were included as covariates, and loss of weight was included as a grouping factor. A Chi-square test was used to test the nominal variables. Analysis of variance (ANOVA) was used in the lung cancer group to test the effect of cancer stages and Karnofsky points.

RESULTS

Table I shows the median baseline characteristics of the cancer patients and the controls. The loss of weight and symptoms of reduced appetite in both groups is shown. The differences of age and cholesterol between the groups were handled with the subsequent analyses of the data.

Table II shows the mean values, standard deviations, confidence intervals and p-values of plasma TRAP and its individual components in both groups.

Loss of weight > 1 kg was reported in 34% of the cancer patients and 17% ot the controls. Loss of weight during six months prior to blood sam-

TABLE I Characteristics of untreated cancer patients and healthy controls

Baseline variable	Cancer n = 57	Control n = 76	
Age (range)	65.3 (44–84)	60.2 (45–87)	0.004*
Sex (male/female)	47/10	68/8	0.24**
Smoking (pack-years)	27.7 (±17.2)	29.2 (±17.7)	0.62*
Plasma cholesterol	4.9 (±1.1)	5.4 (±1.0)	0.006*

Smoking and plasma chlosterol expressed as mean ±SD. P-values for the difference of unpaired T-test* and Chi-square test**.

ling had a significant reducing influence on plasma ascorbate levels within the cancer group, but not in the control group, while it had *no* effect on TRAP and the remaining components in either group. Thus loss of weight was included as a grouping factor for plasma concentrations of ascorbic acid (Table II).

Mean TRAP values were significantly lower in the cancer patients than in the control group 1143 vs 1273 umol/l ($p = 0.0002$) with an estimated 95% CI for the difference (–197 to –64). The sum of the concentrations of known antioxidant components of the plasma, multiplied by their stoichiometric factors i.e. $TRAP_{calc}$, did not significantly differ between the two groups. However, the 95% confidence interval for the difference (–99 to 3 umol/l) indicates a clear tendency that the level of $TRAP_{calc}$ among cancer patients was lower than among healthy controls. There were no significant differences between concentrations of uric acid, which was the largest contributor to plasma TRAP in both groups. However, all other components of TRAP were significantly lower in the cancer patients than in the control group: protein SH-groups 442 vs 571 umol/l ($p < 0.0001$); ascorbic acid 34.0 vs 46.5 umol/l ($p < 0.0003$); vitamin E (including cholesterol as a covariate) 25.0 vs 33.8 ($p < 0.0001$) and the plasma unidentified peroxyl radical trapping antioxidants, i.e. UNID 310 vs 387 umol/l ($p = 0.002$) (Table II). The percentage contributions of the individual antioxidants to TRAP in each group and the 95% CI for the difference are shown in Table III.

The effects of smoking on TRAP and its components was estimated in both groups by correlation analysis. We found a negative correlation between concentrations of uric acid ($r = 0.26$, $p = 0.03$) and ascorbic acid ($r = –0.31$, $p = 0.007$) and the amount of smoking, expressed in pack-years in the control group. No correlation was found between elapsed time after the latest cigarette before blood sampling and plasma TRAP or its

TABLE II Plasma concentrations (umol/l) of TRAP and its components in lung cancer patients compared to healthy controls

umol/l	Cancer patients Mean ± SD (n = 57)	Healthy controls Mean ± SD (n = 76)	p-value	95% CI between for the difference groups
TRAP	1143 ± 181	1273 ± 199	0.0002	–197 to –64
$TRAP_{calc}$	824 ± 150	890 ± 144	0.06	–99 to +3
Urate	296 ± 296	280 ± 69	0.22	–9 to +39
Protein SH-groups	442 ± 80	571 ± 80	p < 0001*	–140 to –87
Vitamin E	25.0 ± 9.8	33.8 ± 9.5	<0.0001**	–10 to –4
Vitamin C	34.0 ± 19.5	46.5 ± 18.4	0.0003**	–19 to –6
UNID	310 ± 149	387 ± 103	0.002	–120 to –32

P-value of unpaired t-test or of analysis of covariance (ANCOVA) where * age, ** cholesterol or *** smoking and weight loss as a grouping factor, is included as a covariate.

TABLE III The percentagewise contributions of individual components to TRAP in lung cancer patients compared to healthy controls

Component	Cancer patients Mean ± SD (n = 57)	Control persons Mean ± SD (n = 76)	95% confidence interval for the difference
Urate	51.3 ± 9.9	43.6 ± 6.9	4.8 to 10.6
Protein SH-groups	15.8 ± 3.5	18.3 ± 3.6	−3.8 to −1.3
Vitamin E	4.4 ± 1.5	5.3 ± 1.3	−1.4 to −0.5
Ascorbate	2.1 ± 1.2	2.6 ± 1.2	−0.9 to −0.1
UNID	26.5 ± 10.9	30.2 ± 5.7	−6.6 to −0.8

components (data not shown). No correlation was found between TRAP or its components and pack years smoked in the cancer group.

Age correlated negatively with the plasma concentrations of protein SH-groups ($r = -0.54$, $p < 0.0001$), while it had *no correlation* between total TRAP and the other components.

The stage of the cancer and the Karnofsky status had no significant effect on plasma TRAP or its components (data not shown). SCLC patients had lower levels of plasma TRAP than the non small cell cancer patients, but this difference was not statistically significant.

DISCUSSION

Although several previous reports have indicated a reduction in antioxidant vitamins such as ascorbic acid[25] and vitamin E,[26] in cancer patients compared to their controls, according to present understanding of pro-oxidant and antioxidant metabolic pathways it is important to evaluate the major plasma antioxidant defence systems simultaneously. Our results clearly indicate differences between the peroxyl radical trapping antioxidants of human plasma between lung cancer patients and their controls matched for age, sex and smoking history. To our knowledge this is the first clinical study comparing plasma peroxyl radical trapping capacity (TRAP) and its individual components between lung cancer patients and their controls. The present data supports the concept of "persistent oxidative stress in cancer"[2] and suggests that it extends to clinical situations. We observed that plasma peroxyl radical trapping capacity (TRAP) was decreased, in the cancer patients compared to the control group. Plasma TRAP seems to be quite stable even under conditions potentially very pro-oxidant such as radiotherapy (Erhola et al, submitted to Free Radical Biology & Medicine). Thus the detected difference between groups represents a significant finding. However, one must keep in mind that the TRAP values *provide* only a crude estimate of the plasma peroxyl radical trapping capacity and must always be interpreted through its individual components.

The decrease of plasma TRAP was due to the reductions of all other components except uric acid which had rather a tendency to increase. Uric acid acts as a scavenger of singlet oxygen, peroxyl radicals and hydroxyl radicals[27] but its role as an antioxidant *in vivo* is still controversial. The interaction of uric acid in stabilizing ascorbate may be very important.[28] It has been proposed also that the increased concentrations of uric acid may even be a responsive compensating mechanism.[27] Furthermore, malignancies are known to increase concentrations of uric acid, which leads to speculation that this could be due to adaptive responses to the disequilibrium between pro-oxidant and antioxidant defence systems.

Reactive oxygen species, hypothetically produced by cancer cells, have been proposed to lead to protein damage[2] and could thus be targeting the protein SH-groups readily available in plasma. Previous studies have reported weak reactivity of protein SH-groups towards oxidants

to the extent that 3/4 of them are wasted during auto-oxidation.[29] However, it has been postulated by others[30] that the high percentagewise contribution of protein SH-groups to plasma antioxidant capacity reflects that protein-free radical interactions might be common and have considerable significance *in vivo*. The latter theory is relevant to the findings of the current study.

The lipophilic antioxidant vitamin E blood levels is highly correlated to total serum lipids.[26] Cigarette smoking has been associated with decreased alveolar fluid vitamin E concentrations,[31] however, many studies have reported similar plasma vitamin E levels among smokers and nonsmokers.[32,33] Nevertheless, high doses of this vitamin might actually reduce enhanced lipid peroxidation in smokers.[34] The highly significant difference in vitamin E (even when plasma cholesterol was included in the analysis as a covariate) concentrations between the two groups in the present study is in good accord with previous knowledge,[26] though the difference reported here may also be due to the metabolic consequences of the disease itself. Vitamin E's importance is further enhanced through its recycling by ascorbic acid. Despite its low contribution to plasma TRAP, vitamin E is probably an efficient antioxidant in lungs.[31] Thus, with regard to lung cancer patients whose lungs are in most cases exposed to the repeated, extensive burden of reactive oxygen species by cigarette smoke,[19] the combined local effects of smoking and the possible production of reactive oxygen species by cancer cells might lead to serious deficiencies in lung antioxidant defence mechanisms, including vitamin E.

The powerfully reducing agent ascorbic acid has been claimed to be the major plasma antioxidant.[29] It is noteworthy that in the presence of transition metal ions (iron and copper) ascorbate may become a pro-oxidant. Normally, since these metal ions are very limited *in vivo*, the antioxidant functions predominate. However, in disease and tissue injury (such as cancer), transition metal ions might increase in presence and thus the possibility of the pro-oxidant function of ascorbate occurring should not be ignored.[35,36]

The negative correlation of ascorbate to the pack-years smoked in the control group fits well with previous reports of dose-dependent reductions in plasma ascorbic acid in smokers[37] and greater antioxidant consumption in response to sustained smoke-related oxidant loads.[37,38] The weight loss within the cancer patients was associated with lower plasma ascorbate, however when the reduction of weight and smoking history were included in the model, there still remained a highly significant difference in ascorbate concentrations between the groups.

Previously it has been reported by us[12–14] and Ghiselli and co-workers[39] that there is an as-yet-unidentified component(s) of plasma that exert(s) a peroxyl radical trapping action. This relatively large proportion of plasma seems to exhibit reactivity under oxidant forming conditions such as infection and anthracyclin based chemotherapy.[12,13] We have previously proposed that at least part of this UNID could be endogenously formed antioxidants.[12,13] Bilirubin, ubiquinol and flavonoids have contributed only for a minor proportion of TRAP (Metsä-Ketelä et al unpublished data). The role of melatonin action as a peroxyl radical scavenger *in vivo* is not yet clear.[40]

CONCLUSION

The challenging hypothesis of "persistent oxidative stress in cancer"[2] was tested in this clinical study. In view of previous results from basic research,[6] the few clinical surveys[12,16] and the present study it seems likely that cancer patients have oxidative disturbances that might lead to subsequent imbalances between the body's oxidant and antioxidant defence systems. Considering that plasma is in intimate contact with all body tissue, it can be seen as an important window to comprehensive body antioxidant

mechanisms. When evaluating the effects of possible vitamin supplements *after* diagnosis of cancer, it should be kept in mind that vitamin E and C together contribute to only 6–9% of the total peroxyl radical trapping capacity of plasma while a large unidentified proportion of plasma antioxidant capacity remains. Active lung cancer treatments might cause alterations in plasma antioxidant components[15] and, in the light of current knowledge, it would seem to serve the interest of patients to withhold any high dose vitamin supplementation—at least in those receiving active treatment—until there is clear evidence of their efficacy.

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