Pro-inflammatory cytokines and oxidative stress/antioxidant parameters characterize the bio-humoral profile of early cachexia in lung cancer patients

NICOLETTA FORTUNATI1, ROBERTA MANTI1, NADIA BIROCCO2, MARIATERESA PUGLIESE1, ENRICO BRIGNARDELLO1, LIBERO CIUFFREDA2, MARIA G. CATALANO3, MANUELA ARAGNO4 and GIUSEPPE BOCCUZZI1,3

1Oncological Endocrinology and 2Medical Oncology, ASO San Giovanni Battista, Corso Bramante 88, Turin; 3Department of Clinical Pathophysiology, University of Turin, Via Genova 3, Turin; 4Department of Experimental Medicine and Oncology, University of Turin, Corso Raffaello 30, Turin, Italy

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Abstract. Cancer-related cachexia, that is present in about 50% of cancer patients and accounts for 20% of all cancer deaths, is clinically characterized by progressive weight loss, anorexia, metabolic alterations, asthenia, depletion of lipid stores and severe loss of skeletal muscle proteins. The main biochemical and molecular alterations that are responsible for the syndrome are prematurely present in the progress of the disease and the identification of the early stages of cachexia can be useful in targeting patients who will benefit from early treatment. The aim of the present study was to delineate the bio-humoral profile of a group of lung cancer patients either non-cachectic or cachectic by evaluating serum pro-inflammatory cytokines and oxidative stress/antioxidant parameters (both recognized to be involved in cachexia pathogenesis) and pro-inflammatory cytokine gene expression in PBMC (Peripheral blood mononuclear cells) of cancer patients. All serum pro-inflammatory cytokines and oxidative stress/antioxidant parameters significantly increased in neoplastic patients, but only TNF-α, ROS, GSH and vitamin E showed a significantly greater increase in cachectic patients. Pro-inflammatory cytokine gene expression mirrored serum level behaviour except for IL-6 that was increased in serum but not as gene expression, suggesting its provenience from tumour tissue. Our data support that the simultaneous determination of ROS, GSH, vitamin E, together with TNF-α allows the identification of a lung cancer patient developing cancer-related cachexia. This bio-humoral profile should be used for the early diagnosis and follow-up of the syndrome. Moreover, the evaluation of gene expression in patient PBMC was helpful in differentiating tumour vs host factors, therefore being useful in the study of pathogenetic mechanisms in neoplastic cachectic patients.

Introduction

Cancer-related cachexia is a life-threatening paraneoplastic syndrome present in about 50% of cancer patients. It is clinically characterized by progressive weight loss, anorexia, metabolic alterations, asthenia, depletion of lipid stores and severe loss of skeletal muscle proteins and accounts for ~20% of all cancer deaths (1,2). Its prevalence is higher in patients with tumour of the gastrointestinal tract and the lung, than in those with other solid or haematologic malignancies (3). The pathogenesis of cancer-related cachexia is still not fully understood, even though several different pathways and catabolic mediators (humoral and tumoural) have been recognized (4-6).

Since cachexia is present in most terminally ill cancer patients, it has long been considered a very late and ineluctable event in the natural history of the neoplastic disease. Many of the biochemical and molecular alterations that are thought to be responsible for the syndrome are already present prematurely in the disease progress and therefore, cancer-related cachexia should be considered as an ‘early-phenomenon’ (7,8). Furthermore, soon after the diagnosis of cancer takes place, the troublesome and long therapy pathway the patient has to follow will increase factors leading to muscle wasting and cachexia (9). Unfortunately, the currently available therapeutic tools for cancer-related cachexia have offered only partial results, mainly because the intervention is very late and the development of an early and more effective intervention is sought (3).

In addition to the effort to look for a new therapy and different treatment timing, the identification of biological markers able to identify which patients to treat and when to treat them as well as to monitor treatment outcome is of increasing importance.

Proinflammatory cytokines, mainly TNF-α, IL-6 and IL-18, are recognized to be involved in the pathogenesis of cancer-
related cachexia (5,10). The serum levels of IL-6 were reported to be elevated in many patients with cancer and cancer-related cachexia but results for other cytokines and their relationship with the severity of clinical status are still discrepant (11-17). Reactive oxygen species and related oxidative stress markers are associated with muscle wasting in animals (18,19) and have also been demonstrated to be altered in cachectic patients making them useful markers in monitoring the response to antioxidant treatment (20,21).

Nevertheless, a precise bio-humoral profile able to identify the very first development of cachexia in order to target patients who will benefit from early treatment is still lacking. The aim of this study is to evaluate a panel of serum proinflammatory cytokines and oxidative stress parameters in order to delineate a useful bio-humoral profile able to detect the development of the disease at an early stage. Since in the tumours of the gastrointestinal cachexia seems to be influenced early by the site of the tumour, we enrolled only patients with lung cancer, that present cancer-related cachexia with a significantly high prevalence. Moreover, because it was reported that host response to cancer-related cachexia development is not always mirrored by serum cytokine levels (22), which cannot be used to discriminate between tumour versus host production, we also decided to evaluate cytokine gene expression on peripheral blood mononuclear cells.

Materials and methods

Patients. The study was conducted on 33 patients (mean age 65.0 years, range 54-75 years; M/F ratio 30/3) with recently diagnosed lung cancer. Patients were not affected by any other serious disease (e.g. diabetes, liver cirrhosis, heart, renal or pulmonary failure) and were not receiving any antineoplastic treatment or any treatment interfering with cytokine production (e.g. steroidal drugs, somatostatin analogues, non-steroidal anti-inflammatory drugs) at the time of the study. All patients were referred to the S.C. Medical Oncology, ASO San Giovanni Battista, Torino. Written informed consent was obtained from all patients.

Samples of peripheral blood and serum were collected from each patient right after diagnosis, at 8:00 a.m. For each patient the following data were also obtained: tumour histology, stage of disease, performance status (ECOG), height (cm), weight (kg), BMI (body mass index). Patients were divided into two groups on the basis of a weight loss >5% of initial weight in the six months preceding the study (weight loss >5%, cachectic patients, n=14; weight loss <5%, non-cachectic patients, n=19).

Clinical characteristics of patients are reported in Table I. Peripheral blood and serum samples from 23 age-sex-matched normal subjects were used as controls.

Table I. Clinical characteristics of lung cancer patients (n=33).

<table>
<thead>
<tr>
<th>Lung cancer histology</th>
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<tbody>
<tr>
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<tr>
<td>Small cell</td>
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<td>Adenocarcinoma</td>
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<table>
<thead>
<tr>
<th>Disease stage</th>
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<tbody>
<tr>
<td>IIIB</td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>IV</td>
<td>19</td>
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<tr>
<th>ECOG</th>
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<tr>
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<td>18</td>
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<table>
<thead>
<tr>
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<tr>
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</tr>
<tr>
<td>19-20</td>
<td>4</td>
<td>12</td>
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<td>21-25</td>
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<td>26-29</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>&gt;30</td>
<td>2</td>
<td>6</td>
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Table II. Expression of IL-6, IL-6R, TNF-α, TNF-RI and TNF-RII genes.

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<th>RNA extraction from peripheral blood mononuclear cells (PBMC)</th>
<th>BM</th>
<th>40</th>
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</table>
| PBMC were separated on a Fycoll-Hypaque density gradient (Lymphoprep™, Axis-Shield PoC AS, Oslo, Norway), starting from 14.0 ml of peripheral heparinized blood. After separation, cells were thoroughly washed with ice-cold saline and frozen at -80°C until the experiment. Total RNA was extracted from PBMC with the Trizol reagent (Invitrogen, Groningen, The Netherlands) following the method developed by Chomczynski and Sacchi (23).

RT-PCR for gene expression evaluation. Total RNA was reverse-transcribed at 42°C for 40 min using AMV reverse transcriptase (Finnzymes, Finland) and oligoDT primer (Invitrogen). The PCR reaction system contained 5 μl of 10X PCR buffer, 10 μl of RT product, 0.2 mM dNTP (Finnzymes), 1.25U Taq DNA polymerase (Finnzymes), 50 ng each of sense and antisense primers in a total volume of 50 μl. Primer sequences are reported in Table II. Amplification was carried out as follows; for TNF-α: 1 x 95°C for 3 min; 35 x 94°C for 1 min, 35 x 94°C for 1 min, 38°C for 1 min and 72°C for 1 min; and 1 x 72°C for 7 min; for IL-6: 27 x 94°C for 40 sec, 65°C for 40 sec and 72°C for 40 sec; 10 x 94°C for 40 sec; 56°C for 40 sec; 72°C for 40 sec; and 1 x 72°C for 7 min; for TNF-RI: 1 x 94°C for 3 min; 35 x 94°C for 30 sec; 52°C for 1 min and 72°C for 1 min; and 1 x 72°C for 7 min; for TNF-RII: 1 x 94°C for 3 min; 35 x 94°C for 30 sec; 60°C for 30 sec and 72°C for 30 sec and 1 x 72°C for 7 min; for β-actin: 1 x 94°C for 3 min; 35 x 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and 1 x 72°C for 7 min. PCR products were electrophoresed.
on 1.5% agarose gel in the presence of ethidium bromide. Gels were analyzed with the PC program Kodak 1D Image System. The net intensity of bands in each experiment was normalized for the intensity of the corresponding β-actin band.

**Evaluation of serum oxidative stress and anti-oxidant parameters**

**Nitrite/nitrate.** The nitrite/nitrate concentration in serum was used as an indicator of nitric oxide synthesis. Nitrates in serum samples were stoichiometrically reduced to nitrites by incubation of 250 μl of the sample for 15 min at 37˚C, in the presence of 1 IU/ml nitrate reductase, 500 μM NADPH and 50 μM FAD in a final volume of 400 μl. When nitrate reduction was complete, unused NADPH, which interferes with subsequent nitrite determination, was oxidized by 100 IU/ml lactate dehydrogenase and 100 mM sodium pyruvate in a final reaction volume of 500 μl and incubated for 5 min at 37˚C. Subsequently, total nitrites in the serum were assayed by adding 500 μl of Griess reagent (4% sulphanilamide and 0.2% naphtylendiamide in 10% phosphoric acid) to each sample (24).

**End-products of lipid peroxidation.** Hydroxynonenal (HNE) concentration was also determined on fresh cytosolic fractions by the Esterbauer et al method (25,26). An aliquot of cytosol (200 μl) was extracted in an equal volume of a solution of acetic acid: acetonitrile (4:96, v:v). After centrifugation at 250 x g for 20 min at 4˚C, 50 μl of supernatant were injected into an HPLC Symmetry C18 column (5 mm, 3.9x150 mm). The mobile phase used was acetonitrile:bidistilled water (42%, v:v). The HNE concentration was calculated by comparison with a standard solution of HNE (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) of known concentration.

**ROS.** Reactive oxygen species (ROS) were measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a probe. DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is rapidly oxidized in the presence of peroxides to highly fluorescent 2',7'-dichlorofluorescein (DCF), which is then measured fluorimetrically (27).

**GSH.** Reduced glutathione (GSH) levels were evaluated as described (28). Briefly, a mixture was directly prepared in cuvette: 2.25 ml of 0.1 M K-phosphate buffer, pH 8.0; 0.2 ml of the sample (plasma or PBMCs cytosol fractions) and 25 μl of 10 mM DTNB in methanol (Ellman's reagent). After 1 min the assay absorbance was measured at 412 nm and the GSH concentration was calculated by comparison with a standard curve.

**Vitamin E.** α-tocopherol was assayed by the method described by Burton et al (29): after extraction of the sample (0.5 ml aliquot of plasma) with 1 ml n-heptane and brief centrifugation, the heptane phase was collected for HPLC analysis. A Supercosil-LC-Si column (25 cm x 4.6 mm, Supelco Inc., PA, USA) was used, the mobile phase being n-hexane-isopropanol (99:1, v:v) and the flow rate 2.0 ml/min; the fluorescence detector was set to 298 nm excitation and 325 nm emission.

**Statistical analysis.** Results are expressed throughout the text as mean ± 1SD and the significance of the difference between the mean values of patients and controls was determined with the two-tail Student's t-test. Significance was attained for P<0.05.

**Results**

**Evaluation of pro-inflammatory cytokines.** The levels of serum TNF-α, TNF-RI, TNF-RII and IL-6 measured in the groups of patients and controls are reported in Table II.

<table>
<thead>
<tr>
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<th>Controls (ctrl)</th>
<th>Non-cachectic patients (nc)</th>
<th>Cachectic patients (c)</th>
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</table>
| TNFα (pg/ml)     | 1.887±0.26 (n=14) | 2.646±0.53 (n=17) | 4.182±0.6 (n=13) | ctrl vs nc, P<0.05  
|                  |                  | 1573±68 (n=13)         | nc vs c, P < 0.05   |
|                  | 1024±90 (n=15)   | 1661±159 (n=17)        |                         |
| TNF-RI (pg/ml)   | 1834±150 (n=15)  | 2449±181 (n=17)        | 2996±286 (n=13)       |
| TNF-RII (pg/ml)  | 0.438±0.102 (n=13) | 8.186±1.77 (n=17)  | 11.61±1.56 (n=13)    |

Table II. Serum concentration of pro-inflammatory cytokines in lung cancer patients.
Serum soluble TNF-RI was also increased in both neoplastic patient groups, but no significant difference was observed between cachectic and non-cachectic patients. Similarly, TNF-RI RNA expression in PBMC (Fig. 2) was increased in patient groups in comparison with controls, without any difference between cachectic and non-cachectic patients.

Furthermore, serum soluble TNF-RII was increased in both neoplastic patient groups, but no significant difference was observed between cachectic and non-cachectic patients. TNF-RII RNA expression in PBMC (Fig. 3) was also increased in patient groups, without any difference between cachectic and non-cachectic patients.

Finally, serum IL-6 level was significantly higher in both neoplastic patient groups in comparison with controls, being however similar in cachectic and non-cachectic subjects. On the contrary, the level of IL-6R RNA expression in PBMC, (Fig. 4), showed no difference among neoplastic patients, cachectic or not and control subjects. No difference was observed among the three groups as far as IL-6R RNA expression in PBMC was concerned (Fig. 5).

Evaluation of oxidative stress and anti-oxidant parameters. The levels of oxidative and anti-oxidant parameters are
reported in Table III. In both groups of neoplastic patients the levels of HNE, ROS and the NO₂/NO₃ ratio were significantly higher than in the controls, while GSH and vitamin E were significantly reduced. The increase of ROS as well as the reduction of vitamin E were more pronounced in cachectic than in non-cachectic patients.

**Discussion**

The present study reports the behavior of several biochemical parameters, such as proinflammatory cytokines, oxidative stress and antioxidant markers, in lung cancer patients and try to delineate a bio-humoral profile able to identify the very first development of cancer-related cachexia. We observe here that cachectic patients present higher serum levels of TNF-α, ROS, higher PBMC expression of TNF-α gene and lower serum GSH and vitamin E levels.

Cachexia is generally defined as the loss of >5% of an individual’s baseline body weight over 2 to 6 months (30,31). Nevertheless, several authors reported that the choice of weight loss >10% of the body weight is a better cut-off point to distinguish cachectic from non-cachectic patients (17,32). In the present study we decided to identify our cachectic patients using the loss of >5% of body weight in the last 6 months, since we wanted to identify patients in the early stages of cachexia in order to recognize any early biochemical change.

All our cancer patients presented a significant increase in serum TNF-α, TNF-RI and TNF-RII levels. Higher TNF-α serum level has been reported in patients with advanced stage cancer (16,20,21) and a decrease in TNF-α level has been described after combined cachexia treatment (33). In NSCLC patients, TNF-α serum level was increased with respect to controls but no difference was reported between cachectic and non-cachectic patients (17). Reports about soluble TNF receptor determination in serum from cancer patients are few and not definitive. In general, the level of both soluble receptors is increased in neoplastic and cachetic patients regardless of tumour site (34,35-37). In addition, in experimental models of cancer-related cachexia the up-regulation of TNF-RI expression in muscle was reported as a putative pathogenic mechanism (22,38). We report for the first time in lung cancer patients that both soluble TNF receptors and TNF-α serum levels are significantly increased; furthermore, we also demonstrated that the gene expression of the three molecules in host PBMC is significantly higher than in the controls. It is therefore conceivable that serum TNF-α and its receptors are mainly coming from host production and they reflect the chronic inflammation status occurring in advanced cancer. However, serum levels of both receptors is not different in cachectic with respect to non-cachectic patients; their expression level in PBMC is also
similar in both groups of cancer patients. On the contrary, TNF-α serum level as well as PBMC gene expression is significantly more increased in cachectic patients confirming that its determination is a good marker for cachexia even in the early stages.

Serum IL-6 level was increased in all our cancer patients, but we did not observe any difference between the cachectic and non-cachectic group. The increase in serum IL-6 was previously reported in advanced cancer patients (20,21) as well as in terminally ill patients about one week before death (39) and was also related to the tumour size (34). Furthermore, treatment of cachetic patients with progestagen, idomethacin and anti-cycloxygenase-2 determined a significant reduction of IL-6 level (33,40). Kayacan and coworkers (17) confirmed the increase of serum IL-6 in NSCLC patients, but failed to observe any difference between cachectic and non-cachectic patients in accordance with our observation. The increase in serum IL-6 level is not related to an increase of host production, since no modification was observed in IL-6 and IL-6R expression in host PBMC. We therefore suggest that serum IL-6 is mainly produced by lung tumours and this is also consistent with the observation from other authors (34). In conclusion, IL-6 is not a good marker of cachexia, at least in lung cancer patients, since its serum levels are mainly determined and influenced by tumoural mass (34).

Oxidative stress has been often reported to be involved in carcinogenesis (41-43) and is thought to be responsible for the chronic inflammation status occurring in advanced cancer patients and for the up-regulation of cytokine production that is pivotal for the induction of cancer-related cachexia (5). Furthermore, the excessive production of reactive oxygen species (ROS) in tumour-bearing rats was also suggested to be associated to cachexia development (18). Actually, serum ROS levels were repeatedly reported to be significantly higher in advanced cancer patients (stage IV) and also that treatment with different antioxidants caused a sensitive reduction of their level (20,21). In the present study, we assessed a more complete panel of oxidative stress and antioxidant parameters.

All our cancer patients showed a significant increase of ROS, HNE and NO2/NO3 together with a reduction of both GSH and vitamin E, as expected. It is noteworthy that among these parameters we observed a significant difference in cachectic patients with prostate carcinoma. The increase in serum IL-6 was increased in all our cancer patients, in accordance with our observation. The increase in serum IL-6 level is not related to an increase of host production, since no modification was observed in IL-6 and IL-6R expression in host PBMC. We therefore suggest that serum IL-6 is mainly produced by lung tumours and this is also consistent with the observation from other authors (34). In conclusion, IL-6 is not a good marker of cachexia, at least in lung cancer patients, since its serum levels are mainly determined and influenced by tumoural mass (34).

In conclusion, the simultaneous determination of several oxidative stress and antioxidant parameters are helpful in identifying patients switching towards cachexia.

Acknowledgements

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References