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Original article

Stage dependent destructuration of neuro-endocrine-immune system components in lung cancer patients

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ABSTRACT

Background: Close relationships among the nervous, endocrine and immune system components maintain body homeostasis. Alteration of time-related prophile of variation of system components and loss of integrated function may favour the developing of cancer and may be aggravated in the presence of neoplastic disease. The aim of our study was to evaluate the prophiles of time-related variation of neuro-endocrine-immune system components in lung cancer patients.
Methods: Peripheral blood samples were collected at intervals of 4 hours for 24 hours from 11 healthy subjects (age range 35–53 years, mean age ± s.e. 43.6 ± 1.7) and nine patients suffering from nonsmall cell lung cancer (age range 43–63 years, mean age ± s.e. 51.0 ± 2.4). In each blood sample, lymphocyte subpopulations (CD3, CD4, CD8, HLA-DR, CD16, CD20, CD25, γδTcR) were analyzed and melatonin, cortisol, TRH, TSH, free thyroxine, GH, IGF1 and interleukin IL2 on serum were measured.
Results: In our I–II stage lung cancer patients CD8+ lymphocytes ($P = 0.01$), and in particular the T suppressor subset ($P < 0.0001$), CD20+ cells ($P = 0.05$), γδTCR expressing cells ($P < 0.01$) and IGF1 ($P = 0.004$) were diminished, whereas CD16+ cells ($P < 0.0001$), CD25+ cells ($P = 0.03$), free thyroxine ($P = 0.001$) and GH ($P < 0.0001$) were increased in respect of healthy subjects. In our III–IV stage lung cancer patients CD8+ lymphocytes ($P = 0.003$) and in particular the T suppressor subset ($P < 0.0001$), CD20+ cells ($P = 0.05$), γδTCR expressing cells ($P = 0.01$), melatonin ($P = 0.03$), TSH ($P = 0.006$) and IGF1 ($P < 0.0001$) were diminished, whereas CD4+ cells ($P = 0.002$), CD16+ cells ($P < 0.0001$), CD25+ cells ($P = 0.002$), cortisol ($P = 0.003$), TRH ($P = 0.004$), free thyroxine ($P = 0.001$), GH ($P < 0.0001$) and IL2 ($P = 0.0002$) were increased in respect of healthy subjects. A statistically significant difference was evidenced between the two groups of cancer patients for the values of CD16+ cells ($P < 0.0001$), free thyroxine ($P = 0.001$) and IGF1 ($P < 0.0001$) higher in I–II stage lung cancer patients and for the values of CD4+ cells ($P < 0.0001$), γδTCR expressing cells ($P = 0.002$), TRH ($P = 0.002$) and IL2 ($P = 0.01$) higher in III–IV stage lung cancer patients. Lung cancer patients showed alteration of the pattern of circadian variation of CD3+, CD8+, CD8+ dim, CD16+, CD20+ and γδTCR expressing cells and of cortisol, TSH and GH serum levels. Pair-wise comparisons showed severe and stage dependent alterations in lung cancer patients.
Conclusions: The prophiles of time-related variation of neuro-endocrine-immune system components are altered in a stage dependent manner in lung cancer patients and this alteration may impair the customary integrated system function.

1. Introduction

The neuro-endocrine and immune system function is characterized by time-related variations of their components with biological rhythms in different frequency ranges that show a well-defined time relation to each other and the rhythms of the same frequency may have the same phase or different phases [1–3]. Immune cells and neuro-endocrine structures show time-related variations of quantitative (peripheral blood lymphocyte number/percentage or hormone serum levels) and qualitative (functional) characteristics and this temporal organization allows for cohordination of neuro-endocrine axes and influences innate and adaptive immune system function [4–9]. The co-ordinated relationships among these multifrequency structures and external

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time cues or zeitgebers are a characteristic of the healthy organism and the alteration of the organism's time structure may lead to functional disturbances and to alteration of the anatomic integrity [10], as suggested by epidemiologic studies in shift and night workers that have evidenced an increase in the incidence of pathologies such as cardiovascular disease and metabolic syndrome. On the other hand, chronodisruption or internal desynchronization are recognized as risk factors for the development of neoplastic disease and the disruption or the reinforcement of the host circadian timing system, respectively, accelerates or slows down cancer growth through modifications of host and tumor circadian clocks [11–16]. In previous studies, we have evidenced alterations in the circadian organization of the neuro-endocrine and immune system in cancer patients [17–22]. Periodic regression models are probably the most useful tools in chronobiology, as they are able to reproduce a biological rhythm but, in the same time, they are not able to make comparisons between different estimated models. In this study, we have evaluated the prophiles of variation of neuro-endocrine-immune system components in lung cancer patients compared to healthy control subjects using a novel statistical method that fits bivariate periodic regressions jointly modelling paired variables in a multivariate longitudinal fashion.

2. Methods

Subjects gave written informed consent and the study was approved by the local Scientific and Ethical Committee. Peripheral blood samples were collected at intervals of 4 hours for 24 hours from 11 healthy subjects (age range 35–53 years, mean age ± s.e. 43.6 ± 1.7) and nine patients suffering from lung cancer (age range 43–63 years, mean age ± s.e. 51.0 ± 2.4). Inclusion criteria for healthy subjects were sex (male), age (< 80 years), body mass index (BMI) (> 20 and < 30), normal physical activity level, no psychiatric disorder, no alcohol intake, no chronic conditions, normal blood pressure level. In the control group, healthy status was assessed by medical history and physical examination, basal screening blood and urine test, ECG, chest X ray, upper and lower abdominal ultrasound scan. Inclusion criteria for subjects suffering from lung cancer were sex (male), age (< 80 years), BMI (> 25 and < 30), normal physical activity level, no treatment (surgery, chemotherapy, radiotherapy), performance status > 80% by Karnofsky performance status scale or < 2 by ECOG score, no psychiatric disorder, no alcohol intake, no chronic conditions, normal blood pressure level, tumor cell type (nonsmall cell lung cancer) and the extent of the tumor was evaluated by clinical examination, bronchoscopy, computed tomography (CT) of the brain, chest, upper abdomen and ultrasonography of the liver.

There were three cases of squamous cell carcinoma and six of adenocarcinoma. The pathological diagnosis was based on light microscopy according to the WHO classification. Tumors were staged according to the TNM classification of the International Union Against Cancer staging system after reviewing the clinical, radiologic, and pathologic data. The numbers of pT1, pT2, and pT3–4 cases were 2, 4, and 3, respectively. All nine cases showed metastasis to regional lymph nodes. Regarding stage, the numbers of stage II, III and IV cases were 5, 2, and 2, respectively. The groups were matched closely to avoid sex, BMI and seasonality of sampling related bias and all subjects were submitted to the same social routine, with identical mealtimes and sleep/wakefulness rhythm in the week preceding the sampling (lights on at 07:00 h and lights off at 23:00 h, thereby supplying the subjects with 16 hours of light and 8 hours of darkness per day, 16:8 L:D). Sleep was allowed between 23:00 h (lights off) and 07:00 h (lights on). During daytime (between 07:15 h and 20:15 h), subjects stayed in the Department and standardized meals were provided at appropriate times for breakfast (07:30 h), lunch (12:30 h), and dinner (18:30 h). During the overnight sampling period, a dim blue light (< 100 lux) was used.

In each blood sample, the lymphocyte subpopulations (CD3, CD4, CD8, HLA-DR, CD16, CD20, CD25, γδTcR) were analyzed in peripheral blood anticoagulated with sodium ethylenediamine tetra-acetic acid (EDTA). Analyses of lymphocyte subpopulations were performed on unfixed cell preparations with a 5-parameter flow cytometer (FACSCalibur, Becton-Dickinson FACS Systems, Sunnyvale, CA, USA) and a panel of monoclonal antibodies (mAbs) to lymphocyte surface antigens (CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC, HLA-DR FITC, CD16 PE, CD19 PE, CD25 PE Cy7, HLA-DR FITC, PAN γδTcR: Becton-Dickinson Biosciences, San Jose, CA, USA). We used fluorochrome-labelled mAbs conjugated with phycoerythrin (PE), PE Cy7, peridinin chlorophyll protein (PerCP), allophycocyanin (APC) and fluorescein isothocyanate (FITC) and 10 μl mAbs were added to 100 ml EDTA blood in Trucount tubes (BD Biosciences, San Jose, CA, USA). After a 15-min incubation, at room temperature, the erythrocytes were disintegrated and after centrifugation, the supernatants were washed with PBS. Non-lymphocytic cells contaminating the preparations were excluded from analysis using scatter gates set on the 90° light scatter profile. At least, 10,000 cells were acquired on the FACScan. Absolute counts of T cell subsets were calculated based on the proportion of the respective T cell subpopulation and on absolute counts obtained by the procedure. The number of fluorescent cells was expressed as a percentage of the total lymphocytes. We elected to evaluate rhythmic changes in the percentages of cells rather than in the absolute counts based on total lymphocytes because blood samples age gradually over time, which may result in a decreased reliability in the cytometric counting of leukocytes in the stored blood [23]. On the other hand, erythrocyte lysing procedures cause substantial cell loss and the absolute counts determined by a "lyse no-wash" flow cytometry procedure may also be influenced by the cell loss determined by the lysis process [24].

We measured melatonin, cortisol, TRH, TSH, GH, IGF1 and interleukin (IL) 2 on serum. To measure hormone and cytokine serum concentrations, blood samples were centrifuged immediately after collection and frozen at −20 °C for later determination. All samples were analyzed in duplicate in a single assay; the intra-assay and inter-assay coefficients of variation were below 4% and 7% respectively for melatonin, 3% and 5% for cortisol, 5% and 6% for TRH, 5% and 8% for TSH, 3% and 6% for FT_4, 5% and 3% for GH, 4% and 7% for IGF1, 5% and 7% for IL-2. We measured melatonin by radio-immuno-assay (Melatonin RIA kit, Buehlmann Laboratories AG, Schonenbuch, Switzerland), cortisol by Immuno-assay in Elettro-Chemiluminescence (Cortisol Cobas Roche, Burgess Hill, West Sussex, England), TRH by radio-immuno-assay ("Frederic Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary), TSH by Immuno-assay in Elettro-Chemiluminescence (TSH Cobas Roche, Burgess Hill, West Sussex, England), FT_4 by Immuno-assay in ElettroChemiluminescence (FT4 Cobas Roche, Burgess Hill, West Sussex, England), GH by immunoenzymometric assay (AIA-PACK HGH, Tosoh, Japan), IGF1 by radioisotopic assay (IGF1 Immulite 2000, Medical Systems, Genoa, Italy), IL-2 by immunoenzymatic assay (IL-2 EIA, Technogenetics, Milan, Italy).

3. Statistical analysis

Statistical evaluation of the lymphocyte percentages and hormone serum levels was performed by an inferential temporal descriptive biometric analysis using the methods named Single Cosinor and Population Mean Cosinor, which fit the best sinusoid to individual and group data, testing the occurrence (whether the zero-amplitude assumption is rejected at a probability P value ≤ 0.05) and quantifying the parameters MESOR, Amplitude