Molecular Medicine

Supplemental Data

Redox Imbalance in Lung Cancer of Patients with Underlying Chronic Respiratory Conditions

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METHODS

Study Design and Patients

This is a prospective, cross-sectional study, in which patients were recruited consecutively from the Lung Cancer Clinic of the Respiratory Medicine Department at Hospital del Mar (Barcelona, Spain). For the purpose of the investigation, a group of 17 Caucasian control subjects and 80 Caucasian patients with LC were recruited consecutively before having received any treatment for their lung neoplasm from the weekly LC board meeting. Blood samples were obtained in all 17 control subjects and at the time of diagnostic confirmation for LC in all 80 patients. These patients were further subdivided *post hoc* into two groups according to the presence of underlying COPD, which was diagnosed on the basis of current guidelines(1,2): 1) 59 patients with LC who also had COPD (LC-COPD group, 2 females) and 2) 21 patients with LC without COPD (LC group, 7 females). A group of control subjects (n=17, 9 females), who underwent bronchoscopy for non-tumor lesions, was also recruited in the study, and blood samples were drawn on the same day. In all 80 patients, blood samples were obtained at the time of diagnostic confirmation for LC. Moreover, from the same study cohort, in the group of patients who underwent thoracotomy for the surgical resection of their lung neoplasms (clinical indication according to guidelines for diagnosis and management of lung cancer (3)), specimens from the tumor and non-tumor lung parenchyma were also obtained in all cases (n=35, 44%) and were further subdivided *post hoc* as follows: 1) 23 patients with LC who also had COPD (LC-COPD group, all males) and 2) 12 patients with LC without COPD (LC group, 4 females). Therefore, in these two groups of patients, blood and lung specimens were available for the study (n=35).

Histological diagnosis and staging, (tumor, node, metastasis, TNM) (4,5) of LC were confirmed in all patients. Exclusion criteria were as follows: chronic cardiovascular disorders, chronic metabolic diseases, clot system disorders, signs of severe bronchial inflammation and/or infection (bronchoscopy), current or recent invasive mechanical ventilation, and chronic oxygen therapy. In the present investigation, approval was obtained from the institutional Ethics Committee on Human Investigation (Hospital del Mar–IMIM, Barcelona) in accordance with the World Medical Association guidelines (Helsinki Declaration of 2008) for research on human beings. Informed written consent was obtained from all patients.

Clinical Assessment

Lung function parameters were assessed in all patients following standard procedures. Body composition evaluation included the assessment of body mass index (BMI) and fat-free max index (FFMI) by bioelectrical impedance. Nutritional parameters were also evaluated through conventional blood tests.

Sample Collection

Blood sample specimens were obtained in all the recruited patients (n=80) from the arm vein after an overnight fasting period, and conventional analytical parameters were analyzed together with the target study redox markers. Moreover, in all patients undergoing thoracotomy (n=35), lung specimens were obtained from both tumor and non-tumor surrounding parenchyma during the surgery, in which standard technical procedures were followed by the specialized thoracic surgeons. In all cases, the expert pathologist selected a fragment of lung tumor and non-tumor specimens of approximately 10x10 mm2 size from the fresh samples after a careful collection of the specimens required for diagnosis purposes. Importantly, a minimum amount of 50% of cancer cells was similarly identified in all tumor types from all the study patients. The remaining cell components were inflammatory and stromal cells in all the analyzed tumors.

Sample Preservation

Lung specimens (tumor and non-tumor) were snap-frozen in liquid nitrogen and stored at −80 °C until further use. Blood samples were centrifuged

(588*g* at 4ºC during 15 min) and were immediately frozen at −80 °C until further analyses.

Molecular Biology Analyses

Immunoblotting of 1D electrophoresis. Protein levels of the different molecular markers in lung specimens were explored using methodologies previously published (6,7,8). Briefly, frozen samples from all the patients were homogenized in a buffer containing HEPES 50 mM, NaCl 150 mM, NaF 100 mM, Na pyrophosphate 10 mM, EDTA 5 mM, Triton-X 0.5%, leupeptin 2 μg/ml, PMSF 100 μg/ml, aprotinin 2 μg/ml and pepstatin A 10 μg/ml. Samples were then centrifuged at 1,000*g* for 30 min. The pellet was discarded and the supernatant was designated as a crude cytoplasmic homogenate. The entire procedures were always conducted at 5ºC (on ice). Protein levels in each homogenate were spectrophotometrically determined with the Bradford technique using triplicates in each case and bovine serum albumin (BSA) as the standard (Bio-Rad protein reagent, Bio-Rad Inc.). The final protein concentration in each sample was calculated from at least two Bradford measurements that were almost identical. Equal amounts of total protein from crude parenchyma homogenates (ranging from 20 to 30 μg per sample/lane) were always loaded onto the gels, as well as identical sample volumes/lanes. In order to carry out the comparisons among the different groups, sample specimens were always run together and kept in the same order.

SDS-PAGE gels were used to detect protein levels of the target redox markers in the lung specimens (tumor and non-tumor) from both groups of patients (LC-COPD and LC). The gels were always run in the same electrophoresis box for each of the antigens and the experiments were confirmed at least twice. Fresh gels were specifically loaded for each of the antigens in lung specimens of both patients with LC. Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes, blocked with non-fat milk 5% or with bovine serum albumin 1% and incubated with selective primary antibodies overnight. The following antibodies were used to detect the different molecular markers: total protein carbonylation (OxyBlot Protein Oxidation Detection Kit, Chemicon International Inc.), malondialdehyde protein adducts (anti-MDA-protein adducts antibody, Academy Bio-Medical Company, Inc.), total protein nitration (anti-3-nitrotyrosine antibody, Invitrogen), Mn-superoxide dismutase (SOD) and CuZn-SOD (anti-Mn-SOD and CuZn-SOD antibodies, Santa Cruz Biotechnology), catalase (anti-catalase antibody, Calbiochem Merck Group) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, anti-GAPDH antibody). Antigens from all samples were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescence kit. The specificity of the different antibodies was confirmed by omission of the primary antibody, and incubation of the membranes only with secondary antibodies.

PVDF membranes were scanned with the Molecular Imager Chemidoc XRS System (Bio–Rad Laboratories) using the software Quantity One version 4.6.5 (Bio–Rad Laboratories). Optical densities of specific proteins were quantified using the software Image Lab version 2.0.1 (Bio-Rad Laboratories). Values of total reactive carbonyl groups, total MDA-protein adducts and total protein tyrosine nitration levels in a given sample were calculated by addition of optical densities (arbitrary units) of individual protein bands in every case. Final optical densities obtained in each specific group of patients corresponded to the mean values of the different samples (lanes) of each of the antigens studied. To validate equal protein loading among various lanes, SDS-PAGE gels were stained with Coomassie Blue and the glycolytic enzyme GAPDH was used as the protein loading controls in all the immunoblots.

Identification of carbonylated proteins in lung specimens. Two-D electrophoresis and silver staining. Carbonylated lung proteins were separated and identified in the samples as published elsewhere (9,10). Briefly, 2 volumes of 10 mM DNPH and the same volume of SDS 12% were first added to crude bronchial homogenates (400 μg protein/sample) and incubated for 20 min at room temperature in order to specifically identify carbonylated proteins. The reaction was stopped by adding the neutralization solution. Crude parenchyma homogenates (800 μg protein/sample) were prepared for 2D-electrophoresis with the 2D Clean up kit (Amersham Biosciences) following the manufacturer's instructions. The samples were then incubated for 15 min on ice, centrifuged for 5 min at 13,000*g* and the pellets were then washed three times and centrifuged at 13,000*g* for 5 min. The pellets were re-suspended in 2D re-hydration buffer (8M urea, 2% CHAPS, 20 mM DTT, and 0.002% bromophenol blue). Each parenchyma sample was then separated into two portions (400 μg total each) and both portions underwent 2-D electrophoresis. First-dimensional protein separation was performed with the the Ettan IPGPhor 3 (GE Healthcare Biosciences AB). Samples were applied to immobilized pH gradient strips (18-cm nonlinear pH 3-10, GE Healthcare Biosciences AB) for 30 min at room temperature. The strips were then covered with mineral oil overnight and isoelectric focusing was performed at a maximum of 10,000 V/h for up to a total of 35,200 V-h.

For the second dimension, the IPG strips were equilibrated at room temperature for 30 min in equilibration buffer (6 M urea, 2% SDS, 50 mM tris-HCl, 30% glycerol, and 0.002% bromophenol blue) to which 1% DTT was added prior to use. An additional 30 min equilibration period was then used with equilibration buffer to which 2.5% iodoacetamide was added. The strips were then embedded in 0.5 % agarose on the top of 30% acrylamide gels. The second dimension SDS/PAGE was performed for 5 h, 70 mA per gel at 250 V. One of the resulting 2D gels for each parenchyma sample was then stained with silver stain. Gels were fixed overnight in a fixation solution (50% acetic acid, 50% methanol), then rinsed twice in water, sensitized for 1 min in 0.2% sodium thiosulfate,

followed by rinsing in water and immersion for 30 min in a silver nitrate solution (2% silver nitrate). Gels were then rinsed twice in water and developed in a developer solution (20% sodium carbonate, 0.05% formaldehyde, 0.004% sodium thiosulfate). A stop solution (6% acetic acid) was then added for 15 min followed by rinsing with water for 5 min. Gels were then stored in 1% acetic acid. The second gel derived from a given sample underwent electrophoretical transfer to PVDF membrane and immunoblotting with anti-DNP antibody as described above. Gels and PVDF membranes were imaged with a digital camera and aligned (Adobe Photoshop 8.0.1) so as to identify positive carbonylated protein spots on the gels.

Identification of carbonylated proteins in lung specimens. Mass spectrometry. Identification of carbonylated proteins was conducted in the Proteomics Laboratory at Universitat de Barcelona following the quality criteria established by ProteoRed standards (Instituto Nacional de Proteómica, Spain) and procedures previously published (9,10). Protein carbonylated spots from silver-stained gels were manually excised for in-gel digestion in a 96-well ZipPlate placed in a Multiscreen vacuum manifold (Millipore). Proteins were reduced, alkylated, and digested with sequence grade trypsin (Promega). Peptides were eluted with 15-25 μL of 0.1% TFA in 50% ACN. 2.5 μL of tryptic digest were deposited onto Mass Spec Turbo 192 type 1 peptide chips pre-spoted with CHCA (Qiagen) and left for 3 min for peptide adsorption. Then each spot was washed for 5 s with 1 μl of finishing solution (Qiagen) and left until dryness. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed in a Voyager DE-STR instrument (Applied Biosystems) using a 337-nm nitrogen laser and operating in the reflector mode, with an accelerating voltage of 20 kV. Samples were analyzed in the m/z 800-3000 range and were calibrated externally using a standard peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA, USA). Peptides from trypsin autolysis were used for the internal calibration. Protein identification from MALDI-TOF and/or TOF-TOF results was done with the MASCOT search engine (Matrix Science) using human proteins available in the SwissProt database as well in the NCBI non-redundant database for the identification of the human bronchial proteins. Moreover, the following parameters were used for database searches: one missed cleavage allowed, plus Cys carbamidomethylation as fixed and Met oxidation as variable modifications selected, respectively.

Protein carbonyl enzyme-linked immunosorbent assay (ELISA) in blood. Plasma levels of protein carbonylation were determined using the Oxiselect protein carbonyl ELISA kit (Cell Biolabs, Inc.) following the manufacturer's instructions and previous studies (9,11). All reagents used in these experiments were part of the specific ELISA kit. In brief, protein levels of each plasma sample were spectrophotometrically determined with the Bradford technique as mentioned above. For all the individual specimens, equal amounts of total protein plasma [10 μg/mL in 100 μL total volume diluted in phosphate-buffered saline (PBS)] were added to the assigned pre-coated ELISA-plate wells, which were covered with a sealing tape. Plates were incubated at 4ºC overnight, subsequently washed with PBS three times and incubated with 100 μL of the DNPH working solution in the dark at room temperature for 45 min. Then, after several washes with PBS, samples were incubated with 200 μL blocking solution for 1.5 h. Subsequently, several additional washes were performed, and samples were incubated with 100 μL anti-DNP antibody (dilution 1:1000) on an orbital shaker at room temperature for one hour. Samples were then washed with wash buffer several times and incubated with 100 μL horseradish peroxidase (HRP)-secondary antibody at room temperature for one hour. Finally, after several washes with wash buffer, the samples were incubated with 100 μL substrate solution for 30 min, and the enzyme reaction was stopped by adding 100 μL stop solution to the wells. A standard curve was always generated with each assay run. Absorbances were read in a microplate reader at 450 nm using as a reference filter that of 655 nm. Intra-assay and inter-assay coefficients of variation for blood protein carbonyl formation ranged from 1.5% to 7.33%, respectively. The minimum detectable concentration of protein carbonyls in plasma was set to be 0.1 nmol/mg (Cell Biolabs Inc.).

Protein nitrotyrosine ELISA in blood. Plasma from all individuals was used in this assay (Nitrotyrosine ELISA, Cell Biolabs, Inc. San Diego, CA, USA) following the manufacturer's instructions and previous studies (9,11). Briefly, 50 μL of each sample were added to the assigned pre-coated ELISA-plate wells, which were covered with an adhesive tape and incubated at room temperature for ten minutes. Samples were then again incubated with 50 μL diluted anti-nitrotyrosine antibody at room temperature for one hour. Thereafter, samples were washed with wash buffer and incubated with 100 μL diluted secondary antibody-enzyme conjugate for an additional one hour. Finally, samples were washed several times with wash buffer and incubated with 100 μL tetramethylbenzidine (TMB) substrate solution in the dark for 30 min. The enzyme reaction (peroxidise) was stopped by the addition of 100 μL stop solution. A standard curve was always generated with each assay run. Absorbances were read in a microplate reader at 450 nm using as a reference filter that of 655 nm. Intra-assay and inter-assay coefficients of variation for blood protein nitrotyrosine formation ranged from 2.2% to 6.7%, respectively. The minimum detectable concentration of nitrotyrosine in plasma was set to be 8 μM (Cell Biolabs, Inc.).

Glutathione ELISA in blood. Plasma levels of the ratio of oxidized (GSSG) to reduced glutathione (GSH) were determined using the OxiSelect total glutathione GSSG/GSH ELISA kit (Cell Biolabs, Inc.) following the precise manufacturer's instructions and

previous methodologies(11). Briefly, 25 μL of diluted samples were added to the designated wells, and plates were then incubated with 25 μL nicotinamide adenine dinucleotide (NADH) and 25 μl glutathione reductase (GR) at room temperature for 2 min. Thereafter, 50 μL chromogen were added to each well and the absorbances were recorded in a microplate reader at 405 nm, every minute during 10 min. A standard curve was always generated with each assay run. Intra-assay and inter-assay coefficients of variation for the measurements of blood total glutathione quantification ranged from 3.8% to 6.9%, respectively. The minimum detectable concentration of total glutathione in plasma was set to be 8 nMM (Cell Biolabs, Inc.).

Measurement of superoxide anion radicals using lucigenin-derived chemiluminescence in blood. The reagents employed in these methodologies were all purchased from Sigma (Sigma). In order to quantify superoxide anion production, lucigenin-derived chemiluminescence (LDCL) signals in a xanthine oxidase/xanthine system, were determined in all blood (serum) samples from both groups of patients and control subjects using a luminometer (Lumat LB 9507, Berthold Technologies GmbH) as also formerly described (9,10,12). As previously reported in a study (12), in which they concluded that in XO/xanthine system the LDCL reached a plateau at concentrations of lucigenin above 20 μM, the concentration of lucigenin used in the present investigation was 20 μM. Briefly, 50 μL of serum samples were poured in a tube containing 950 μL Krebs-HEPES buffer. The mixture was incubated at 37ºC in a water bath for 10 min. Twenty μM lucigenin was immediately added after the 10-min incubation period, and the tubes were subsequently placed into the luminometer. The luminometer output was read during 10 min. In all the samples, LDCL signals were measured in the presence of lucigenin alone (baseline levels) and in the presence of 0.5 U/mL SOD.

Statistical Analyses

All statistical analyses were performed using the software statistical package for the social sciences (SPSS) 15.0 (IBM). Data are expressed as mean (standard deviation). The normality of the study variables was explored using the Shapiro-Wilk test. Variables detected in either blood or lung sample specimens from both groups of patients were analyzed independently using appropriate statistical approaches. LC-COPD patients were further subdivided into those with moderate (n=24) and heavy (n=35) exposures to cigarette smoke in order to determine the potential influence of smoking history on the study results. Median value of 60 pack-years in both subgroups of LC-COPD patients was used as the cut-off value to subdivide these patients according to their smoking history. Furthermore, comparisons of oxidative stress markers were also explored in both lung tumor and non-tumor specimens of LC (n=12) and LC-COPD (n=23) patients who underwent thoracotomy. Again, a post hoc analysis according to their smoking history was also conducted in LC-COPD patients, in both tumor and non-tumor lung specimens: moderate smokers (n=8) and heavy smokers (n=15).

In the different sets of comparisons, for the quantitative variables, differences between groups were assessed using one-way analysis of variance (ANOVA) and Tukey's *post hoc* analysis was used to adjust for multiple comparisons. Differences in qualitative variables between the study groups were explored using the Chi-square test. Statistical significance was established at $P \leq 0.05$.

The potential predictive value of all the study variables was evaluated using receiver operating characteristic curves (ROC) and their associated areas under the curve with 95% confidence intervals (CI). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), with 95% CI were also calculated for the best cut-off points of the study variables that best identified underlying COPD among all LC patients (superoxide anion, protein carbonylation and nitration in blood). Furthermore, multivariate logistic regression analyses were performed with categorized variables of the biological parameters protein carbonylation and nitrotyrosine in order to estimate the variables potentially related to COPD among all LC patients.

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