Cathepsins D, B, and L in Malignant Human Lung Tissue

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INTRODUCTION

Lung cancer is the most common tumor in the world, with increasing incidence in many countries (1, 2). Despite advances in cancer treatment, the overall outlook for patients with this disease remains poor, with approximately 10% of all patients diagnosed with the early stage of disease often relapse despite complete surgical resection. More reliable biological markers are needed to improve detection and to predict the risk of relapse for patients treated for early stage lung cancer.

Cats D, B, and L belong to a group of proteolytic enzymes, which are mostly localized to lysosomes under physiological conditions. In various human and animal tumors, altered expression and trafficking of Cats has been observed (3–5). The final control of their activity by endogenous inhibitors may be compromised in tumors (3, 6–9). The complex mechanisms of Cats regulation in malignant cells still remain to be elucidated, but it seems that there are differences in some of their regulatory pathways (3).

In human tumors, increased expression of various Cats compared to the surrounding nonmalignant tissue has been observed in several organs. In breast carcinomas, high levels of Cat D was demonstrated and correlated with poor prognosis (4, 10). However, the role of Cat D in the development of breast tumor metastasis is still controversial (10, 11). The expression of Cat D in some other human tumors was found to be elevated (3), but, to date, it has not been studied in lung carcinoma.

Cat B contributes to biochemical processes underlying tumor metastasis (3). High levels of Cat B in matched pairs of breast (6), colon (13), colorectal (14), and lung (15) carcinomas versus normal tissues were measured. Recently, Knoch et al. (8) studied 65 matched pairs of lung tumors and normal lung parenchyma and found a 4.4-fold increase in median Cat B activity in tumor samples. Moreover, the ratio of Cat B and the inhibitory activity of endogenous cystatins (CPIs) was significantly shifted in favor of increased Cat B activity. Both Cat B and Cat C. CPI activity ratios were found to be related to survival probability (8, 9). This finding is strongly supported by immunohistochemical analysis for the presence of Cat B protein in lung tumor sections: higher expression of Cat B was associated with significantly shorter survival in non-small lung cancer (16).

The expression of Cat L, another cysteine proteinase, has also been found to be elevated at mRNA levels in various human carcinomas, including lung (5, 17). In the most recent study (18), the median Cat L activity was found to be significantly higher in tumors than in normal lung, but the Cat L protein concentration has not been measured so far.

The aim of this study was to determine the activity and enzyme protein levels of all three lysosomal enzymes (Cat D, Cat B, and Cat L) in lung tumors compared to surrounding histologically normal lung tissue. Possible correlation between

ABSTRACT

The levels of cathepsins in malignant and surrounding nonmalignant lung tissue were determined in 17 non-small cell lung cancer specimens. Cathepsin (Cat) D activity was assayed using hemoglobin, whereas Cat B and Cat L activities were assayed using fluorometric substrates, benzyloxycarbonyl-Ala-Arg-Arg-7-amino-4-methylcoumarine and benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarine, respectively. Cat protein concentrations were determined using ELISAs. In malignant tissues, the activities of Cat B and Cat L were significantly higher than the activities in nonmalignant tissues (P < 0.0012 and P < 0.0003, respectively), whereas Cat D concentration was not. There was also a 5.6-fold increase in median Cat B protein (P < 0.054) and a 2.2-fold increase in Cat L protein (P < 0.069). By contrast, the aspartic proteinase, Cat D protein, was not significantly increased in tumors versus control lung tissues. Moderate but significant correlation (r = 0.5, P < 0.045) between Cat B and Cat L expression was observed, but neither correlated with Cat D. The relative increase in median Cat L activity (P < 0.037) and protein (P < 0.0005) was greater in poorly differentiated tumors than in moderate ones. Cat L activity (P < 0.003) and protein (P < 0.005) increases were higher in adenocarcinoma than in squamous cell carcinoma. We conclude that in lung cancers the three lysosomal enzymes are regulated in a noncoordinate manner and that there is specific induction of cysteine cathepsins. Whether Cat B and/or Cat L would be of diagnostic and/or prognostic value requires further study in a larger patient population.

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3 The abbreviations used are: Cat, cathepsin; AMC, 7-amino-4-methylcoumarine; C, control lung tissue; CPI, cysteine proteinase inhibitor; EU, Enzyme Units; AU, Anson Units; RISA, relative increase in specific enzyme activity; T, tumor tissue; RIP, relative increase in protein; Z, benzyloxycarbonyl; Z-A-R-R-AMC, benzyloxycarbonyl-alanine-arginyl-arginyl-aminomethyl coumarine; Z-F-R-AMC, benzyloxycarbonyl-phenylalanine-arginyl-aminomethyl coumarine.
individual Cat expression and correlation with histological grade, tumor type, and the extent of inflammation and tumor necrosis were considered.

**MATERIALS AND METHODS**

**Patient Selection and Sample Preparation**

Seventeen patients (9 females and 8 males), ages 51-84 (median, 66) years, underwent lobectomy for presumed lung cancer between October 1992 and August 1993. Pathological diagnosis by bronchoscopy or postoperative histology confirmed non-small cell lung cancer in 16 of 17 specimens and endobronchial carcinoid in 1 of 17 (Table 1). Only two patients had a family history of cancer. Only one patient had an occupational history of exposure to potential carcinogens because he was exposed to asbestos for 5 years. All patients, except one, were smokers. Smoking habits were scored as the number of packages consumed per day multiplied by the years of smoking.

Routine staging procedures were performed preoperatively in all patients. Fourteen patients were UICC stage I and one patient each was UICC stages II, III, and IV. The patients studied had lung cancers of different histological types, including squamous (n = 9), adenocarcinoma (n = 5), non-small lung carcinoma (poorly differentiated, n = 1), adenosquamous (n = 1), and endobronchial carcinoid (n = 1). Tumors were graded from well to poorly differentiated, corresponding to histological grades 1-3, respectively. Lung tumor sections were often infiltrated by inflammatory cells and were therefore independently evaluated by two pathologists for the presence of macrophages and lymphocytes and scored as low, 1(+); moderate, 2(+); and abundant, 3(+). The presence of inflammation was scored as high and low when the cumulative index, representing the sum of macrophage and lymphocyte scores, was above and below 2(+), respectively.

In some lung tumor sections, large necrotic areas were observed and hence the presence of necrosis was also considered and scored 0, 1(+), 2(+), and 3(+), as indicated in Table 1.

Malignant and histologically benign tissue samples were obtained from each lobectomy specimen and one biopsy material. Homogenizing buffer was prepared with 10 mm Tris buffer (pH 6.9) containing 0.05% BRIJ 35, 0.5 mm EDTA, 0.5 mm diethioerithritol, and 1 mm benzamidene. Each tissue segment was combined with a triple volume of buffer solution and homogenized in an Omni mixer (Sorvall, Inc.) at 7000 rpm for 5 min and further with a Potter-Elvehjem homogenizer. The homogenates were then centrifuged at 40,000 rpm for 5 min, and the supernatant was further centrifuged at 100,000 rpm for 30 min. Clear supernatants were used for analysis of Cats. Protein concentration in the supernatants was determined using a Bio-Rad kit for protein determination with BSA as a standard, as suggested by the manufacturer.

**Enzyme Activities**

**Cat B Activity.** Cat B activity was determined as described previously (6). In brief, 20 μl supernatant were added to 280 μl activation buffer [e.g., 0.4 mm phosphate buffer (pH 6.0)] containing 4 mm EDTA/Na and 1.2 mm DTT and then incubated at 37°C prior to the addition of the substrate Z-A-R-R-AMC (100 μμ). The mixture was then incubated for 150 min until the reaction was terminated with cold 1 mm iodoacetic acid (500 μμ). Blank assays were prepared in the same fashion but without the addition of the enzyme, e.g. tissue sample. Control assays contained 1 mm L-epoxy succinyl leucyl-lamido(4-guanidine) butane (E-64). All assays were carried out in duplicates. Fluorescence was read at 370-nm excitation and 460-nm emission wavelength in SLM Aminco spectrophotometer standardized against AMC. Cat B activity was expressed in

**Table 1**  

Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Histology</th>
<th>Grade</th>
<th>Macrophage</th>
<th>Lymphocyte</th>
<th>Risk: family</th>
<th>Smoking (packs × yr)</th>
<th>Occupation</th>
<th>TNM stage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. J.</td>
<td>82</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>Negative</td>
<td>30</td>
<td>Negative</td>
<td>1 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>H. M.</td>
<td>60</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>2</td>
<td>/</td>
<td>/</td>
<td>Negative</td>
<td>30</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>B. J.</td>
<td>53</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>Negative</td>
<td>30</td>
<td>Negative</td>
<td>2 0 1</td>
<td>IV Lobectomy</td>
</tr>
<tr>
<td>T. S.</td>
<td>82</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>/</td>
<td>1</td>
<td>1</td>
<td>Negative</td>
<td>35</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>W. A.</td>
<td>51</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>Negative</td>
<td>35</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>D. J.</td>
<td>82</td>
<td>F</td>
<td>Adeno-squamous</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>Negative</td>
<td>15</td>
<td>Negative</td>
<td>2 0 0</td>
<td>III(A) Lobectomy</td>
</tr>
<tr>
<td>W. G.</td>
<td>63</td>
<td>M</td>
<td>Squamous</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>Negative</td>
<td>100</td>
<td>Negative</td>
<td>1 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>O. P.</td>
<td>69</td>
<td>F</td>
<td>Squamous</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>Negative</td>
<td>0</td>
<td>Negative</td>
<td>1 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>M. K.</td>
<td>75</td>
<td>M</td>
<td>Squamous</td>
<td>3</td>
<td>/</td>
<td>/</td>
<td>Negative</td>
<td>40</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>F. A.</td>
<td>62</td>
<td>M</td>
<td>Squamous</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>Positive</td>
<td>45</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>D. D.</td>
<td>79</td>
<td>F</td>
<td>Squamous</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>Negative</td>
<td>20</td>
<td>Negative</td>
<td>1 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>S. H.</td>
<td>65</td>
<td>F</td>
<td>Squamous</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>Positive</td>
<td>80</td>
<td>Negative</td>
<td>1 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>B. I.</td>
<td>69</td>
<td>F</td>
<td>Squamous</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>Negative</td>
<td>40</td>
<td>Negative</td>
<td>1 1 0</td>
<td>II Lobectomy</td>
</tr>
<tr>
<td>F. J.</td>
<td>66</td>
<td>M</td>
<td>Squamous</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Negative</td>
<td>100</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>K. W.</td>
<td>84</td>
<td>M</td>
<td>Squamous</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>Positive</td>
<td>30</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>S. C.</td>
<td>47</td>
<td>F</td>
<td>Non-small</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>Negative</td>
<td>30</td>
<td>Negative</td>
<td>2 0 0</td>
<td>I Lobectomy</td>
</tr>
<tr>
<td>M. H-L.</td>
<td>37</td>
<td>M</td>
<td>Carcinoid</td>
<td>/</td>
<td>3</td>
<td>2</td>
<td>Negative</td>
<td>40</td>
<td>Positive</td>
<td>1 0 0</td>
<td>Biopsy (asbestos)</td>
</tr>
</tbody>
</table>

"Inflammation was evaluated by the relative abundance of tumor-infiltrating macrophages and lymphocytes both scored from 0 to 3 and was validated as cumulative index as described in "Materials and Methods.""  
"F, female; M, male.  
*Family history of larynx carcinoma.
EU/mg protein, where one EU represents the amount of enzyme releasing 1 nM AMC/min. In comparing the differences between tumor and control tissue, the RISA was defined as the normalized difference between the activity in tumor and the activity measured in control tissue: \((T - C)/C\) as described (6).

**Cat L Activity.** Cat L activity was determined by a modified procedure (6) from Mason et al. (19) as follows: Samples (100 µl) were preincubated for 180 min at 37°C in 0.34 M acetate buffer (100 µl), pH 4.2, containing 2 mM DTT and 1 mM EDTA/Na. Cat L activity was measured on Z-phenylalanine-arginyl-AMC substrate in the presence (control assay) and absence (sample assay) of the selective inhibitor Z-Phe-Phe-CNH₂ (6, 18). Blank assays were prepared in the same fashion but without the addition of the enzyme, e.g., tissue sample. After preincubation in the activation buffer, acetate buffer (pH 5.5) containing 1 mM EDTA and 1.3 mM dithioerithritol was added (100 µl), followed by the addition of 2 mM inhibitor solution (100 µl). The reaction was started by adding 100 µl of the substrate solution to each assay. The reaction was terminated after 180 min incubation at 37°C by adding 1 mM iodoacetate (500 µl). Fluorescence was measured as described for Cat B (above). Cat L activity was calculated from the difference between the fluorescence in the sample assay (due to Cat B and Cat L activity) and fluorescence in the control assay (due to Cat B activity).

**Cat D Activity.** The degradation of hemoglobin at pH 3.5 was assayed according to the Anson procedure, modified as described previously (6). In brief, 25 µl of the sample were incubated with 2.5% hemoglobin solution (100 µl) in 0.4 M acetate buffer (pH 3.5) for 240 min at 37°C. In the control assay, 7 µM pepstatin (25 µl) was added prior to the substrate. The reaction was terminated with 200 µl ice-cold trichloroacetic acid (4.5% final concentration). The pellets were removed by centrifugation, then the hydrolyzed products were measured in clear supernatants using Fenol-Ciocaulte reagent (Sigma). The absorbancy at 750 nm was standardized with Tyr, and Cat D activity was expressed in AU, corresponding to Tyr equivalents produced per min and per ml.

**ELISAs**

A microtiter plate reader (EL 312e bio-kinetics reader; Bio-Tek Instruments, VT) was used to measure absorbency in ELISAs. The Cat proteins were expressed in ng/mg cytosolic protein. The RIP in tumors was calculated as the normalized difference between the tumor and the control sample, \((T - C)/C\), similarly as described above for the Cat activities.

**Cat D Protein.** Cat D protein, e.g., Cat D antigen, was measured in tissue extracts using an ELISA (Ciba-Corning, CA) with the antibodies that recognized all molecular forms of the Cat. Test tubes were precoated with streptavidin to bind the catin-conjugated monoclonal antihuman Cat D antibody. The monoclonal antibody and a rabbit polyclonal antibody, both specific for Cat D, were simultaneously incubated with specimen tissue cytosols (and/or with Cat D standards and/or the control) to form stable immunocomplexes which attached to the test tube surfaces. Unbound material was washed away four times with PBS buffer. In the second incubation an antirabbit antibody, conjugated with horseradish peroxidase, was added to the tube. Unbound conjugate was washed away with PBS, while the bound form reacted with 3,3',5,5'-tetramethylbenzidine solution in the presence of hydrogen peroxide to develop a color. The intensity of the absorbency, measured at 450 nm, was correlated to the amount of Cat D in the sample using the standard curve.

**Cat B Protein.** Cat B protein was determined using the double sandwich enzyme immunoassay (ELISA; BioAss, Dienesen, Germany). Polystyrene microtiter plates were coated with antihuman Cat B antibodies, and the specimens (Cat B standard and the control) were incubated for 3 h at 37°C to attach. After washing, the second antihuman Cat B antibody, which was raised in another species and conjugated with horseradish peroxidase, was added and allowed to bind to immobilized Cat B. The intensity of the color reaction, developed after the addition of a peroxidase substrate, was related to the amount of Cat B in the sample using the standard curve.

**Cat L Protein.** Cat L protein was determined using the double sandwich enzyme immunoassay (ELISA; BioAss) in a similar manner as described above for Cat B. According to the manufacturer’s protocol, both immunoassays (Cat B and Cat L) detected all enzyme forms and the complexes with the endogenous inhibitors.

**SDS-PAGE and Immunoblotting**

Slab gel electrophoresis was carried out on 10% polyacrylamide gels in the presence of SDS and 10% mercaptoethanol. For immunoblotting, proteins separated by SDS-PAGE were transferred electrotheretically to nitrocellulose membranes using a Bio-Rad Trans-Blot apparatus, as described by Towbin et al. (20). The blots were incubated with rabbit primary antibodies and subsequently with goat secondary antibodies, which were conjugated with horseradish peroxidase (Pierce, Rockford, IL) and detected using a silver-gold enhancement system (Amersham, Arlington Heights, IL), as recommended by the manufacturer. Primary polyclonal antibodies were used in a 1:250 dilution for Cat B and 1:100 dilution for Cat D. Immunoactive polyclonal Cat B antibodies were a generous gift from Dr. Joza Babnik (Jozef Stefan Institute, Ljubljana, Slovenia). Monoclonal antibody to mature human brain Cat D was a generous gift from Dr. Ralph Nixon (McLean Hospital, Harvard Medical School, Boston, MA).

**Statistical Analysis**

The data were analyzed using the SPSS statistical package (21). Distribution of the six variables, e.g., the Cat activities and proteins, were determined in malignant and control lung tissue extracts of the same patient using box plot and frequency histograms. The distributions in most cases did not reach criteria for normal, Gaussian distribution. Therefore, nonparametric tests were used for comparative analysis such as the Wilcoxon rank, Kruskal-Wallis, and Spearman rank tests.

**RESULTS**

Cat D, B, and L Activities and Proteins in Tumor and Control Samples

The median values of Cats D, B, and L activities and proteins (masses) in tumor and control samples are presented in Fig. 1. The median difference between Cat D activities in tumor
Fig. 1  a. cathepsin activities in lung tumor and control tissues. Median values of cathepsin activities in tumor (■) and control (□) tissues are presented. Specific activity of Cat D is expressed in AU per mg protein in tissue homogenates, as described previously (6). Cat B and Cat L activities were determined as described (6) and in ‘Materials and Methods’ and are presented in EU per mg protein. The corresponding ranges, expressed as 25th to 75th percentiles, are: Cat D, 0–1.17 AU/mg in control samples and 0.76–2.19 AU/mg in tumor samples; Cat B, 0.023–0.203 EU/mg in control samples and 0.107–0.784 EU/mg in tumor samples; Cat L, 0.180–0.518 EU/mg in control samples and 0.029–0.169 EU/mg in tumor samples. b. cathepsin protein in lung tumor and control tissues. Median values of cathepsin protein in tumor (■) and control (□) tissues were determined by ELISA as described in ‘Materials and Methods.’ The corresponding ranges, expressed as 25th to 75th percentiles, are as follows: Cat D, 442–1530 ng/mg of total protein in control samples and 850–1758 ng/mg in tumor samples; Cat B, 0–237 ng/mg protein in control samples and 177–720 ng/mg in tumor samples; Cat L, 799–5201 ng/mg protein in control samples and 1834–5836 ng/mg in tumor samples.

and control tissues was 0.470 AU/mg, representing a 1.9-fold increase in tumors (P < 0.061). The difference in Cat D protein was 13 ng/mg, corresponding to 1.8-fold but not a significant increase (P < 0.079).

The median increase of Cat B activity was 0.244 EU/mg protein, representing a 9.2-fold increase in the median tumor value. Similarly, tumor Cat B protein increased for 355 ng/mg protein, representing a 5.6-fold increase in the median tumor value. The increase in Cat B activity was highly significant (P < 0.001), while borderline significance (P < 0.054) was observed for Cat B protein.

Cat L activity was higher in tumor tissue compared to the control for 0.260 EU/mg, representing a 4-fold increase in the median tumor value. Tumor tissue also contained more Cat L protein, the difference between the medians was 177 ng/mg, representing a 2.2-fold increase in median tumor value. The increase in Cat L activity was highly significant (P < 0.0003), while the increase of Cat L protein was not (P < 0.069).

Correlation between Cats’ Expression

Correlation between Tumor and Control Tissues. Cat B activities in tumor samples correlated with the activities in their control counterparts (r = 0.72, P < 0.001). Similarly, Cat L activities in tumors correlated with the activities in their control tissue counterparts (r = 0.74, P < 0.001). Cat D activ-
Correlation between Relative Increase in Cats. The relative increase in the three Cats in tumors was compared for the three Cats, and the significance of the correlation coefficients between their increase (RISA or RIP values) is presented as described in "Materials and Methods".

Table 2. Relative increase in Cat activities in matched pairs of lung carcinomas and control lung tissues

The mean and median RISA, e.g., (T − C)/C, as defined in "Materials and Methods" and previously (6), is presented. χ² and the significance as obtained by the Kruskal-Wallis test was used to evaluate the statistically significant differences among the groups.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Cat D (T−C)/C</th>
<th>Cat B (T−C)/C</th>
<th>Cat L (T−C)/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2 (n = 17)</td>
<td>Mean ± SE</td>
<td>Median</td>
<td>25th–75th Percentile</td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.1</td>
<td>4.5</td>
<td>1.6–13.5</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>1.1</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>25th–75th Percentile</td>
<td>0.4–2.5</td>
<td>1.6–13.5</td>
</tr>
</tbody>
</table>

Table 3. Relative increase in Cat proteins (RIP) in matched pairs of lung carcinomas and control lung tissues

The mean and median of RIP, e.g., (T−C)/C, as defined in "Materials and Methods," is presented. χ² and the significance as obtained by Kruskal-Wallis test was used to evaluate the statistically significant differences among the groups.

<table>
<thead>
<tr>
<th>Histological grade</th>
<th>Cat D (T−C)/C</th>
<th>Cat B (T−C)/C</th>
<th>Cat L (T−C)/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 2 (n = 17)</td>
<td>Mean ± SE</td>
<td>Median</td>
<td>25th–75th Percentile</td>
</tr>
<tr>
<td></td>
<td>0.8 ± 0.5</td>
<td>3.9</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.7</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>25th–75th Percentile</td>
<td>0.4–1.3</td>
<td>1.4–7.4</td>
</tr>
</tbody>
</table>

Table 4. Correlation between the relative increase in cathepsin activity (RISA) and relative increase in cathepsin proteins (RIP)

The RISA, e.g., (T − C)/C, was defined in "Materials and Methods" and previously (6). The RIP, e.g., (T − C)/C, was defined in "Materials and Methods." The Spearman rank test was used to calculate the significance (Sig.) of correlation coefficients.

<table>
<thead>
<tr>
<th>Cat D activity (r, significance)</th>
<th>Cat B activity</th>
<th>Cat L activity</th>
<th>Cat B protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat B activity</td>
<td>−0.95 Sig. 0.82</td>
<td>−0.83 Sig. 0.0001</td>
<td>−0.18 Sig. 0.06</td>
</tr>
<tr>
<td>Cat L activity</td>
<td>0.055 Sig. 0.88</td>
<td>0.18 Sig. 0.97</td>
<td>−0.00 Sig. 0.79</td>
</tr>
<tr>
<td>Cat D protein</td>
<td>−0.18 Sig. 0.63</td>
<td>0.18 Sig. 0.97</td>
<td>−0.00 Sig. 0.79</td>
</tr>
<tr>
<td>Cat B protein</td>
<td>−0.26 Sig. 0.37</td>
<td>0.24 Sig. 0.46</td>
<td>0.8 Sig. 0.015</td>
</tr>
<tr>
<td>Cat L protein</td>
<td>−0.26 Sig. 0.37</td>
<td>0.24 Sig. 0.46</td>
<td>0.8 Sig. 0.015</td>
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</tbody>
</table>

Correlation between Cat Activities and Cat Proteins. The correlation of the enzymatic activities with the respective enzyme proteins showed that Cat activities correlated with their protein content in a coordinate fashion with the ratios between the median tumor and median normal values, which are shown in Fig. 1. As also shown in Tables 2 and 3, mean values differ significantly from the median values, indicating the nonparametric distribution.
markedly higher concentrations of Cat B precursor (M_r 46,000) and Cat B mature forms (M_r 30,000 and a doublet of 25,000–27,000). In contrast, the concentration of the Cat D heavy chain (M_r 30,000) was similar in tumor and control tissues.

Correlation with Histopathological Parameters

Histological Grade. Most tumor samples (n = 14) were stage I and therefore no correlation with clinical stage could be made. The samples were stratified according to the histological grade, grades 2 and 3 having the same sample size (n = 8). Median values of the relative increase in matched pairs (RISA and RIP) are shown in Tables 2 and 3. A significant increase in Cat L activity (P < 0.04) and Cat L protein (0.005) was observed. No significant differences between moderately and poorly differentiated tumors were observed with respect to Cat B and Cat D activity and protein.

Correlation with Lung Tumor Histology. In adenocarcinoma, the median relative increase in Cat L activity (P < 0.01) and Cat L protein (0.009) content were both found to be significantly higher compared to squamous carcinoma (Tables 2 and 3). Although the relative increase of median Cat B activity was higher in adenocarcinoma, the difference between the median values between the two histologies was not significant. The relative increases in Cat D activities and Cat D protein in adenocarcinoma versus squamous carcinoma were not significantly different.

Correlation with Inflammation. Inflammation of the lung tumors was validated by the presence of tumor-infiltrating inflammatory cells, lymphocytes, and macrophages, which were scored as described in “Materials and Methods.” In the groups of tumors with abundant infiltration of inflammatory cells, an increase in Cat D activity, Cat B activity, Cat L activity, and Cat L protein was observed, as shown in Table 5, but none of these differences were statistically significant. Only the relative increase in tumor Cat L protein (RIP), which was higher in tumors with inflammation compared with tumors with no or low inflammation, was significant.

DISCUSSION

Lysosomal enzymes, Cats, presumably play a role in tumor progression (3). In this pilot study, the levels of three Cats (Cat D, Cat B, and Cat L) were measured in the tumor and control samples taken from patients undergoing surgery for lung cancer. Both Cat-specific activities and the protein concentrations were measured. In comparing the Cat activity and Cat protein, we found good correlation for Cat D followed by Cat B and a moderate correlation for Cat L. Enzyme activities might not necessarily reflect the amount of the enzyme protein for two major reasons: First, in contrast to protein, the activity may not be stable under the condition of tissue extraction. In our previous study of breast carcinoma (6), we have shown that higher pH may cause partial inactivation of cysteine Cats B and L, while Cat D is more alkaline stable (23), which may explain better correlation between Cat D activity and protein content. Second, the discrepancies between activity and protein levels may be due to the presence of endogenous inhibitors (CPIs). Cysteine Cats, but not Cat D, are regulated by a variety of
endogenous inhibitors, such as stefins A and B, cystatin C, and kininogen (3, 25), which bind to Cat B and Cat L with different rates and affinities. The presence of inhibitors would influence the activity, but not the immunochemical determination of Cat protein. This study has shown that the Cat activities, reflecting the concentrations of the functional enzymes, in matched pairs of lung tumors were more increased and with higher significance compared to the Cat proteins. Similar findings were reported in breast carcinoma (6, 22) and may be explained by a decrease in the activities of CPIs, adding to the increase in the activities of cysteine proteinases. The imbalance between Cat B and CPI activities in lung tumors was reported recently (8, 9), suggesting a deficiency of the inhibitors in tumors. This imbalance was also of prognostic significance for early recurrence in patients with lung carcinoma (9).

Cat D activity and protein content were not significantly increased in lung tumors. This is in contrast to numerous studies of Cat D in breast carcinoma (6, 4, 10) and may be explained by different regulatory mechanisms of Cat D expression by steroid hormones and growth factors in different tissues (4, 11). The lack of correlation with histological grade as well as with tumor histology also suggests that Cat D is not associated with cell dedifferentiation and malignant progression of lung tumors.

In the first study of Cat B in lung tumors, Krepeia et al. (15) measured a 2–3-fold increase in mean Cat B activity in primary lung tumors over that of matched normal parenchyma. In a recent study of 69 matched pairs of lung tumors, Knoch et al. (8) found a 4.4-fold increase in the median Cat B activity in lung tumors. In our study, the relative increase in tumors was 4.6-fold for Cat B activity and 3.9-fold for Cat B protein, as also shown by immunoblotting. Increased Cat B activity was not significantly higher in poorly differentiated tumors compared to moderately differentiated, as also reported by Knoch et al. (8). Since this increase was not significant and not observed for Cat B protein, we cannot conclude that Cat B expression was higher in poorly differentiated tumors, confirming recent results by Werle et al. (18, 24). Cat B activity, but not protein increase, was not significantly higher in adenocarcinoma compared to squamous cell carcinoma. Previously, we have found that patients with squamous cell carcinoma and small cell carcinoma secreted considerably less Cat B activity into the lavage fluid compared to patients with adenocarcinoma (26). In an immunohistochemical study of lung tumors, Ozeki et al. (27) also found that Cat B expression was highly elevated in adenocarcinoma (Clara cells), correlating with positive lymph node status, presence of distant metastasis, and poor clinical outcome. However, in the most recent study, Cat B was not found to vary across different histologies (18), and additional studies are needed to confirm the possible association of Cat B with the histological type of lung cancer.

Of significance, a 4.6-fold increase in Cat L activity and 1.7-fold increase in Cat L protein in tumors was found in this study. Most recently, Werle et al. (18) found a 1.6-fold increase in median Cat L activities in lung tumors compared to normal lung tissues. In our study, Cat L activity and protein levels were both significantly increased in poorly differentiated tumors compared to moderately differentiated tumors, while such an increase was not observed by Werle et al. (18). The authors also reported on much lower levels of Cat L activity in lung tumor homogenates compared to Cat B, and provided evidence that a considerable amount of Cat L, but not Cat B, was associated with endogenous kininogens. As was suggested by the authors (18), the complexes might have formed during the homogenization procedure, and we believe that the discrepancies between these two studies are due mainly to different preparation of the homogenates. On the other hand, Cat L activity was found low in pulmonary carcinoids which are low-grade malignant neoplasm (18), supporting our results on a higher relative increase of Cat L expression in poorly differentiated tumors. This strongly suggests that Cat L may be a marker for cell differentiation. Cat L expression was markedly increased in adenocarcinoma. Corroborating this in vivo data in human lung tumors (5, 17, 24), Heidtmann et al. (28) reported that cultured non-small lung cancer cell lines secreted the precursor form of Cat L and some of them also secreted the active form of this enzyme in contrast to the small lung cell carcinoma lines. The secretion was stimulated by tumor promoters, indicating a possible role of Cat L in tumor progression (28).

Macrophages, like metastatic tumor cells, are involved in local proteolysis during pathological processes and contain high concentrations of Cats (3). Most malignant tumors contain macrophages, associated in particular with inflammatory and necrotic areas within the tumors. Although we found that tumors with a higher amount of infiltrating inflammatory cells tend to express higher levels of all three Cats, no significant differences
were observed, except for the relative increase of Cat L protein in these tumors. It has been reported that activated alveolar macrophages contained high levels of Cat L (30), but a larger number of lung tumors have to be examined to assess the contribution of macrophage-associated Cat L in lung tumors.

The present study shows that cysteine Cats B and L are associated with malignant lung tumors, whereas the aspartic proteinase Cat D was not. A correlation between the relative increases in Cat B and Cat L activity and protein levels suggests some similarities in their regulation, as proposed previously for lung (18) and for breast carcinoma (29, 31). This pilot study strongly suggests to further evaluate the expression of cysteine Cats in larger patient populations to establish their correlation with tumor cell differentiation and tumor histology as well as to prove the prognostic significance of Cat B and possibly of Cat L for survival of lung cancer patients, as has been suggested recently by Ebert et al. (8, 9, 18) and Sukoh et al. (16).

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