ABSTRACT

A panel of murine monoclonal antibodies was generated against a high-molecular-weight glycoprotein produced by human lung cancer cells. This lung cancer-associated protein (LCAP) has been shown to circulate in the plasma of patients with lung cancer. Various combinations of MAbS were used in solid-phase enzyme-linked sandwich immunoassays to optimize the detection of LCAP in the plasma of these patients. One of these monoclonal antibodies, designated DF-L1, used both in the solid phase as well as the tracer, was selected to evaluate circulating levels of LCAP in normal subjects and in patients with lung cancer. In 341 normal subjects, the mean LCAP level was 7 units/ml, with 47 (13.8%) and 18 (5.3%) subjects having levels ≥15 units/ml and 23 units/ml, respectively. In contrast, 27 of 35 (77.1%) patients with lung cancer had LCAP levels ≥23 units/ml. A total of 16 of 19 (84.2%) patients with adenocarcinoma, four of seven (57.1%) patients with squamous cell carcinoma, and four of six (66.7%) patients with small cell carcinoma had levels ≥23 units/ml. Moreover, in a small group of patients, serial LCAP levels correlated with clinical course during therapy. The LCAP assay is technically reproducible and unaffected by interfering substances in the blood or by variations in the handling of samples. These results indicate that LCAP is a new and potentially useful marker for the evaluation of patients with lung cancer.

INTRODUCTION

There are presently few sensitive circulating biochemical markers for carcinoma of the lung. Several candidates, including carcinoembryonic antigen, squamous cell carcinoma-associated antigen, calcitonin, ferritin, and glycosyl transferase, have been described previously (1-5). However, there is a need for more sensitive and specific markers to monitor the course of these tumors (6).

Antibody-based assays that detect circulating antigens are useful in the clinical evaluation of patients with other epithelial malignancies. Several of these antigen markers have been identified as mucins or mucin-like molecules (7-10). We and others have previously described the identification of a family of high-molecular-weight mucin-like carcinoma-associated antigens (11). These antigens are detectable at low levels on the apical surface of normal epithelial tissues, as well as in normal secretions and plasma (11-13). However, in malignant epithelial cells, this family of antigens is found at higher levels in the cytosol and in the cell membrane. Circulating levels of these glycoproteins are significantly elevated in the plasma of patients with breast and ovarian malignancies (14, 15). Moreover, circulating mucin-like antigens have been found to be sensitive and reliable markers for monitoring the clinical course of patients with metastatic lesions from these diseases (10, 16).

We have recently generated a monoclonal antibody against a human lung adenocarcinoma, designated MAbS DF-L1 (17). Immunoblot analyses demonstrate that this antibody detects increased levels of a lung cancer-associated antigen in the plasma of patients with lung cancer. This antigen is a member of the family of mucin-like proteins previously identified in breast and ovarian carcinomas (11). In this study, we report the development of a solid-phase ELISA using MAb DF-L1 to detect this antigen, designated LCAP. LCAP levels in patients with lung cancer were significantly elevated compared with those in normal controls. The LCAP assay provides a reliable method to evaluate and monitor patients with lung cancer.

MATERIALS AND METHODS

MAb Production. A panel of MAbS was generated by immunizing BALB/c mice with an extract of a human lung adenocarcinoma as previously described (17). The antibodies were purified from ascites, aliquoted, and stored at −20°C.

Peroxidase Conjugation. Purified MAb was conjugated to HRP by a modification of methods (18, 19) using the two heterobifunctional reagents, SPDP and SMCC (Sigma Chemical Co., St. Louis, MO). Briefly, SPDP was conjugated to HRP, and the SPDP was reduced by dithiothreitol. MAbS were conjugated to SMCC in dimethyl formamide. The two protein derivatives were then conjugated through the SPDP-SMCC bridge. Aggregated material was removed by Aa 34 (LKB, Poitier Girard, France) molecular sizing column chromatography.

Assay Format. MAbS (50 µg/ml) were adsorbed to 96-well microtiter culture plates in a 0.1 M NaHCO₃/0.5 M NaCl buffer (pH 8.5) for 1 h. This concentration was chosen following antibody titration to optimize the signal:noise ratio of the high, 200-unit/ml calibrator (see “Assay Calibrators”). The solution was aspirated, and the wells were incubated for 1 h with 5% BSA in 0.01 M PBS to block nonspecific protein binding sites on the plastic. The BSA was removed, and samples containing antigen were added to the wells. After a 1-h incubation, the wells were washed 3 to 4 times with a solution of 0.1% Tween 20 in PBS. The plates were then incubated with HRP-conjugated MAb for 1 h and washed again. O-Phenylenediamine was used as substrate, and the reaction was stopped after 30 min by the addition of 4 N H₂SO₄. Absorbance was read spectrophotometrically at a wavelength of 490 nm. All steps were carried out at room temperature.

Assay Calibrators. Spent tissue culture medium of the human lung carcinoma cell line CALU-3 (American Type Culture Collection, Rockville, MD) was pooled, concentrated, and used as a source for calibration of the assay. An arbitrary value of 200 units/ml was assigned to the pooled medium, and appropriate calibrators were prepared by dilution in PBS. Calibration curves of absorbance at 490 nm versus units were run for each plate, and the values of unknown samples were determined by a point-to-point linear interpolation of the calibration curve.

Plasma Samples. Plasma samples from normal subjects were obtained from the American Red Cross Greater Ozarks Blood and Tissue Services Blood Bank in Springfield, MO. Plasma samples were collected from patients at the Dana-Farber Cancer Institute, Boston, MA, and Terumo Medical Corporation, Elkton, Maryland 21921 [S. S.]
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according to protocols approved by the Institutional Review Board. Samples were collected in evacuated tubes containing disodium EDTA. Plasma was separated by centrifugation at 1000 × g for 15 min, aliquoted, and stored at −70°C.

RESULTS

Development of LCAP Assay

A panel of 10 MAbs was generated on the basis of reactivity with purified LCAP derived from CALU-3 cells. As determined by direct immunoassay, only 3 MAbs retained reactivity with purified LCAP after conjugation to HRP. Moreover, only 4 of the MAbs adsorbed reliably to microtiter plates and retained activity. These MAbs were evaluated in various combinations to detect LCAP levels in solid-phase ELISAs. The five “sandwich” immunoassay combinations that detected soluble LCAP from cultured cell supernatants were then evaluated for optimal circulating LCAP detection with a small panel of plasma samples from lung cancer patients and from normal individuals (Table 1). The combination DF-L1, DF-L1-HRP distinguished cancer patients from normal controls to the greatest extent and was thus chosen for a more comprehensive analysis.

Preparation of LCAP Calibrators

Spent tissue culture medium from CALU-3 human lung adenocarcinoma cells, containing high concentrations of LCAP as determined by Western blot analysis, was pooled and diluted to make arbitrarily defined calibrator solutions, ranging from 0 to 200 units/ml. The LCAP assay was quite reproducible, as demonstrated in a series of calibration curves run on different days (Fig. 1). Serial dilutions of plasma samples from normal subjects and from lung cancer patients were evaluated using the DF-L1, DF-L1-HRP assay (Fig. 2). In undiluted plasma, absorbance was at the upper limits of the assay for both normal and cancer samples. However, when the plasma was diluted, absorbance levels rapidly decreased to the baseline signal for normal samples, while those for samples from patients with advanced lung cancer remained elevated until 1/200 or more dilutions. The optimal dilution at which absorbance levels for normal samples were near baseline while those for cancer samples remained elevated was 1/50, and it was therefore chosen as the standard dilution for all subsequent samples.

Table 1  Comparison of assay formats for the detection of circulating LCAP

<table>
<thead>
<tr>
<th></th>
<th>DF-L1-HRP</th>
<th>DF-L3-HRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capture MAb</td>
<td>Normal subjects</td>
<td>Cancer patients</td>
</tr>
<tr>
<td>DF-L1</td>
<td>7</td>
<td>92</td>
</tr>
<tr>
<td>DF-L2</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>DF-L4</td>
<td>NE*</td>
<td>NE</td>
</tr>
</tbody>
</table>

* Mean units/ml of 5 samples.

Table 2  Intraassay reproducibility of LCAP assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean ± SD at A490 (n = 12)</th>
<th>Coefficient of variation (%)</th>
<th>LCAP (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 units/ml</td>
<td>0.05 ± 0.002</td>
<td>3.90</td>
<td></td>
</tr>
<tr>
<td>20 units/ml</td>
<td>0.38 ± 0.020</td>
<td>5.04</td>
<td></td>
</tr>
<tr>
<td>100 units/ml</td>
<td>1.55 ± 0.090</td>
<td>5.57</td>
<td></td>
</tr>
<tr>
<td>200 units/ml</td>
<td>2.64 ± 0.070</td>
<td>2.67</td>
<td></td>
</tr>
<tr>
<td>Specimen 1</td>
<td>1.34 ± 0.060</td>
<td>4.14</td>
<td>85.6</td>
</tr>
<tr>
<td>Specimen 2</td>
<td>0.85 ± 0.050</td>
<td>5.74</td>
<td>52.1</td>
</tr>
<tr>
<td>Specimen 3</td>
<td>0.46 ± 0.020</td>
<td>4.88</td>
<td>25.5</td>
</tr>
</tbody>
</table>

Fig. 1. Reproducibility of LCAP calibrator curves. LCAP calibrators containing 0, 20, 100, and 200 units/ml were assayed in duplicate on 4 consecutive days. Absorbances (ABS) at 490 nm were determined for each calibrator. O, Day 1; ◆, Day 2; ■, Day 3; ▲, Day 4. Points, mean; bars, SD.

Fig. 2. The effect of dilution on LCAP levels. Plasma samples from normal subjects and patients with lung cancer were assayed for LCAP levels as a function of dilution. O and ◆, normal subjects; ■ and ▲, patients with metastatic lung cancer. Points, mean; bars, SD. ABS, absorbances.

Assay Characterization

The LCAP assay was optimized for routine use and then characterized for inter- and intraassay variation, antigen recovery, interference effects, and sample handling.

Reproducibility Studies. Intra- and interassay reproducibility were assessed for the calibrators and three serum specimens containing different concentrations of LCAP. The intraassay reproducibility was determined by one individual running the calibrators and serum specimens in replicates of 12, calculating the mean absorbance value for each and determining the percentage of the coefficient of variation. Interassay reproducibility was determined by running the calibrators and serum samples in duplicate by two individuals over seven assay runs. Intraassay coefficients of variation for the calibrators ranged from 2.67 to 5.57% and from 4.14 to 5.74% for the serum samples (Table 2). Interassay coefficients of variation ranged from 4.29 to...
9.52% for the calibrators and 4.13 to 7.61% for the samples (Table 3). Thus, the reproducibility of the assay was satisfactory.

Antigen Recovery. Known quantities of LCAP were added to four plasma samples containing baseline levels of endogenous LCAP. These samples were then assayed, and recovery was determined by dividing the observed value by the expected value and multiplying by 100. Recovery ranged from 96.4 to 106.0% of added LCAP (Table 4).

Interference Studies. The ability of the assay to quantitate LCAP in the presence of potentially interfering, circulating substances was investigated. Known quantities of LCAP were added to plasma specimens with baseline levels of endogenous LCAP that contained different levels of bilirubin (up to 22.1 mg/dl), rheumatoid factor (≥1:160), or triglycerides (up to 1026 mg/dl). These substances are frequently elevated in plasma specimens and often interfere, by nonimmunological mechanisms, in the performance of some immunoassays. The specimens were then reasayed in the LCAP assay, and the percentage of recovery was calculated as above (antigen recovery). The data (Table 5) indicated little if any quantitative interference by these substances in the assay.

Freeze/Thaw. To assess the stability of LCAP in samples that had been frozen and thawed, specimens were cycled between freezing (−20°C) and thawing (18°–25°C) 9 times and assayed along with aliquots of these same samples that had only been frozen and thawed once. No apparent effect on LCAP levels was observed through 9 freeze-thaw cycles compared with one cycle, as all sample recoveries were >90% of control.

Matched Serum-Plasma Correlation. Serum and EDTA plasma samples were obtained from 156 volunteer donors and assayed for LCAP levels. The values obtained for the plasma samples were regressed on the serum values. The correlation coefficient obtained was 0.966, and the slope, 0.979. The overall concordance using 23 units/ml as a reference cut-off (see following section) was 94%.

Distribution of Circulating LCAP Levels in Normal Subjects

Plasma samples from 341 normal blood bank donors were evaluated for LCAP levels (Fig. 3). The mean value of the normals was 7 units/ml (SD ± 8) with a median value of 5 units/ml. The levels ranged from a low of 0 units/ml to a high of 43 units/ml. Thirty-five % of the samples had LCAP levels of 2 units/ml or less, while only 13.8% of the samples had levels above 15 units/ml. A level of the mean plus 2 SDs was chosen as a reference cut-off value. Although the mean LCAP level for smokers was slightly higher than for nonsmokers (10 units/ml versus 7 units/ml), the ranges of LCAP levels for smokers (0 to 34 units/ml) and nonsmokers (0 to 43 units/ml) overlapped. Nonetheless, the small difference between LCAP levels in the two groups was statistically significant (Mann-Whitney test, P = 0.01). More studies are currently being performed to assess the effect of smoking on LCAP levels and the clinical significance of the results.

Distribution of Circulating LCAP Levels in Lung Cancer Patients

A panel of 35 plasmas from patients with metastatic lung cancer was screened to determine circulating LCAP levels. Patients with all four major histological types of lung cancer (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and small cell carcinoma) were studied. LCAP levels were elevated in 27 of 35 (77.1%) lung cancer patients with a mean of 127 units/ml and a range of 8 units/ml to >1000 units/ml (Fig. 4). The difference in LCAP levels from lung cancer patients and those from normal controls was highly
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Fig. 4. LCAP levels in patients with lung cancer. Plasma samples from normal subjects and patients with lung cancer were assayed for LCAP levels. A normal cut-off value of 23 units/ml was used. NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma.

statistically significant \( (P < 0.001) \). Moreover, LCAP levels were elevated in patients with each histological type of lung cancer: adenocarcinoma, 16 of 19 (84.2%); squamous cell carcinoma, 4 of 7 (57.1%); other undifferentiated non-small cell carcinoma, 3 of 3 (100%); and small cell carcinoma, 4 of 6 (66.7%).

Serial LCAP Levels

LCAP levels were monitored in selected patients with lung cancer during treatment for metastatic disease. For example, one patient with adenocarcinoma was considered by clinical criteria to have stable disease for the 5 mo (Fig. 5A). However, LCAP levels increased continually for 5 mo prior to clinical documentation of disease progression. Serial LCAP levels from a second patient, also with adenocarcinoma, correlated with a clinically documented response to therapy over an initial 6-mo period (Fig. 5B). As the patient responded to therapy, LCAP levels decreased, and conversely as disease progressed, LCAP levels increased. Serial levels of LCAP from a patient with small cell carcinoma also paralleled clinical response to therapy (Fig. 5C). Elevated levels of LCAP prior to chemotherapy rapidly decreased to normal and remained below the normal cut-off as the patient entered a complete clinical response.

DISCUSSION

In this study, we have used MAb DF-L1 to develop a solid-phase sandwich enzyme immunoassay that detects a circulating high-molecular-weight lung cancer-associated protein. In a prior study, we have reported that MAb DF-L1 detects a peptide epitope of the core protein of LCAP (17). Immunoperoxidase studies demonstrate reactivity of MAb DF-L1 with formalin-fixed paraffin-embedded sections of human lung cancer. Furthermore, the DF-L1 reaction epitope is present on the surface of lung cancer cells, and the reactive antigen is secreted into tissue culture medium (17).

Normal, healthy individuals had low levels of circulating LCAP. We have previously reported that LCAP is detectable by immunoperoxidase staining on apical surfaces of several normal tissues (17). Furthermore, circulating LCAP is detectable by Western blot analysis at low levels in the plasma of normal individuals (17). The mean \( \pm \) SD LCAP level in normal subjects was \( 7 \pm 8 \) units/ml. Therefore, 23 units/ml, or the mean plus 2 SDs, also defined as the 95% confidence limit, are an appropriate cut-off value to distinguish significantly elevated levels. Only 2% of all the samples had elevated LCAP levels above 30 units/ml. This small percentage of patients with elevated LCAP levels is probably due to simple statistical
distribution of values around the mean. Alternatively, these elevated levels may be due to occult benign or malignant disease. The LCAP assay proved to be sensitive in detecting patients with lung cancer. Seventy-seven % of all lung cancer samples had elevated LCAP levels (≥23 units/ml). These included certain samples with levels greater than 1000 units/ml that were from patients with advanced metastatic disease. Although elevated in patients with all histological subtypes of lung cancer, LCAP was most commonly increased in those with adenocarcinoma, with 84% of samples having elevated levels. These results are consistent with our previous immunoperoxidase staining results of lung cancer tissue sections using MAB DF-L1 (17). LCAP levels were also frequently elevated in small cell carcinoma samples. These results were unexpected since preliminary immunoperoxidase studies of formalin-fixed small cell carcinoma tissue failed to demonstrate reactivity of MAB DF-L1 with this histologic subtype in a small number of samples (17). Nonetheless, immunoblot studies of plasma, as well as of pleural effusions from patients with small cell carcinoma, demonstrated elevated levels of the appropriate molecular weight antigen (data not shown). Our initial immunoperoxidase studies were performed on bronchoscopic biopsies that may not have included representative areas. Two additional small cell lung cancer tissue sections, obtained by surgical excision, have now been analyzed, and reactivity with DF-L1 has been seen in both.

In selected patients with metastatic adenocarcinoma of the lung, serial LCAP levels paralleled the clinical course of disease. A lead time of several months in predicting clinical response was observed, as well as accurate correlation with clinical course. Thus, as demonstrated with serial levels of the circulating breast carcinoma-associated antigen, CA15-3 (14), LCAP could be useful in monitoring the clinical course of patients with lung cancer.

The specificity of this assay for lung cancer as compared with other types of cancer is not absolute. Although LCAP levels are elevated in patients with certain other tumor types, the sensitivity was highest in patients with lung cancer. These results are in concert with our previous findings that showed that MAB DF-L1 reacts with other members of this antigen family expressed in nonlung carcinomas (17). Our data demonstrate that the LCAP ELISA is sensitive and the results reproducible. Antigen recovery, interference studies, and inter- and intraassay reproducibility analyses were all within acceptable limits of variation. Matched plasma and serum samples exhibited no difference in LCAP levels, and plasma LCAP levels were unaffected by multiple freeze-thaw cycles. These findings indicate a certain degree of latitude in the handling and storage of blood samples to be monitored for LCAP levels and, thus, add to the convenience of the assay. In summary, we have described the design and performance of a new plasma assay for a circulating glycoprotein that may be useful in the evaluation of patients with lung cancer. Larger trials are currently under way to determine the clinical utility of monitoring serial LCAP levels as a marker to evaluate the clinical course of patients with this disease.

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REFERENCES


* Unpublished data.
Lung Cancer-associated Protein: Development and Characterization of a New Assay That Detects a Circulating Lung Cancer Marker

Peter Maimonis, Daniel F. Hayes, Steven Schaffel, et al.


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