An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer

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The identification of circulating tumor antigens or their related autoantibodies provides a means for early cancer diagnosis as well as leads for therapy. The purpose of this study was to identify proteins that commonly induce a humoral response in lung cancer by using a proteomic approach and to investigate biological processes that may be associated with the development of autoantibodies. Aliquots of solubilized proteins from a lung adenocarcinoma cell line (A549) and from lung tumors were subjected to two-dimensional PAGE, followed by Western blot analysis in which individual sera were tested for primary antibodies. Sera from 54 newly diagnosed patients with lung cancer and 60 patients with other cancers and from 61 noncancer controls were analyzed. Sera from 60% of patients with lung adenocarcinoma and 33% of patients with squamous cell lung carcinoma but none of the noncancer controls exhibited IgG-based reactivity against proteins identified as glycosylated annexins I and/or II. Immunohistochemical analysis showed that annexin I was expressed diffusely in neoplastic cells in lung tumor tissues, whereas annexin II was predominant at the cell surface. Interestingly, IL-6 levels were significantly higher in sera of antibody-positive lung cancer patients compared with antibody-negative patients and controls. We conclude that an immune response manifested by annexins I and II autoantibodies occurs commonly in lung cancer and is associated with high circulating levels of an inflammatory cytokine. The proteomic approach we have implemented has utility for the development of serum-based assays for cancer diagnosis as we report in this paper on the discovery of antiannexins I and/or II in sera from patients with lung cancer.

There is increasing evidence for an immune response to cancer in humans, demonstrated in part by the identification of autoantibodies against a number of intracellular and surface antigens in patients with different tumor types (1–3). For example, somatic alterations in the p53 gene elicit a humoral response in 20–40% of affected patients (4). The detection of anti-p53 antibodies can predate the diagnosis of cancer (4). The identification of circulating tumor antigens or their related autoantibodies provides a means for early cancer diagnosis as well as leads for therapy. The purpose of this study was to identify proteins that commonly induce a humoral response in lung cancer by using a proteomic approach and to investigate biological processes that may be associated with the development of autoantibodies. Aliquots of solubilized proteins from a lung adenocarcinoma cell line (A549) and from lung tumors were subjected to two-dimensional PAGE, followed by Western blot analysis in which individual sera were tested for primary antibodies. Sera from 54 newly diagnosed patients with lung cancer and 60 patients with other cancers and from 61 noncancer controls were analyzed. Sera from 60% of patients with lung adenocarcinoma and 33% of patients with squamous cell lung carcinoma but none of the noncancer controls exhibited IgG-based reactivity against proteins identified as glycosylated annexins I and/or II. Immunohistochemical analysis showed that annexin I was expressed diffusely in neoplastic cells in lung tumor tissues, whereas annexin II was predominant at the cell surface. Interestingly, IL-6 levels were significantly higher in sera of antibody-positive lung cancer patients compared with antibody-negative patients and controls. We conclude that an immune response manifested by annexins I and II autoantibodies occurs commonly in lung cancer and is associated with high circulating levels of an inflammatory cytokine. The proteomic approach we have implemented has utility for the development of serum-based assays for cancer diagnosis as we report in this paper on the discovery of antiannexins I and/or II in sera from patients with lung cancer.

2-D PAGE and Western Blotting. After excision, the tumor tissue was frozen immediately at −80°C, after which an aliquot was lysed...
Protein Identification. For protein identification by mass spectrometry, 2-D gels were stained by using a modified silver-staining method and excised proteins were digested as described previously (15). A peptide mass profile was obtained by using a PerSeptive Biosystems matrix-assisted laser desorption ionization–TOF Voyager-DE Mass Spectrometer (Framingham, MA). The peptide masses obtained were used for database searches for protein identification.

Annexin Deglycosylation. Annexin I was purified from the A549 cell line by immunoadfinity chromatography. Briefly, the mouse mAb EH17a was purified by affinity chromatography with the mAb TrapGII kit and linked to the CNBr-activated Sepharose 4B (Amersham Pharmacia). The immunoadfinity column was used further to purify annexin I from the sonicated A549 cells. Purified annexin I was treated with endoglycosidase F according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis). Treated proteins were separated by SDS/PAGE and visualized by silver staining or transferred to a polyvinylidene fluoride membrane.

Immunohistochemistry. Immunohistochemistry for annexin I and II was performed by using an automated stainer (Ventana Medical Systems, Tucson, AZ). Annexin I antibody (ICN) was used at 1:100 dilution and annexin II (ICN) was used at 1:400 dilution. Formalin-fixed, paraffin-embedded sections of lung and lung tumors were stained by using the Ventana Basic DAB Detection Kit, which employs the avidin–biotin–complex method for the detection of primary annexin antibodies (16).

Cytokine and C Reactive Protein (CRP) ELISAs. The serum concentrations of IL-1β, IL-6, and TNFα were determined by using ELISA kits (Chemicon). The serum concentration of CRP was measured by indirect ELISA by using rabbit anti-human–CRP antibody and peroxidase-conjugated rabbit anti-human–CRP antibody (Dako). In all cases, a standard curve was constructed from standards provided by the suppliers.

Statistical Analysis. A comparison of cytokine and CRP serum levels was performed between patients with lung cancer (anti-annexin autoantibody-positive or antiannexin autoantibody-negative), healthy subjects, and patients with chronic lung disease. Results were expressed as means ± SEM. The statistical significance of differences between groups was determined by using the Wilcoxon two-sample test. Data were considered statistically significant if P < 0.05.

IL-6 Treatment. A549 cells were incubated with or without IL-6 (10 ng/ml) for 24 h in DMEM without FCS. The culture supernatant was harvested and concentrated by using Centrifrap 3 and Centricon 3 centrifugal filter units (Millipore). Cultured cells were washed three times with PBS, and the proteins bound to the cell membrane were EDTA-extracted for 30 min at 4°C in PBS supplemented with 1 mM EDTA and a mixture of protease inhibitors (Roche Molecular Biochemicals) and concentrated. Cultured cells were lysed by the addition of 300 μl of solubilization buffer and scraped. Protein concentrations were determined by means of the Bradford assay (Bio-Rad) before SDS electrophoresis and protein transfer to an Immobilon-P polyvinylidine fluoride membrane for Western blotting analysis with antiannexin I, antiannexin II, and anti-α-tubulin (Sigma) antibodies. Annexin I and II band intensities were normalized to α-tubulin intensities.

Results

Reactivity of Sera from Lung Cancer Patients with Annexins I and II. Sera obtained at the time of diagnosis from 54 patients with lung cancer including 30 with adenocarcinoma, from 60 patients with other types of cancer, and from 61 additional controls consisting of 51 healthy subjects and 10 subjects with chronic lung disease were investigated for the presence of antibodies to A549 adenocarcinoma cell line proteins (Table 1). Most lung cancer patient sera reacted against multiple proteins. The reactive proteins most commonly observed with lung cancer patient sera, but not with noncancer controls, consisted of two groups of contiguous proteins. The first group, with a pI between 7.6 and 8.2 and molecular mass of ~36 kDa, was identified as annexin II by mass spectrometry (Figs. 1 and 24). Annexin II reactivity was observed with sera from 18 of 54 (33%) patients with lung cancer, including sera from 11 of 30 (37%) adenocarcinoma patients (Table 1). A second group of contiguous protein spots, with a pI between 6.6 and 7.2 and molecular mass of 37 kDa, identified as annexin I by mass spectrometric analysis (Figs. 1 and 34), was observed with sera from 16 of 54 (30%) lung cancer patients, including 12 of 30 (40%) with adenocarcinoma. Antibodies against both annexins I and II were observed with sera from 6 on 54 (11%) lung cancer patients, including 4 of 30 (13%) with adenocarcinoma and 2 of 18 (11%) with squamous cell carcinoma. Positive sera generally were reactive against annexins I and II. Protein spots that reacted with
patient sera and that were identified as annexins I and II by mass spectrometry also reacted with the corresponding mAb (Figs. 2B and 3B). In total, 18 of 30 (60%) sera from patients with lung adenocarcinoma exhibited reactivity against annexin I and/or annexin II (Table 1). None of the sera exhibited immunoreactivity against other identified annexins in lung adenocarcinoma 2-D patterns, specifically annexins IV and V (Figs. 1, 2A, and 3A). Reactivity was not limited to patients with advanced-stage disease. Sera from 51% (19 of 37) of patients with stage I lung cancer contained autoantibodies to annexin I and/or II. Likewise, sera from 67% (6 of 9) of patients with stage II and 43% (3 of 7) of patients with stage III contained autoantibodies to annexin I and/or II. There was no correlation between smoking status and the occurrence of autoantibodies to annexin I and/or II in patients with lung cancer; 89% were smokers among patients with autoantibodies, and among patients without autoantibodies against annexins, 96% were smokers. For healthy subjects, 41% (21 of 51) were smokers. The occurrence of autoantibodies was not correlated with age of the patients (64.1 ± 1.5 for patients with sera containing antiannexin autoantibodies and 65.1 ± 1.7 for patients without antiannexin antibodies). Sera showed similar reactivity against annexins I and II in autologous tumor protein blots and in blots prepared from normal lung tissue, and in A549-derived blots (data not shown). Sera from lung cancer patients that exhibited IgG-based reactivity against annexin I and/or II exhibited reactivity that was specific to IgG1 among the IgG subtypes examined (IgG1–4) and also exhibited IgM-based reactivity (data not shown). None of the sera from other cancer types or from noncancer controls exhibited autoantibodies against annexin II. Annexin I autoantibodies were found in sera of 6 of 60 patients with other types of cancers, namely 4 of 17 with esophageal cancer, 1 of 14 with brain tumor, and 1 of 11 with breast cancer.

Expression of Annexins I and II in Tumor Tissue. Annexin expression in lung tumors was assessed by immunohistochemistry, using monoclonal anti-annexin I and II antibodies. We have analyzed 18 lung tumors, including 4 from patients with autoantibodies against annexin I, 4 with autoantibodies against annexin II, 4 with autoantibodies against annexins I and II and 6 without anti-annexin autoantibodies. Annexin I was abundantly expressed in a diffuse manner in most adenocarcinomas (9 of 11) and squamous cell carcinomas (7 of 7) (Fig. 4). Intense annexin II immunoreactivity was also detected in a majority of tumors in a predominantly membranous pattern (8 of 11 adenocarcinomas and 5 of 7 squamous cell carcinomas) (Fig. 4). Lower expression levels for annexin II were observed in the other tumors (3

Fig. 1. Silver staining of A549 lung adenocarcinoma cell proteins separated by 2-D PAGE. Arrows point to the location of annexins I, II, IV, and V (A1, A2, A4, and A5) spots in the pattern.

Fig. 2. (A) IgG1-based reactivity against annexin II protein in a Western blot of A549 proteins, using a lung cancer patient serum. (B) Close-up of a Western blot showing reactivity with antiannexin II mAb, confirming identity of the reactive protein shown in A. (C) Matrix-assisted laser desorption ionization–time of flight spectra obtained from protein A2 after trypsin digestion and tryptic peptide sequences from annexin II matching with peaks obtained from the spectra.

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adenocarcinomas and 2 squamous cell carcinomas), but the staining was also predominantly membranous. There were no appreciable differences in annexins I and II expression, by immunohistochemical analysis, between autoantibody positive and negative lung cancer patients.

**Role of Glycosylation in Annexin Antigenicity.** We sought to determine whether annexin glycosylation contributed to immunogenicity. After purification, annexin I was subjected to N-deglycosylation. The resulting products were separated by SDS electrophoresis and analyzed by Western blotting (Fig. 5). N-deglycosylation by endoglycosidase F induced a basic shift of the protein without an apparent large molecular mass difference (Fig. 5B) compared with untreated annexin I (Fig. 5A). Two sera were tested that exhibited IgG-based immunoreactivity against annexin I. These sera did not react against endoglycosidase F-treated annexin but exhibited IgG-based immunoreactivity

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**Fig. 3.** (A) IgG1-based reactivity against both annexin I and II proteins by using a patient serum. (B) Close-up of a Western blot showing reactivity of annexin I protein with antiannexin I mAb. (C) Mass spectrometry identification of annexin I after trypsin digestion of the protein A1.

**Fig. 4.** Immunohistochemical analysis of annexin I and II expression in lung carcinomas. Representative immunoreactivity of annexins I and II in lung adenocarcinoma (ACA) and squamous cell carcinoma (SCC) (×300). Annexin I staining showed a mixture of nuclear, cytoplasmic, and membranous immunoreactivity, whereas annexin II staining showed immunoreactivity localized to the cytoplasmic membrane. T and S denote tumor and stroma tissue, respectively.

**Fig. 5.** (A) Western blot analysis of purified annexin I before enzymatic treatment. (B) Western blot analysis of purified annexin I after endoglycosidase F treatment, showing a basic shift in annexin I migration. A and B were 2-D PAGE Western Blot-hybridized with antiannexin I mAb. (C) One-dimensional SDS/PAGE Western blot of purified annexin I, hybridized with a serum from an annexin I antibody-positive lung cancer patient. Hybridization was observed before (−endo F) but not after (+endo F) N-deglycosylation by endoglycosidase F.
against annexin I, which we had observed already by 2-D PAGE Western blot with this patient’s serum (Figs. 3A and 5C).

Assays of IL-1β, IL-6, TNFα, and CRP in Subject Sera. High serum levels of IL-6 have been reported in some patients with lung cancer (12, 13). We therefore determined whether patients that exhibited immunoreactivity against annexins I and II exhibited different serum levels of IL-1β, IL-6, TNFα, and CRP from nonreactive patients and controls. Sera from a total of 40 patients with lung cancer (20 with antiannexin I and/or II autoantibodies and 20 without antiannexin autoantibodies), from 39 healthy subjects, and from 10 patients with chronic lung disease were investigated. Compared with healthy subjects, patients with lung cancer had significantly higher serum levels of IL-6 (healthy subjects, 23.14 ± 1.88 pg/ml; patients, 60.53 ± 6.27 pg/ml; P = 0.003) (Fig. 6) and higher CRP levels (healthy subjects, 124.19 ± 26.64 ng/ml; patients, 529.33 ± 102.47 ng/ml; P = 0.001). In addition, patients with autoantibodies against annexin I and/or II had significantly higher IL-6 serum levels (74.25 ± 9.75 pg/ml) than patients without antiannexin autoantibodies (46.82 ± 6.58 pg/ml; P = 0.029) (Fig. 6). No statistically significant differences in IL-1β and TNFα serum levels were observed between the different groups.

Effect of IL-6 Treatment on the Expression of Annexins I and II. IL-6 has been identified as a major cytokine expressed by tumor-infiltrating cells in lung cancer (17). We therefore examined the effect of IL-6 treatment on the expression of annexins I and II in lung cancer cells. A549 cells were treated with IL-6 for 24 h, and the cytosolic, membrane-associated, and secreted protein fractions were analyzed by Western blotting with antiannexin I and II mAbs. IL-6 treatment resulted in an increase in membrane-associated annexins I and II (3.2- and 2.3-fold increase in band intensity, respectively; Fig. 7) and in faintly detectable annexin I and II bands in the secreted protein fraction (Fig. 7). No appreciable change was observed in annexin levels in the cytosolic fraction after IL-6 treatment.

Discussion

In this study, sera from more than half of the patients with lung cancer exhibited IgG1 and IgM autoantibodies to annexin I and/or II. Annexin II autoantibodies were found only in lung cancer patients in our series, whereas annexin I autoantibodies also were observed in a few patients with other cancers. The annexins belong to a family of multifunctional, calcium-dependent, phospholipid-binding proteins (18). Prior studies have shown that, in the lung, annexins I and II were expressed in cilia and pleural mesothelial cells but not in Clara cells (19). Annexin I but not annexin II was found to be expressed in epithelial. In addition, annexin II was expressed in type I and II alveolar cells where no expression of annexin I was observed. We have shown that annexins I and II were highly expressed in lung cancer cells. Annexin I is a 37-kDa protein that has been implicated in glucocorticoid-induced inhibition of cell growth (20, 21). Annexin II is a 36-kDa protein that occurs in a monomeric form or as a tetramer, associated with the annexin II light chain (p11), which is a member of the S100 family (22). Annexin II has been implicated in cell–cell adhesion and in plasminogen activation and may function as a cell surface receptor (23). Annexin II tetramers have been shown to interact with procathepsin B on the surface of tumor cells and may be involved in extracellular proteolysis, facilitating tumor invasion and metastasis (24). Interestingly, annexin I is a target of autoantibodies in autoimmune diseases such as systemic lupus erythematosus (25) and rheumatoid arthritis (26). Annexin II, specifically, has not been implicated previously as a target of autoantibodies in any disorders.

Annexins are known to undergo posttranslational modification including glycosylation (27). Annexins I and II are both phosphorylated by various kinases (28). In our study, immunoreactivity against annexin I was found to be dependent on N-glycosylation. A potential N-linked glycosylation site is present at positions 42 and 61 from the N terminus of annexins I and II, respectively (29, 30). Glycosylation may contribute to protein stability and may enhance signal transduction (27). Although immunoreactivity was dependent on N-glycosylation, there was no indication that such glycosylation or any other posttranslational modification associated with immunoreactivity was restricted to cancer cells or to immunoreactive patients, because sera from immunoreactive patients also reacted with annexins I and II from normal lung. This suggests that an altered immune response and/or altered levels and cellular distribution of annexins I and II in lung cancer patients account for the development of antiannexin autoantibodies. Nevertheless, our data clearly indicate that identification of some antigenic proteins, as in the case of glycosylated annexins, necessitates the screening of proteins in their modified state. This is difficult to achieve with other screening approaches that rely on recombinant proteins that may lack antigenic and other modifications necessary for reactivity with autoantibodies (31).
An increased level of serum IL-6 in some patients with lung cancer has been reported previously and shown to be part of an inflammatory response (12, 32). Also, cytokine-expression analyses of tumor-infiltrating cells in non-small-cell lung cancer have shown that IL-6 was the predominant cytokine expressed (17). We have shown that patients that exhibited autoantibodies to annexins I and II had significantly higher IL-6 serum levels than any of our comparison groups, most importantly lung cancer patients without demonstrable antiannexin autoantibodies. This finding suggests that host factors such as cytokines may affect the immune response to potentially antigenic proteins. We also have shown that IL-6 treatment of A549 resulted in an increase in membrane-bound annexin I and II, supporting a prior finding of an IL-6-stimulated translocation of annexin I to the cell surface (33). An increase in membrane-associated annexins I and II may enhance immunogenicity.

A prerequisite for an immune response against a cellular protein is its presentation as an antigen. Interestingly, it has been shown previously in a melanoma cell line that annexin II-derived peptides are bound to MHC class II molecules (34). Expression of MHC molecules in lung cancer cells also may be induced as a result of cytokine secretion by tumor-infiltrating cells (35). Thus, inflammatory cytokine(s) may contribute to an increased expression of MHC molecules and to annexin I and II translocation to the cell surface, resulting in a humoral immune response.

Although in our study autoantibodies to annexin I were observed in some patients with tumors other than lung cancer, annexin II autoantibodies were restricted to lung cancer patients. Our findings led us to propose a mechanism for the development of autoantibodies against certain proteins in cancer, whereby host factors such as cytokines may affect the level and cellular distribution of a potential antigen, the presentation of specific polypeptides, and also modulate the immune response in other ways. Given the high frequency with which autoantibodies to certain proteins may occur in cancer, as we have demonstrated for autoantibodies to annexins I and II in lung cancer, assays for panels of such circulating antibodies and/or their corresponding antigens may have clinical utility.

The mouse monoclonal antiannexin I antibody, EH17a, developed by J. D. Ernst, was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City. We thank Drs. M. B. Orringer, M. D. Iannettoni, and D. A. Arenberg for their assistance in sample procurement. This work was supported in part by research funding from the National Cancer Institute Early Detection Research Network Program and by a fellowship for Franck Brichoy from the French Association for Cancer Research.