Tumor-infiltrating B Lymphocytes as a Potential Source of Identifying Tumor Antigen in Human Lung Cancer

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ABSTRACT

Functional studies using freshly isolated tumor-infiltrating B lymphocytes (TIB) are difficult to perform and interpret. Here we document a novel function of TIB using fresh human lung cancer tissues engrafted in SCID mice; they are at activated state and produce tumor-specific antibodies in tumor microenvironment: (a) TIB engrafted in SCID mice produced human IgG; (b) IgG derived from TIB highly bound intracellular and membrane-bound antigens of autologous cancer cells; and (c) less recognition of autoantigens on normal lymphocytes by IgG derived from TIB compared with IgG from the serum of the patient. On the basis of the novel findings presented in this study, we modified the original serological analysis of antigens by recombinant cDNA expression cloning in a patient with lung cancer who expressed unusually favorable clinical evolution and analyzed humoral immunity against identified mutated p53 antigen. This study provides the first demonstration that antibodies derived from TIB recognize tumor antigens by serological analysis of antigens by recombinant cDNA expression cloning methodology and circulating auto-p53 antibodies in sera derived from TIB in tumor microenvironment. Our approach using TIB may allow the identification of key antigens in the humoral cancer-related immune system.

INTRODUCTION

Interpretation of the specificity of an observed humoral immune response to cancer cells has always been a critical issue in human tumor immunology. The recent development of a general method to analyze the humoral immune response in cancer patients has provided a powerful new tool to detect the immune response to cancer. This method, known as SEREX (1,2), combines molecular cloning procedure of autologous cancer cells with patient serum including tumor-specific antibodies. More than 1000 antigens have thus far been identified in various malignancies, including lung cancer, by using SEREX (1–5); however, some of these antigens can be classified as naturally occurring autoantigens (3), because many gene products are recognized by the immune system of cancer patients including autoantigens expressed in normal cells and tissues.

Anti-p53 serum antibodies have been detected in up to 30% of individuals with cancer and a range of different tumors (6–8). Previous studies of the immune response against p53 in cancer patients have relied on serum analysis (9–11). These studies have yielded important information on the clinical significance, epitope dominance, and the role of protein overexpression in the development of anti-p53 immune response. However, several critical questions remain unanswered. Although the mechanisms underlying the generation of these anti-p53 antibodies are not fully understood, it seems that overexpression and subsequent release at minute amounts into the bloodstream are prerequisites for a B-cell response (12, 13).

Lymphocytes are the major components of mononuclear cell infiltrates in many solid tumors. The immune system is known to act against tumors, and the presence of TILs is considered to reflect a tumor-related immune response (14). In this regard, the majority of TILs are T cells, which play a key role in the local immune response at tumor microenvironment (15). However, B cells, plasma cells, and natural killer cells are also present in cancer tissues (16, 17). To our knowledge, functional analysis of TIBs in lung cancer has been reported in only a few studies because of difficulties in obtaining freshly isolated cells and long-term culture (18, 19). To investigate the potential role of TIBs as a source of cells-producing tumor-specific antibodies, we (18) and others (19) previously examined antibody production by TIBs in fresh human lung cancer tissues engrafted in SCID mice (coengrafting TILs and tumor cells without disrupting the microenvironment), and characterized the tumor specificity of these antibodies. We reported that TIB xenografts actively produced human IgG until 20 weeks after engraftment and seemed to survive exclusively within the xenografts (18). However, we examined the specificity of these antibodies using only Western blotting analysis.

Using two-color flow cytometry, we demonstrated in the present study that the expression level of cell surface functional molecules in TIBs was higher than that in peripheral blood B lymphocytes. To characterize antigens recognized by IgG produced by TIBs, we used modified SEREX methodology. We provide the first evidence for specificity of TIBs in lung cancer microenvironment and the possible role of TIBs as a source of tumor-specific antibodies in human tumor immunology.

MATERIALS AND METHODS

The study protocol was approved by the Human and Animal Ethics Review Committee of the University of Occupational and Environmental Health, Japan, and a signed consent form was obtained from each subject before taking tissue samples used in the present study.

Engraftment of Human Lung Tumor Tissue into SCID Mice. Female SCID mice (BALB/c×C57BL/6J-Scid-scid) were obtained from Charles River Inc. (Tokyo, Japan), and maintained in specific pathogen-free conditions throughout the study. Surgically resected specimens from patients with lung cancer (including A904) were used to prepare tissues for implantation as described previously (18). Engrafted SCID mice were bled by retro-oral venipuncture every 2 weeks, and the serum was collected (~200 μl from every venipuncture) for analysis of human immunoglobulin. These experiments were performed in duplicates in each case (i.e., same tumor tissue was engrafted in two different mice), and human immunoglobulin titers were measured by the latex agglutination method (18).

Patients and Collection of Sample Material. Samples were obtained from seven patients with primary lung cancer who underwent surgery at our department. None of the patients had received any anticancer treatment before surgery. The histopathological type comprised five adenocarcinomas, one squamous cell carcinoma, and one large cell carcinoma. Resected tumor tissues from the patients were obtained during surgery; TILs and PBLs were prepared...
as described previously (17, 20). One of our patients, Patient A904, a 51-year-old nonsmoker male, presented in September 1996 with a large cell carcinoma of the lung. No metastatic lesions were found in other locations, and the primary tumor (pt2; N0) located in the upper-middle lobe of the right lung was resected surgically. A904L cell line was established from the tumor (18, 21, 22). After the first operation, a metastatic tumor appeared in the right adrenal gland, which was subsequently resected in October 20, 1997. The patient received two courses of cisplatin combined with vindesin and mytomycin C, as an adjuvant chemotherapy. Serum samples from Patient A904 were collected at the time of surgery, as well as 3 months, and 2 years later, and stored at −20°C. Serum samples from SCID mice engrafted with the primary A904 tumor tissue were obtained as described previously (18). The patient shows no evidence of the disease at present.

**Antibodies and Reagents.** The following mAbs were used as purified immunoglobulins in cell surface analyses: CD19 was used as a marker of B cells, and HLA-DR, CD40, and CD80 were used for markers of activated B cells. CD19 mAb J4.119, HLA-DR mAb I3 (Beckman Coulter Company, Marseille, France), CD40 mAb LOB7/6 (Serotec, Oxford, United Kingdom), antilymphocytin mAb 10F7 (American Type Culture Collection, Rockville, MD), control murine IgG1 (Becton Dickinson, San Jose, CA), and CD80 mAb L307.4 (BD PharMingen, San Diego, CA). Isolated lymphocytes (2 × 10⁶) were stained by the mAbs.

**FACS Analysis and in Vitro Binding Assay.** Staining and flow-cytometric analysis of lymphocytes were conducted by standard procedures, as described previously (21, 23), using FACScan (Becton Dickinson, Mountain View, CA). Briefly, cells (2 × 10⁶) were incubated with specific mAbs and subsequently phycoerythrin-conjugated CD19 mAb J4.119 at saturating concentrations in FACS medium consisting of HBSS (Nissui, Tokyo, Japan), 0.5% human serum albumin (Green-cross, Osaka, Japan), and 0.2% NaN₃ (Sigma Chemical Co., Aldrich, St. Louis, MO) for 30 min at 4°C. After three washes in FACS medium, the cells were analyzed with FACScan.

In vitro binding assay has been described previously (24). We modified the assay as follows: briefly, cells (1 × 10⁶) were incubated with 4% paraformaldehyde for 20 min at room temperature in the dark. After centrifugation and decantation of the supernatant, FACS permeabilizing solution (Beckman Coulter Co.) was added to the cells and incubated from 10 min at room temperature in the dark. After three washes with FACS medium, cells were incubated with 2 μl of diluted serum for 90 min at 4°C. After washing twice with FACS medium, the cells were treated with 2 μl of FITC-conjugated F(ab')² fragment of rabbit anti-human IgG (Beckman Coulter Co.), then incubated for 60 min at 4°C. After three washes in FACS medium, the cells were analyzed with FACScan. Amplification of mAb binding was performed in a three-decade logarithmic amplifier. Quantification of cell surface antigens on one cell was performed using beads, QIFKIT (Dako Japan, Kyoto, Japan) as reported previously (21, 23).

**Immunoscreening and DNA Sequencing.** Total RNA was extracted from A904L cells. The cDNA libraries of these cells were constructed in a λ ZAP Express vector using a cDNA library kit (Stratagene, La Jolla, CA), and immunoscreening of the libraries was performed as described previously (2–5), with the following modifications. Serum from tumor-bearing SCID mice (primary engrafted A904 tumor tissue) were pooled and examined. The pooled serum was diluted (final dilution, 1:200) in Tris-buffered saline containing 1% BSA and 0.02% NaN₃, and used to screen A904L cells library (2.3 × 10⁹ plaque-forming units). Immunoreactive clones were subcloned, purified, and excised in vitro to pBK-CMV plasmid forms (Stratagene). Plasmid DNA was prepared using the Wizard Miniprep DNA purification system (Promega, Madison, WI). The inserted DNA was evaluated by EcoRI-XhoI restriction mapping, and clones representing different cDNA inserts were sequenced. The sequencing reactions were performed using ABI PRISM 310 (PE Biosystems, Tokyo, Japan) automated sequencers.

**Screening for p53 Mutations.** Total RNA extracted from A904L cells and A904-EBV cells was converted to cDNA using a First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Tokyo, Japan). The cDNAs were used as templates for PCR amplification using primers OKY95 (5'-ATGAAGCTCTCCAGAATGC-3') and OKY108 (5'-ACACATGTAGTTGATAGTG-3') for 40 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. PCR products were purified using QIA quick PCR purification kit (Qiagen, Tokyo, Japan) and sequenced.

**RESULTS**

High Expression of Functional Molecules on the Surface of TIBs. In the first step, we evaluated the expression of various cell surface molecules on TIBs and paired peripheral blood B lymphocytes from patients with lung cancer. Flow cytometric analysis showed that TIBs constituted about 4–16% of the total number of cells in seven lung cancers (data not shown). CD40 molecule was highly expressed on TIBs compared with matched peripheral blood B lymphocytes (Fig. 1). Among the screened molecules, CD80 and HLA-DR were also more highly expressed on TIBs than those on peripheral blood B lymphocytes (Fig. 1). These data suggested that TIBs were at activated state and might play an important role in immune responses at lung cancer microenvironment.

TIBs Engrafted in SCID Mice Produce Human IgG that Can Potentially Induce Tumor Regression. To investigate the functional significance of TIBs, we examined the production of human immunoglobulin by TIBs in fresh human lung cancer tissues engrafted in SCID mice as described previously (18). SCID mice engrafted with these tissues were bled by retro-orbital venipuncture every 2 weeks, and the blood was collected for analysis of human immunoglobulin. SCID mice inoculated with these tissues had high levels of human IgG in their sera, whereas control mice did not (18). As shown in Fig. 2, serum concentration of human IgG increased gradually after lung
cancer tissue A904 engraftment and reached a peak level at 6 weeks but decreased thereafter with the shrinkage of the tumor tissue. These results suggested that antibodies derived from TIBs might contribute to tumor regression. To determine whether the antibodies derived from TIBs have the specificity of cancer related humoral response, we used antibodies derived from TIBs (A904) in the following experiments, because we have autologous lung cancer cell line A904L. The clinical course of Patient A904 is shown in Fig. 6.

**IgG Derived from Lung Cancer Tissue of SCID Mice Avidly Binds to Autologous Lung Cancer Cells.** To characterize the function of antibodies derived from TIBs in SCID mice, we examined the recognition of autologous lung cancer cells by these antibodies using *in vitro* intracellular binding assay. The histogram in Fig. 3A shows that IgG derived from TIBs highly reacted with autologous lung cancer cells A904L at 1:200 serum dilution, but IgG derived from untreated mice did not (data not shown). At the same dilution. IgG derived from TIBs in SCID mice highly reacted with autologous A904L cells, whereas only marginal reaction was noted by IgG derived from A904 patient serum stored at the time of surgery (Fig. 3B). We also examined the recognition against normal cells using autologous lymphocytes to analyze the tumor specificity of antibodies derived from TIBs. Recognition of autologous lymphocytes by IgG derived from A904 patient serum stocked at the time of surgery was markedly higher than by IgG derived from A904 tumor tissue at the same dilution (Fig. 3C). Interestingly, extracellular binding also showed a high level of recognition of autologous A904L cells by IgG derived from A904 tumor tissue (Fig. 3D). These results indicated that antibodies derived from TIBs avidly bound to intra- and extracellular lung cancer antigens, and only marginally recognized autoantigens expressed in normal lymphocytes.

**Identification of Lung Cancer Antigens by IgG Derived from Lung Cancer Tissue.** We demonstrated previously the reactivity of IgG derived from TIBs to human lung tumors using Western blot
analysis (18). To confirm these findings, we assessed the identification of tumor antigens using SEREX methodology. A cDNA expression library of $2.3 \times 10^6$ primary clones was prepared from A904L cells, and phage plaques were immunoscreened with IgG derived from A904 tumor tissue at 1:200 dilution. A total of 39 positive cDNA clones, which had no false-positive clones encoding immunoglobulin gene fragments, were obtained and sequenced. Comparative analysis showed that these clones represented cDNAs from 37 distinct cDNA clones. A homology search through the Basic Local Alignment Search Tool database revealed that 35 cDNA clones corresponded to previously identified genes, including 11 cDNA clones of unknown function. Two cDNA clones were unknown genes, with no identity in BLAST or limited to short segments of known genes (Table 1).

Among of isolated cDNA clones, we were interested in the most frequently isolated cDNA clones with strong seroreactivity. These clones corresponded to the gene encoding p53 tumor suppressor gene with a single base substitution (CGC to CTC), which alters codon 158 from Arg to Leu (Fig. 4). Sequencing of cloned p53 reverse transcription-PCR products from autologous A904-EBV cells demonstrated wild-type p53 (data not shown). These results indicated that p53 was mutated in A904L cell line. The frequency of mutated p53 antigen response was examined among 11 normal adults and 16 lung cancer patients. Of 16 sera from lung cancer patients, 2 were reacted with the mutated p53 antigen, and all 11 of the normal adults sera were nonreactive (data not shown).

**DISCUSSION**

 Previous studies using human TILs have focused almost exclusively on static, in vitro phenotypic, or functional evaluation of either isolated mononuclear infiltrating cells or on histopathological and immunohistochemical analysis of tumor biopsy tissues (16, 26–28). The purpose of our study was to elucidate the immune response to tumor cells mediated by TIBs in lung cancer. The major findings of our study were as follows: (a) fresh human lung cancer tissues engrafted in SCID mice produced human IgG; (b) IgG derived from autologous TIBs highly bound intra- and extracellular antigens of autologous lung cancer cells; and (c) less recognition of autoantigens expressed in normal lymphocytes by IgG derived from autologous TIBs than from peripheral blood B cells. Considered together, these results indicate that TIBs can recognize lung cancer antigens and for mutated p53 antigen, which was recognized strongly by antibodies derived from TIBs (Fig. 5). Serum of Patient A904, which was collected and stored at the time of surgery, exhibited strong reactivity with mutated p53 antigen up to 1:2 $\times 10^4$ (Fig. 5). Although seroreactivity with the antigen diminished after the first operation, serum samples stored for 3 months and 2 years also showed reactivities with the antigen up to 1:100 (Fig. 6). These results suggested that the maintenance of humoral response against the antigen may require continuous presence of tumor as a source of the antigen. However, no recurrence was demonstrated in the 4-year period after last operation.

**FIG. 5. Seroreactivity of Patient A904 and SCID mice engrafted with A904 lung cancer tissue for mutated p53 antigen.** Assays are scored positive only if test clones are clearly distinguishable from the control phage. Serum dilutions are indicated. Representative data of five similar experiments are shown.

### Table 1 Genes isolated from large cell carcinoma of the lung

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<th>Genes</th>
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* Known genes; identity in database (BLAST), function is known.
* Unknown genes; identity in database (BLAST), function is unknown.
* Novel genes; no identity in database (BLAST).
produce tumor-specific antibodies. On the basis of the novel findings presented in this study, we modified the original SEREX design and reported the correlation between TIBs and circulating anti-p53 antibodies.

The search for antibodies that distinguish cancer cells from normal cells is one of the most challenging tasks in the field of cancer research (29–33). The screening of cDNA expression libraries derived from human tumors with autologous antibody (SEREX) has proven to be a powerful method for defining the structure of tumor antigens recognized by the humoral immune system (1). Because tumor tissue is used to prepare the library, there is no need to establish tumor cell lines. In addition, cDNA clones identified as positive by SEREX can be directly sequenced, thus providing immediate structural definition of the antigenic target. However, about 30–50% of antigens identified by SEREX are recognized as naturally occurring autoantigens expressed in normal cells and tissues (3). In fact, autologous serum of Patient A904 highly recognized autoantigens expressed in normal lymphocytes (Fig. 3B). On the basis of our findings, we modified the original SEREX design. The first modification involved establishment of an autologous lung cancer cell line A904L cells as the pure source of cDNA for SEREX analysis. Another modification involved preparation of IgG derived from autologous TIBs using a TIL/tumor graft system (coengrafting TILs and tumor cells without disrupting the tissue structure and the microenvironment) in SCID mice as the source of immune probes. As shown Fig. 3, IgG derived from cancer tissue acted as a potent powerful probe and showed stronger reactivity than those derived from the patient serum, which is used as a probe in the original SEREX. These results indicated that antibodies derived from TIBs have a high specificity and sensitivity against cancer cells, and our approach using the modified SEREX technique may allow the identification of key antigens that regulate local immune response at tumor microenvironment. Additional screening of antigens recognized by TIBs in Patient A904 is under way in our laboratory, and all of the identified antigens will be evaluated for their clinical significance.

Mutation of the p53 tumor suppressor gene has been observed in 50–60% of human cancers (34) and ~50% of non-small lung cancer (11). Among the isolated antigens in this study, the mutated p53 at codon 158 has been reported in 4 of 65 patients with non-small cell lung cancer (35), and the IARC TP53 Mutation Database indicated that 38 cases of the same mutation in 1893 lung tumors (including large cell carcinoma) had codon 158 mutation from Arg to Leu (36). Anti-p53 antibodies were detected in sera of patients carrying cancer with an accumulated p53 protein in the tumor or a mutant p53 gene, or both. Of those patients with a humoral response to p53, it has been found that most have antibodies against the immunodominant amino- and carboxyl-terminal regions, outside of the core DNA binding domain (13). The secondary structure of these regions remains unchanged in the mutant forms of the protein, suggesting that the immune response is more likely to be because of accumulation of the protein rather than because of the appearance of novel antigenic determinants induced by mutation. However, several studies have identified patients who have anti-p53 antibodies and yet have no identifiable accumulation of p53 in their tumors (9, 10). Whereas intratumoral p53 protein accumulation appears to be a prerequisite for production of anti-p53 antibodies, the exact mechanisms of the self-immunization process have not yet been clarified, and whether the type of gene mutation may influence the production of anti-p53 antibodies is still a matter of debate. Our study demonstrated that antibodies derived from TIBs represent a unique and valuable source of such circulating anti-p53 antibodies, and our strategy was for successful exploitation of this important resource. On the other hand, it has been reported that mutant p53 proteins elicit humoral and cellular immune responses (37, 38). Patients with cancer, bearing mutated and intracellularly accumulated p53 protein, produce anti-p53 antibodies reacting to both mutant and wild-type p53, and they also exhibit a T-cell proliferation response in vitro to wild-type p53 protein (39, 40). These reports suggest that the immune response against mutated p53 does not acquire tolerance, although p53 is a ubiquitously expressed protein. Our results showed circulating anti-p53 antibodies in sera derived from TIBs in the tumor microenvironment. It is likely that the low level of prolonged mutated p53 humoral response reflects persistent antigenic stimulation of B cells by tumor cells in Patient A904. In this regard, Mitsudomi et al. (41) reported that the majority of their 22 patients with lung cancer who had a high titer of p53 antibody preoperatively showed decline of the titer after surgery. They also demonstrated the lack of any relationship between clinical course and changes in antibody titer. To clarify these studies, close follow-up studies of our patient are necessary to adequately determine the clinical prognosis. Although we assessed the humoral immune response of TIBs in a single case in the present study, additional studies of the immune response of TIBs in a broader cohort of lung cancer patients with many clinical stage/grade, histopathology, immune status, and smoking status are necessary for full assessment of the significance of such antibodies produced in the tumor microenvironment.

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