Antibody Response to the Tumor-associated Inhibitor of Apoptosis Protein Survivin in Cancer Patients

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Abstract

Antibody reactivity against survivin, a recently identified tumor-associated protein, was determined in sera from patients with lung (n = 51) or colorectal cancer (n = 49). The same collection of sera was tested for the presence of antibodies against p53. Eleven sera from lung cancer patients and four sera from colorectal cancer patients reacted with purified recombinant survivin in an ELISA (21.6% and 8.2%, respectively), whereas four sera from lung cancer patients and nine sera from colorectal cancer patients contained anti-p53 antibodies (7.8% and 18.4%, respectively). The increase in prevalence when anti-survivin and anti-p53 antibodies were determined in parallel was statistically significant (29.4% versus 7.8%, P = 0.005 in lung cancer population; 26.6% versus 8.2%, P = 0.015 in colorectal cancer population). The high prevalence of anti-survivin antibodies makes these antibodies an attractive novel marker for the diagnosis of lung and colorectal cancer, particularly in patients lacking anti-p53 antibodies.

Introduction

Proteins associated with malignant transformation of cells such as overexpressed proto-oncogenes (1) or mutant tumor suppressor protein p53 can give rise to the production of autoantibodies (2–4) that could serve as parameters to monitor tumor progression (5). An important mechanism involved in cancer formation is the inhibition of apoptosis, which, by extending the life-span of cells, favors the accumulation of transforming mutations (6). Apoptosis pathways are effectively blocked by proteins belonging to the IAP1 family that directly inhibit caspase and pro-caspase molecules (7–9). Recently, survivin has been described as a structurally unique mammalian IAP molecule with a strictly regulated expression during ontogeny, as shown in mouse and man (10, 11). In contrast to other IAPs, survivin is undetectable in normal adult tissues, yet is abundantly expressed in fetal tissue and in a variety of human tumors including lung, colon, breast, prostate, pancreatic, and gastric carcinoma as well as in high-grade lymphomas and neuroblastomas (10–13). In a recently published extensive analysis of human transcriptomes (14), survivin transcripts attracted notice by their strong association with tumor expression of recombinant protein with a NH2-terminal 6xHis tag and the survivin-specific primers. The PCR product was ligated into the prokaryotic expression vector pQE30 (Qiagen), which allows the overexpression of recombinant protein with a NH2-terminal 6xHis tag and the subsequent purification using nickel-nitritotriacetic acid resin (Qiagen). The correct sequence of the cloned PCR product was examined using the ALF-express Auto Read sequencing kit (Pharmacia Biotech, Freiburg, Germany) with cyanine-labeled dATP and pQE30-specific primers. Overexpression in Escherichia coli and purification were performed according to the manufacturer’s protocol (Qiagen). Purity of the recombinant protein was determined by SDS-PAGE and Coomassie Blue staining.

ELISA. Purified recombinant survivin was diluted in 50 mM bicarbonate buffer (pH 9.5) to a final protein concentration of 5 μg/ml as determined by the Bradford assay (Bio-Rad, Munich, Germany). A recombinant His-tagged fragment (amino acids 285–475) of the melanoma-associated protein tyrosinase intended to be used as a control antigen was expressed, purified, and refolded applying the same procedures. The survivin and tyrosinase solutions were dispensed at 5 μg/ml into 96-well plates (100 μl/well) and incubated overnight at 4°C. After removal of the protein solution, plates were blocked with 5% skim dry milk solution (Sigma-Aldrich, Steinheim, Germany) in PBS for 1 h at 37°C (100 μl/well). The plates were washed five times with PBST. Serum samples diluted 1:100 in PBS were added at 100 μl precoated well. After 1 h, serum was removed, and the plates were washed five times with PBST. Each well was then incubated for 1 h with 100 μl of a 1:200 dilution of goat antihuman IgG (Fab’), labeled with horseradish peroxidase (Coulter Immunodiagnostics, Krefeld, Germany), washed five times with PBST, and developed by adding 100 μl of a tetramethylbenzidine solution [1 mg of tetramethylbenzidine (Sigma-Aldrich) diluted in 10 ml of 0.05 mM citrate buffer (pH = 5.0) with 0.006% H2O2]. After a 15-min incubation in the dark, the reaction was stopped with 100 μl of 0.25 mM H2SO4, and the absorbance at 460 nm was measured. All serum samples were run in duplicate and randomly distributed on the plates. Sera from cancer patients and sera from healthy donors were tested simultaneously.

ELISA for Detection of Anti-p53 Antibodies. Serum samples were assayed with solid-phase ELISA using purified recombinant wild-type p53 (Dianova, Hamburg, Germany) according to the manufacturer’s instructions. The specificity of this ELISA has been validated previously (16).

Preabsorption of Sera with Soluble Survivin. Aliquots of sera diluted 1:100 were incubated with 30 μg/ml soluble survivin for 1 h at 37°C and then assayed in a survivin ELISA as described above, with a slight modification. To enhance the strength of the signal, the peroxidase-labeled second antibody was diluted 1:100,000.

Western Blot Analysis. Recombinant survivin protein was separated by SDS-PAGE (10 μg/lane) and then blotted on a polyvinylidene difluoride membrane (PALL, Portsmouth, United Kingdom). The membrane was blocked with a 5% skim dry milk solution (Sigma-Aldrich) in TBS. After removal of the blocking solution, the polyvinylidene difluoride membrane was washed

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3 The abbreviations used are: IAP, inhibitor of apoptosis protein; PBST, 0.05% Tween 20 in PBS; TBS, Tris-buffered saline; TBST, 0.2% Triton X-100 in TBS.

We provide evidence that survivin is a target for antibodies that show a variable prevalence, depending on the type of cancer.

Materials and Methods

Sera. Blood samples from cancer patients and healthy blood donors were obtained with informed consent. After centrifugation, sera were aliquoted and stored at −20°C.

Preparation of Recombinant His-tagged Survivin. Survivin cDNA was obtained by reverse transcription-PCR using total RNA from Jurkat cells and survivin-specific primers. The PCR product was ligated into the prokaryotic expression vector pQE30 (Qiagen, Hilden, Germany), which allows the overexpression of recombinant protein with a NH2-terminal 6xHis tag and the subsequent purification using nickel-nitritotriacetic acid resin (Qiagen). The correct sequence of the cloned PCR product was examined using the ALF-express Auto Read sequencing kit (Pharmacia Biotech, Freiburg, Germany) with cyanine-labeled dATP and pQE30-specific primers. Overexpression in Escherichia coli and purification were performed according to the manufacturer’s protocol (Qiagen). Purity of the recombinant protein was determined by SDS-PAGE and Coomassie Blue staining.

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three times with TBS and cut into strips. Strips were incubated with either 1 ml of serum samples diluted 1:100 or monoclonal anti-His6-antibody (Qiagen) diluted 1:5000 in 3% skim dry milk solution (Sigma-Aldrich) in TBS, respectively. After a 1-h incubation at room temperature, serum was removed, and the strips were washed three times with TBST. Strips were then incubated for 1 h with either 1 ml of 1:2500 diluted goat F(ab’2) antihuman IgG labeled with horseradish peroxidase (Coulter Immunodiagnostics) or 1 ml of 1:2500 diluted rabbit antimouse immunoglobulin labeled with horseradish peroxidase (Dako, Hamburg, Germany) in TBS, respectively. Strips were washed three times with TBST and developed by adding 1 ml of a dianinobenzidine solution [4 mg of diaminobenzidine, 3 mg of NiCl₂, and 3 μl of H₂O₂ (all from Sigma-Aldrich) in 10 ml TBS]. The reaction was stopped by washing the strips with tap water.

**Results**

**Detection of Anti-Survivin Antibodies and Anti-p53 Antibodies by ELISA.** A total of 100 patients with either lung (n = 51) or colorectal (n = 49) cancer were recruited after histopathological confirmation of the tumor. Sera of young and healthy blood donors (n = 300) with an average age ± SD of 19 ± 0.83 years were selected to determine the cutoff for positivity in the ELISA. The average absorbance ± SD seen with the sera of blood donors was 0.048 ± 0.022. The cutoff for positive antibody reactivity against survivin was 0.114, which was defined as an absorbance greater than 3 SDs above the mean value of the controls (99.7 percentile). Sixty healthy blood donors matched for age and gender with the cancer population were selected as a control group.

Sera from 11 lung cancer patients (21.6%) and sera from four colorectal cancer patients (8.2%) were reactive with recombinant survivin in ELISA, whereas none of the control sera from healthy blood donors recognized survivin (Fig. 1). Two specificity controls were included. First, four sera from lung cancer patients as well as
four sera from colorectal cancer patients showing the strongest reactivities to survivin were assayed in an ELISA against the recombinant, His-tagged melanoma-associated tyrosinase that was prepared using the same techniques applied for the generation of survivin. The average absorbance ± SE of the lung and colorectal cancer sera was 0.034 ± 0.004. The average absorbance ± SE of the sera from young and healthy blood donors tested simultaneously was 0.037 ± 0.001 (P > 0.05). Second, the same sera were preabsorbed with recombinant soluble survivin before being tested in the survivin ELISA. Specificity of survivin recognition was evidenced by the marked decrease of signals in ELISA after preabsorption (Fig. 2).

For comparison, the same collection of sera was checked for the presence of anti-p53 antibodies. Four sera from lung cancer patients (7.8%) and nine sera from colorectal cancer patients (18.4%) were found to recognize p53 (Fig. 3). Control sera obtained from healthy blood donors failed to react with p53 (data not shown). The cumulative prevalence of both anti-survivin and anti-p53 antibodies reached 29.4% in lung cancer patients and 26.6% in colorectal cancer patients. The increase in prevalence when assaying anti-survivin and anti-p53 antibodies simultaneously was statistically significant [29.4% versus 7.8% (P = 0.005) in the lung cancer population and 26.6% versus 8.2% (P = 0.015) in the colorectal cancer population, respectively, using a one-tailed Fischer’s exact test; α = 0.05].

Detection of Anti-Survivin Antibodies Reactive against Denatured Recombinant Survivin Protein by Immunoblotting. The 15 sera recognizing survivin in the ELISA were tested against recombinant denatured survivin by immunoblotting. Under the conditions used, 6 of 15 sera (40%) recognized the M, 20,000 denatured recombinant protein survivin in Western blot analysis. No reactivity was found in any of 20 control sera from young and healthy blood donors. To illustrate the immunoblot analysis, Fig. 4 shows the staining pattern of sera from colorectal cancer patients, lung cancer patients, and healthy control donors. In addition, staining of blotted survivin by an anti-His6 antibody is also included.

Discussion

We have examined the presence of antibodies against survivin, a widely occurring tumor-associated protein, in sera from cancer patients. Using an ELISA technique, we show that an anti-survivin antibody response is detected in 21.6% of lung cancer patients and in 8.2% of colorectal cancer patients. Specificity of survivin recognition was confirmed by lack of reactivity with an unrelated recombinant tumor-associated protein prepared and tested using the same procedure described for survivin and by competing-out the anti-survivin reactivity by preincubation of the sera with soluble survivin.

The prevalence of anti-p53 antibodies found in this study in lung (7.8%) or colorectal cancer patients (18.4%) is in accordance with the results reported by others (5). An even higher prevalence was seen for anti-survivin antibodies in patients with lung cancer. In this context, it is interesting that particularly high levels of survivin expression have been found in lung cancer cell lines (8). Thus, survivin may be considered a major cancer antigen.

In lung cancer patients, none of the sera containing anti-p53 antibodies recognized survivin in an ELISA, and none of the sera containing anti-survivin antibodies reacted with p53. These data suggest that the prevalence of cancer patients exhibiting antibody reactivity against tumor-associated antigens is increased when both anti-p53 and anti-survivin antibodies are determined.

Six of 15 sera recognizing recombinant survivin in ELISA were found to stain for recombinant survivin in Western blot analysis, indicating that some sera contain antibodies that also react with denatured survivin. Why the majority of sera showing clear-cut reactivity in ELISA failed to stain survivin in immunoblotting analysis remains to be explained.

Anti-p53 antibodies have been reported to be detectable several years before the clinical manifestation of lung cancer (17). Therefore, whether anti-survivin antibodies could also serve as an early predictive marker in patients at high risk of developing cancer is an intriguing question. By screening a limited number of sera from patients collected at various time points before and after diagnosis of lung cancer, we found anti-survivin reactivity in two patients at 18 and 12 months, respectively, before clinical manifestation of the disease. These preliminary data have to be substantiated by expanding the analysis on a larger number of patients.

In summary, the results clearly show that anti-survivin antibodies can be detected in the serum of patients with lung or colorectal cancer. The high prevalence of anti-survivin antibodies makes these antibodies an attractive novel marker for the diagnosis of these cancers, particularly in patients lacking anti-p53 antibodies.

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