T Cell–Dependent Antibody Responses against Aberrantly Expressed Cyclin B1 Protein in Patients with Cancer and Premalignant Disease

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ABSTRACT

Purpose: Cyclin B1–derived peptides were shown by us to be targets of tumor-specific CD8+ T cells in patients with breast and head and neck cancer. We obtained further evidence of cyclin B1 immunogenicity and its potential to serve as a tumor-specific antigen by analyzing its ability to elicit T cell–dependent humoral immune responses in vivo in patients with different types of tumors.

Experimental Design: Recombinant cyclin B1 protein from two different sources was purified and used as antigen in ELISA assays to test sera from patients with breast, pancreatic, colon, and lung cancer for the presence of anti-cyclin B1 antibody. We also analyzed patients with benign lung disease, premalignant disease, and a known history of heavy smoking.

Results and Conclusions: Cyclin B1 elicits helper T cell–dependent antibody responses in vivo. Tumors with higher level of cyclin B1 expression elicit higher anti-cyclin B1 antibody levels. Antibodies in patients with breast and colon cancer are primarily of the IgG isotype whereas patients with pancreatic and lung cancer have in addition anti-cyclin B1 IgA. Cyclin B1–specific IgG was also detected in long-term smokers and in patients with preneoplastic lung disease. Immune responses to aberrantly expressed cyclin B1 in tumors and premalignant lesions should be further explored as diagnostic and prognostic markers, in addition to their immunotherapeutic potential.

INTRODUCTION

Cell division is a critical event in tumor progression and numerous molecules involved in this process have been the subject of intense investigation in tumor biology. Cyclins, molecules that control the progression through the cell cycle, have been shown to be overexpressed in various human cancers (1–6). Cyclin B1 is important in the cell cycle progression from G2-to-M phase (7). Correlation has been found between the extent of cyclin B1 overexpression in the primary tumor and clinical outcome for patients with cancer of the lung, esophagus, and tongue (8–12). We have a particular interest in cyclin B1 because we recently showed that cyclin B1 can serve as a tumor-specific antigen and that tumor-specific CTL derived from patients with breast and head and neck cancer recognize HLA-A2 presented peptides derived from overexpressed cyclin B1 protein (13). We furthermore showed that in addition to being overexpressed in tumors compared with normal proliferating cells, cyclin B1 is located in the cytoplasm rather than the nucleus, and this aberrant expression (overexpression and in the wrong location) is a result of p53 inactivation (14).

In this paper, we extend our published observations on cyclin B1 as a tumor antigen recognized by human cytotoxic T cells by studying in more detail its ability to elicit T cell–dependent humoral immune responses in patients with various types of tumors. Two previously published studies have shown that antibody against cyclin B1 can be found in patients with various tumors and might be a useful diagnostic marker in combination with antibodies against several other tumor antigens (15, 16). Focusing more closely on lung cancer, we show that antibody is present at both early and late stages of cancer, as well as in sera of heavy smokers that in their lungs have preneoplastic changes that overexpress cyclin B1.

MATERIALS AND METHODS

Patient Samples. Sera and tumor tissues from patients with breast, pancreatic, colon, and lung cancer were collected at the time of diagnosis, under the Institutional Review Board–approved protocol No. 93–34.

ELISA Assay. ELISAs for detecting anti-cyclin B1 antibody were done as previously described (17). Briefly, 50 μL of recombinant cyclin B1 protein (source and isolation procedure described below) at a concentration of 1 μg/mL in PBS were loaded into 96-well microtiter polystyrene base immunoassay plates (Dynex, Chantilly, VA), and incubated overnight at 4°C. Plates were washed with PBS and blocked with 2.5% bovine serum albumin in PBS for 1 hour. After washing, 50 μL of diluted serum were added and incubated for 1 hour at room temperature. Plates were washed with 0.1% Tween 20 in PBS, and the secondary antibody, ALP-labeled polyvalent goat anti-human IgM, IgG, and IgA (Sigma-Aldrich, St. Louis, MO) in 2.5% bovine serum albumin was added and plates incubated for 1 hour at room temperature. The plates were washed and filled with 100 μL per well of ALP substrate solution (Sigma-Aldrich). After 1 hour of incubation in the dark,
the reaction was stopped with 50 μL of 0.5 mol/L NaOH and the absorbance at 405 nm was measured using the plate reader MRX Revelation (Thermo Labsystems, Chantilly, VA). The isotype of the anti-cyclin B1 antibody was determined using the same method described above except that isotype-specific ALP-labeled goat anti-human IgM, IgG, and IgA antibodies were used as secondary antibodies in the ELISA.

Recombinant cyclin B1 protein was obtained from two different sources. Insect cells infected with recombinant baculovirus expressing human cyclin B1 were purchased from BD PharMingen (San Diego, CA). The cell pellet was lysed with a lysis buffer consisting of 1% CHAPS (Sigma-Aldrich), 150 mmol/L NaCl, and 20 mmol/L Tris-Cl (pH 8.0) in the presence of protease inhibitors (Aprotinin, leupeptin, pepstatin phenylmethylsulfonyl fluoride, EDTA, and iodoacetamide). The lysate was centrifuged at 10,000 × g for 20 minutes and the supernatant was run over a column of protein G-Sepharose (Amersham Biosciences, Piscataway, NJ) before it was applied to an affinity column containing anti-cyclin B1 antibody (BD PharMingen) covalently coupled to protein G-Sepharose. Cyclin B1 was eluted from the column in 1 mL fractions using 0.1 mol/L triethylamine (pH 11.5) into a tube containing the same volume of 1 mol/L Tris (pH 6.7), to neutralize the pH. For further purification, ion exchange chromatography was done using a Rainin HPLC separation system (Varian, Woburn, MA). Either lysate or affinity column products were dialyzed into HEPES-buffered saline (10 mmol/L HEPES and 150 mmol/L NaCl) and applied to a Mono Q anion exchange column (Amersham Biosciences). Fractions were eluted with a 30-minute linear gradient of 150 to 500 mmol/L NaCl, at a flow rate of 1 mL/min. To identify column fractions that contained cyclin B1, samples were run on a 10% SDS gel and analyzed for purity by Coomassie blue staining and for specificity by Western blot. The preparation was highly pure as judged by the lack of detection of other protein bands on the gel. When purified recombinant protein became available through Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), that was expressed in Escherichia coli, samples of positive and negative sera were retested and results confirmed with this new commercial preparation.

**Immunohistochemical Staining of Cyclin B1 in Tissue Sections.** Formalin-fixed, paraffin-embedded tissues were sectioned (3-5 μm), air-dried overnight at 37°C, deparaffinized and dehydrated, and stained with the mouse anti-human cyclin B1 antibodies:
B1 antibody, GNS-1 (BD PharMingen). The avidin-biotin peroxidase method was applied according to the manufacturer’s instructions supplied with the Vectastain ABC Elite staining kit (DAKO Co., Carpinteria, CA).

**Statistical Analysis.** A Student’s *t* test was used to detect significant differences in the ELISA assays between absorbance values in the cancer patients’ sera and healthy age-matched controls. Differences with *P* < 0.05 were considered significant.

**RESULTS**

**Anti-Cyclin B1 Antibodies of Different Isotypes Are Present in Sera of Patients.** Figure 1 shows ELISA results obtained on sera from 120 patients, 7 with breast cancer, 17 with pancreatic cancer, 27 with colon cancer, and 69 with lung cancer. The absorbance reading at 405 nm (*A*₄₀₅) corresponds to the amount of specific antibody present in the serum. The *A*₄₀₅ values of various sera ranged from 0.005 to 1.939. Values obtained with the sera from cancer patients were compared with values obtained on 27 healthy controls, 5 to 10 of which were included in every assay. To gain more insight into the similarity or differences between various cancer types and patients responses to cyclin B1, we divided all the antibody positive patients into “positive” (absorbance, 0.5000–0.999) and “strong positive” (absorbance, ≥1). Breast cancer patients were the best responders, 43% of the sera falling into the strong positive group, followed by colon cancer patients (22% strong positive) and pancreatic cancer patients (18% strong positive). The lowest level of antibody was found in lung cancer patients. We considered that this might be due to the stage of the disease at diagnosis, when the sera were collected. Among the lung cancer patients, we had 29 patients at stage I, 11 patients at stage II, 16 patients at stage III, and 4 patients at stage IV at the time of diagnosis. For nine patients, this information was not available. Figure 2A shows that there is no significant difference in antibody levels at different stages of disease. Antibody was found already at stage I, with a trend towards a slight increase with a more advanced stage II disease and then a slight decrease as the tumor progresses. In Fig. 2B, we show that both, non–small cell lung cancers as well as small cell lung cancers can elicit anti-cyclin B1 antibody responses.

We also compared antibody levels in sera from six lung cancer patients with recurrent (metastatic) disease with sera from 65 patients with primary disease (Fig. 3). With the caveat that the number of sera from recurrent disease is small, there seems to be a higher level of antibody present in these patients compared with patients sampled at the time of diagnosis of their primary tumors.

We did antibody isotype analyses on three strong positive sera from each tumor type (Fig. 4). In patients with breast and
colon cancer, the antibody response was primarily of the IgG isotype with low level or no IgM. In contrast, pancreatic cancer patients maintained significant levels of IgM and produced IgA antibody in addition to IgG. IgA isotype was seen in two lung cancer patients as well.

We were unable to obtain tumor sections from most of the patients whose sera we tested for the presence of anti-cyclin B1 antibody. However, in tumor sections that were available from four patients, there was a very good correlation between the level of cyclin B1 expression in tumor tissue (Fig. 5A) and the level of serum anti-cyclin B1 antibody (Fig. 5B).

**Cyclin B1 Overexpression, Antibody Production, and Smoking.** We also tested sera from 20 patients with benign lung disease (5 with granuloma, 6 with inflammatory diseases, 3 with benign tumors, 3 with interstitial lung disease, 2 with metaplasia, and 1 with dysplasia), divided into heavy smokers (n = 9; ≥40 pack-years) or light or nonsmokers (n = 11; <40 pack-years; Fig. 6A). The antibody level in the sera from heavy smokers was higher (absorbance, 0.469 ± 0.17) than in light smokers or nonsmokers (absorbance, 0.308 ± 0.128), and that difference was significant (P < 0.01). Immunohistochemical staining of sections of lung tissue from two heavy smokers, one diagnosed with a displastic lesion (patient 1) and the other with a metaplastic lesion (patient 2), showed that cyclin B1 protein was already overexpressed in both premalignant lesions to levels equal or higher to those seen in fully transformed cancer cells (Fig. 6B).

**DISCUSSION**

Cyclin B1 is an important molecule involved in the transition from G2-to-M phase of the normal cell cycle (7). It regulates cell division and cell arrest by cooperating with cdc2 kinase (18). The cyclin B1/cdc2 complex initiates chromosome condensation, destruction of nuclear membrane, and assembly of the mitotic spindle (19, 20). We and others have shown that cyclin B1 is overexpressed and mislocalized in the cytoplasm of many types of human cancers (4–6) and cancer cell lines (21). Kushner et al. showed that the level of cyclin B1 expression can correlate with staging of head and neck cancer (21), and a correlation between cyclin B1 overexpression and the proliferation marker Ki-67 has also been shown (10). Furthermore, it was reported that the level of expression of cyclin B1 can be correlated with disease prognosis in patients with lung, tongue, and esophageal cancer (10–12). Therefore, the deregulated expression of cyclin B1 seen in many cancers may be linked to carcinogenesis and tumor progression (22).

Recently, we discovered that aberrantly expressed cyclin B1 is recognized by human T cells as a tumor-specific antigen and that patients with breast and head and neck cancer have circulating CD8+ T cells specific for various peptides derived from this molecule. In the current study, we sought to determine if there were also specific antibody responses in patients with tumors known to overexpress cyclin B1. We found that cyclin B1 elicits helper T cell–dependent antibodies as evidenced by high frequency of isotype switching, indirectly demonstrating that in addition to previously detected cyclin B1–specific CTL, helper T cells can also be generated that are specific for this antigen and functional in providing help to B cells.

Whereas the differences in antibody levels and isotypes found in patients with different tumor types are not statistically significant, we nevertheless found them informative. For example, we find in general higher level of antibody in recurrent (metastatic) disease than in nonrecurrent disease. This may be related to our observation that metastatic cells express the highest levels of cyclin B1\(^1\) and thus provide the highest level of antigen. Most of the sera we tested from breast, pancreatic and colon cancer patients were derived in the setting of recurrent disease. The sera from lung cancer patients were primarily derived at the time of diagnosis of the primary disease, the majority being stages I and II.

We also found less isotype switching and highest levels of IgM in patients with pancreatic and colon cancer. This may be a direct result of the profound T-cell suppression in these two

\(^1\)Yu and Finn, unpublished.
tumor types that we and others have published on before (23, 24). In the absence of optimally functioning helper T cells, isotype switching would be expected to proceed at a much slower rate. In pancreatic and lung cancer patients, we also found IgA antibody. Pancreatic, lung, and other cancers produce transforming growth factor-β (24, 25) that is known to be the cytokine that induces isotype switching to IgA (26). Thus, whereas cyclin B1–specific helper T cells promote the switch from cyclin B1 IgM to IgG, switching to IgA may be also due to tumor-produced transforming growth factor-β.

Data presented here, showing that aberrantly expressed cyclin B1 can elicit T cell–dependent antibody responses, confirming and extending the previously published data by Koziol et al. (16), together with our previously published data on cyclin B1 specific human cytotoxic T-cell responses (13), validate the designation of this otherwise normal cell cycle regulatory protein as a tumor antigen.

Our finding that aberrant expression of cyclin B1 characterizes not only primary tumors but also metastatic tumors and premalignant lesions, suggests that it may be a good candidate antigen for cancer vaccines intended to prevent cancer recurrence or progression from premalignant disease to cancer. Detection of anti-cyclin B1 antibodies in long-term smokers and overexpression of this antigen in premalignant lesions, suggests that anti-cyclin B1 antibody may also serve as a serum marker for premalignant disease or for early detection of cancer.

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