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Advances in Brief

cdr2, a Target Antigen of Naturally Occurring Human Tumor Immunity, Is Widely Expressed in Gynecological Tumors[1]

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Abstract

The paraneoplastic neurological disorders provide perhaps the best known example of naturally occurring tumor immunity in humans. For example, patients with paraneoplastic cerebellar degeneration (PCD) appear to suppress the growth of occult breast or ovarian tumors that express a neuronal antigen termed cdr2. PCD patients harbor cdr2-specific CTLs in their peripheral blood, and these cells are likely mediators of the tumor suppression. Whereas cdr2 therefore appears to be the target of an effective immune response in patients with PCD, the general relevance to cancer patients has been unclear, due in part to reports indicating that cdr2 is not expressed in tumors obtained from neurologically normal patients. We have reexamined this question, and we find that cdr2 is widely expressed in such tumors, indicating that cdr2 is in fact an important tumor antigen in the general population of breast and ovarian cancer patients.

Introduction

Given recent efforts to boost the immune system for purposes of immunotherapy, identification and understanding of naturally occurring tumor immune responses in humans are increasingly important. One difficulty in this endeavor is that patients with successful immune responses to cancer are not likely to come to medical attention. An important exception is patients with PND.[3] In these individuals, it is believed that tumor cells expressing neuron-specific proteins successfully trigger an effective antitumor immune response. However, PND patients go on to develop an autoimmune neuronal degenerative disorder, which brings them to clinical attention. Studies of PND patients have led to insights regarding the body's ability to mount an immune response to tumor-restricted antigens (1, 2). For example, in patients with PCD, breast or ovarian tumors express a Purkinje neuronal protein termed cdr2 (3–5). Recently, cells capable of mediating tumor immunity (expanded populations of cdr2-specific CTLs) were detected in five of five HLA-A2.1$^+$ PCD patients (6, 7). In contrast, tumor-specific CTLs have not been found to be expanded in patients with actively growing tumors, even in cases where tumor-restricted antigens are known to be expressed (*e.g.*, the melanoma MAGE/MART antigens; Ref. 8). Studies on PCD patients also demonstrated that dendritic cells were capable of cross-presenting tumor antigen from apoptotic cells, resulting in the activation of class I-restricted tumor-specific CTLs (7) and suggesting that apoptotic tumor cells may be an important source of antigen that triggers tumor immunity. In this study, we examine whether the cdr2 antigen is expressed in tumors obtained from neurologically normal breast and ovarian cancer patients.

Materials and Methods

Reagents. Reagents were obtained from Sigma unless otherwise noted.

Tissue Lysate Preparation. Tumor samples and pathology reports were obtained from the Memorial Sloan-Kettering Cancer Center Tissue Procurement Service. Tissue lysates were prepared by pulverizing tissues in a liquid nitrogen-cooled mortar and pestle, followed by homogenization in lysis buffer [10 mM Tris-HCl (pH 7.4), 15 mM EDTA, 50 mM KF, 50 mM NaH_2PO_4, 10 mM Na PP_i, and 1% NP40 with Complete protease inhibitors (Boehringer Mannheim)] using a Wheaton homogenizer. Protein concentrations were determined by Bio-Rad Protein Assay using BSA as a standard. Purkinje cell extract was prepared as described previously (5). Normal ovary extract was purchased from Clontech.

Preclearing Tumor Lysates. Tumor lysates were microfuged at 10,000 rpm for 10 min, adjusted to 375 μg of protein in 0.55 ml of lysis buffer, and cleared of IgG three times by adding 75 μl of a 50:50 protein A-Sepharose: protein G-Sepharose bead slurry (prepared as a 50% slurry in PBS), followed by shaking at 4°C for 1 h. The final supernatant was analyzed by SDS-PAGE or IEF/SDS-PAGE.

SDS-PAGE and IEF/SDS-PAGE. For one-dimensional analysis, 10% SDS-PAGE gels were prepared and run by standard methods. Twenty μg of protein were loaded per lane, and gels were transferred to nitrocellulose (S&S BA85) for Western blotting.

For two-dimensional gel electrophoresis, tumor lysate or mouse cerebellar lysates were diluted 1:1 with two-dimensional lysis buffer [9.5 M urea (Pharmacia Biotech PlusOne), 2% NP40 (Calbiochem), 5% 2-mercaptoethanol (Pharmacia PlusOne), and 2% Biolyte ampholytes (Bio-Rad) consisting of 75% 3/5 range and 25% 3/10 range Biolytes). IEF gels were performed by the method of O'Farrell (9). IEF slab gels [0.75 mm; 9.2 M urea, 4% acrylamide (Ready Sol IEF; acrylamide:bis ratio of 19:1; Pharmacia), 2% NP40, and 5% Biolyte ampholytes] were loaded with 40 μg of total protein per lane, and the sample was overlaid with buffer (7% urea, 2.5% ampholytes, and 5% 2-mercaptoethanol). The gels were run using 0.01 M H_3PO_4 and 0.02 M NaOH buffers at 4 W for 1800 V/h. Lanes were equilibrated in SDS sample buffer for 5 min and loaded horizontally (with the acidic end on the right) on 1 mm 10% SDS-PAGE gels prepared with a 1-cm flat 3% acrylamide stacking gel.

Biotinylation of PCD Sera. Antibodies were purified from PCD patient and normal human sera, adjusted to pH 8.0 with 1.0 M Tris, on protein A-Sepharose (Sigma) columns, and eluted with 100 mM glycine (pH 3.0) into 0.10 volume 1 M Tris (pH 8.0) to neutralize them. Immunoglobulin-containing fractions were identified by Bio-Rad protein assay and pooled. Pooled antibody was dialyzed against 0.1 M sodium borate buffer (pH 8.8). *N*-Hydroxysuccinimide biotin at 10 mg/ml in DMSO was added at a ratio of 150 μg ester/mg antibody and incubated at RT for 4 h. Twenty μl of 1 M ammonium chloride were added per 250 μg of ester for 10 min at RT. Antibody was dialyzed against PBS extensively to remove uncoupled biotin and stored at a final concentration of 3 mg/ml IgG at 4°C.

Western Blotting. Filters were blocked in 5% nonfat dry milk/PBS (milk block) for 1 h at RT and probed with human sera at a 1:300 dilution overnight at 4°C or for 1–2 h at 25°C. Filters were washed according to Amersham enhanced chemiluminescence protocols and incubated with HRP-conjugated antihuman or mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.; 1:5000) or blotting grade avidin-HRP (Bio-Rad; 1:3000) for 1 h

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[3] The abbreviations used are: PND, paraneoplastic neurological disorder; PCD, paraneoplastic cerebellar degeneration; POMA, paraneoplastic opsoclonus-myoclonus; IEF, isoelectric focusing; RT, room temperature; HRP, horseradish peroxidase.

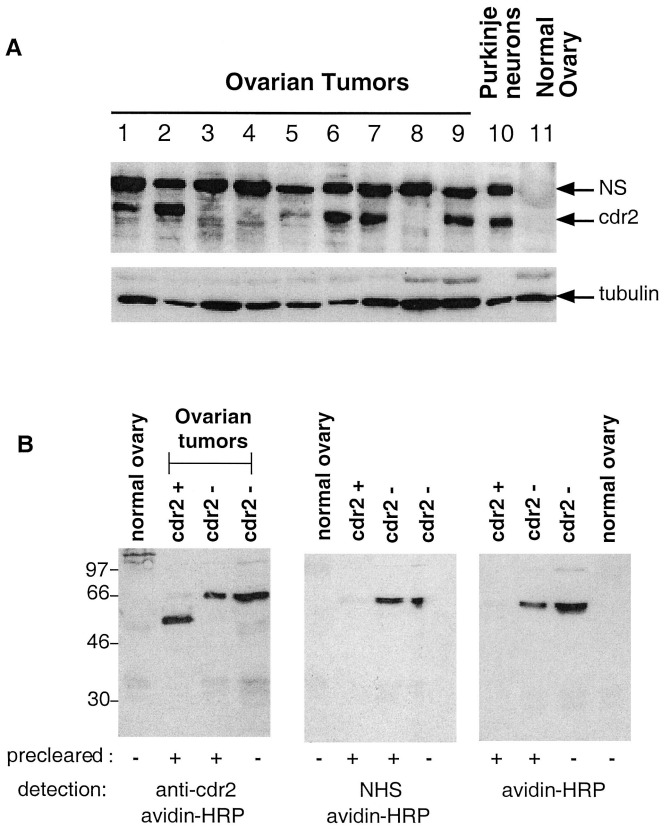

Fig. 1. Expression of cdr2 in ovarian tumors. *A*, protein extracts from nine human ovarian tumors were run on Western blots and probed with biotinylated PCD antisera. Strong cdr2 reactivity was evident in tumors 1, 2, 6, 7, and 9 as well as in extracts of human Purkinje. An anti-tubulin antibody was used as a loading control. An upper band present in Purkinje extracts and ovarian tumors (*NS*) was further characterized (*B*). Identical cdr2 reactivity was seen in two independent experiments in a total of 13 of 21 ovarian tumor specimens (Table 1; data not shown). Each tumor that did not express cdr2 was re-evaluated by Western blot analysis of an independent fragment of the tumor, which confirmed the lack of cdr2 reactivity (data not shown). *B*, extracts of normal ovary and cdr2-positive and cdr2-negative ovarian tumors were either run directly on SDS-PAGE or first precleared of IgG with protein A and protein G beads, as indicated. Western blots were probed with biotinylated PCD antisera (*anti-cdr2*) or normal human sera (*NHS*), followed by avidin-HRP or, as a control, avidin-HRP alone. A M_r 52,000 antigen was specifically recognized by anti-cdr2 antisera, and an unrelated M_r 69,000 band reacted with avidin-HRP alone. Preclearing with protein A/G depleted a faintly detectable M_r 50,000–55,000 species in the cdr2-negative uncleared lane of the anti-cdr2 blot (compare *Lanes 3* and *4* of the *left panel*).

at RT. After a second wash, reactive proteins were detected according to the enhanced chemiluminescence kit protocol.

Anti-β-tubulin antibody (Boehringer Mannheim) was used according to the manufacturer's specifications. Biotinylated antibodies were used at 30 μg IgG/ml milk block.

Results and Discussion

Whereas PCD presents an important example of tumor immunity, its general relevance to cancer patients has been questioned following reports that the cdr2 antigen is only expressed in tumors obtained from PCD patients (4). We have reexamined the relevance of cdr2 as a tumor antigen by carefully evaluating cdr2 expression in gynecological tumors obtained from neurologically normal cancer patients. Whereas cdr2 mRNA is widely expressed, the protein is absolutely restricted at a posttranscriptional level to expression in the brain and testis (5). Therefore, we have examined cdr2 antigen expression by Western blot analysis. Initial attempts to separate cellular proteins using SDS-PAGE gels failed because of high levels of IgG present in the tumors, which reacted with the antihuman secondary antibody and obscured cdr2 reactivity at M_r 52,000. To overcome this problem, lysates of tumor specimens were depleted of IgG by incubation with protein A and protein G coupled beads, run on SDS-PAGE gels, probed with biotinylated sera, and visualized with HRP-avidin as a secondary reagent. Using this method, we were able to clearly identify a cdr2-reactive band (M_r 52,000) that was not detected when probing with normal human serum or secondary reagents (Fig. 1*B*). A M_r 69,000 band evident in some samples was reactive with HRP-avidin alone and was therefore unrelated to cdr2 (Fig. 1*B*, *right panel*).

Primary ovarian tumors from 21 neurologically normal individuals were examined (Table 1*A*). Fig. 1*A* shows representative results from nine tumors. Human Purkinje cell extract served as a positive control, and normal ovary tissue served as a negative control. Five of nine tumors clearly express a M_r 52,000 antigen, which comigrates with the cdr2 from Purkinje cells (Fig. 1*A*). To confirm that the reactive antigen is cdr2, mouse cerebellum and ovarian tumor extracts were resolved side by side on two-dimensional gels (Fig. 2*A*). The reactive species at M_r 52,000 exactly comigrated by isoelectric point as well, indicating that the ovarian tumors express cdr2.

Because PCD antisera from any one patient is a hyperimmune polyclonal serum that could contain multiple antibodies, we probed cerebellar extract and tumor samples with several different PCD sera. As a negative control, antiserum from a patient with a distinct PND, POMA, was used. In cerebellum, three different PCD antisera were strongly reactive with the M_r 52,000 species on two-dimensional gels (Fig. 2*B*). In contrast, POMA antiserum as reactive with the characterized Nova antigens (10) in the cerebellar extract (M_r 46,000–60,000 on *Lane 1-D*) but were not reactive with the cdr2 spot. When ovarian tumor extract was run on five duplicate two-dimensional gels, two different PCD antisera detected cdr2, whereas unrelated antisera did not detect cdr2 (Fig. 3).

We also examined nine breast tumor samples from neurologically normal individuals. Two of nine tumors were clearly cdr2 positive (Table 1). Again, expression was determined using two-dimensional gels and multiple PCD antisera and negative controls, confirming that the antigen was cdr2 (Fig. 3; data not shown).

Our results demonstrate that a significant subset of breast and ovarian tumors up-regulates cdr2 expression. Given the recent observation that cdr2 interacts with c-myc (11), the prevalence of cdr2 expression in gynecological tumors may be relevant to tumorigenesis. Additionally, this finding has great immunological significance because it establishes cdr2 as a potential target tumor antigen for the treatment of neurologically normal patients with breast and ovarian cancer. The finding that PCD patients harbor cdr2-specific CTLs that are capable of targeting tumor cells expressing cdr2 epitopes underscores the importance of cdr2 as a potent tumor antigen. An additional question raised by the data presented here is whether some patients with cdr2-positive gynecological tumors might harbor cdr2-specific CTLs and thereby have an improved clinical outcome. This suggestion stems in part from the finding that 15% of neurologically normal

Table 1 *cdr2 expression in gynecologic tumors*

The indicated tumor samples were analyzed by SDS-PAGE and probed with PCD antisera. cdr2 reactivity was confirmed by two-dimensional IEF/SDS-PAGE and by probing with multiple PCD and negative control antisera.

Tissue diagnosis	Tumor description	No. of tumors expressing cdr2
Ovarian cancer	Epithelial	13/20
	Stromal	0/1
	Met[a] to mesentery	0/2
Breast cancer	Primary tumor	2/9
	Met to ovary	0/1
Fallopian tube cancer	Primary tumor	1/1
Small cell lung cancer	Met to ovary	0/1
Colon cancer	Met to ovary	0/1

[a] Met, metastasis.

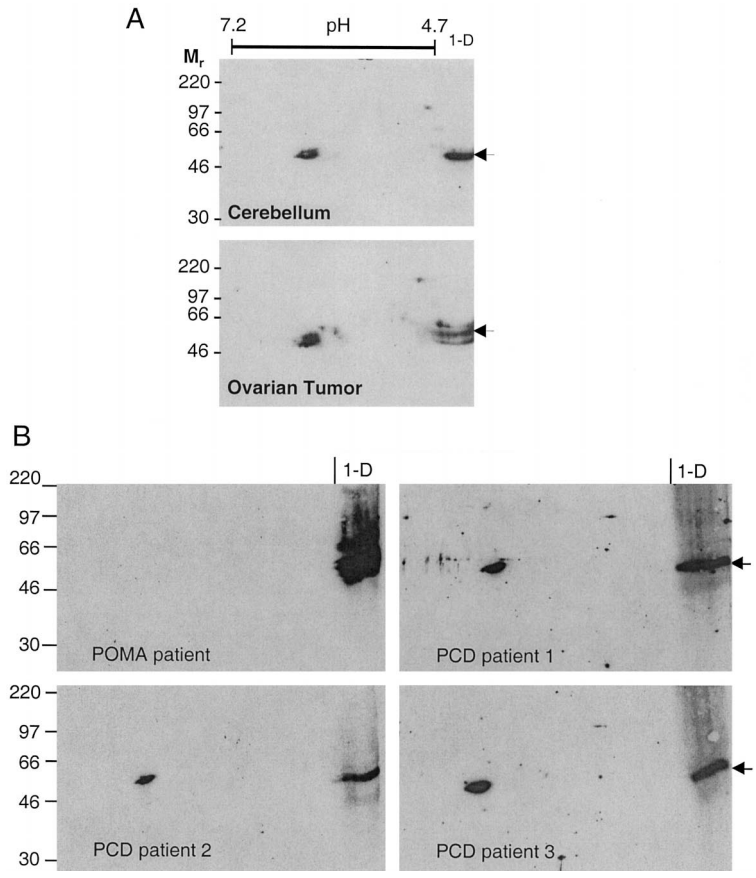

Fig. 2. IEF-SDS/PAGE analysis of cdr2 in cerebellum and ovarian tumor. *A*, extracts of mouse cerebellum and human ovarian tumor were run side by side on two-dimensional gels and probed with PCD antisera and HRP-antihuman IgG secondary antibody. Tumor or cerebellar lysate was loaded in the *right-hand lane* as a control (*1-D*). An immunoreactive band of identical molecular weight and isoelectric point is present in both extracts. The mouse cdr2 cDNA encodes a protein that is 87% identical with human cdr2, and this protein migrates identically with cdr2 detected in human Purkinje extracts (data not shown). *B*, mouse cerebellar extract was resolved on four identical two-dimensional gels and probed with three PCD antisera or antiserum from a POMA patient, which recognizes a characteristic set of M_r 52,000–69,000 bands. A sample of extract was loaded in the *right-hand lane* as a control (*1-D*).

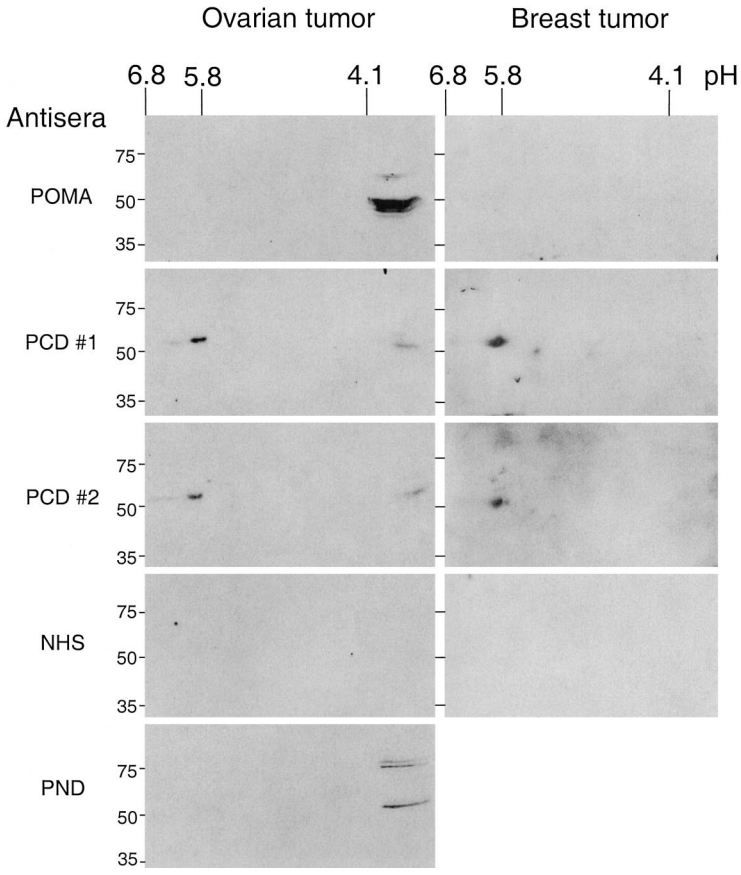

Fig. 3. The cdr2 antigen in ovarian and breast tumors is recognized by multiple PCD antisera. Lysates from an ovarian tumor and a breast tumor were run on duplicate two-dimensional gels, transferred to nitrocellulose, and probed with two different PCD antisera. As controls, blots were probed with normal human serum or POMA or an uncharacterized PND antiserum that recognizes distinct antigens (see *Lane 1-D*) but fail to react with cdr2. Breast tumor gels were not loaded with one-dimensional samples. Identical results were seen with two independent breast and ovarian tumors, four different PCD antisera, and four different hyperimmune control antisera (data not shown).

small cell lung cancer patients have low titer antibodies to the Hu onconeural antigen, and this evidence of an immune response correlates with an improved clinical course (12, 13). Notably, all small cell lung cancers express the Hu onconeural antigen (14). This observation, together with our finding of cdr2 expression in a significant percentage of gynecological tumors, suggests that tumor cell expression of onconeural antigens is necessary but not sufficient for the induction of antigen-specific tumor immune responses.

Several factors may contribute to the development of PND antigen-specific immunity, including tumor cell expression of MHC class I (demonstrated in a high percentage of PND-associated tumors; Ref. 15); the proximity of dendritic cells to apoptotic tumors, which may be important in tumor antigen presentation (16); and the lack of FasL or other suppressive molecules on the surface of the tumor cell (17). Analysis of cdr2-expressing gynecological tumors as well as an investigation of immunity to cdr2 in PCD patients may determine which, if any, of these factors may be important to the development of naturally occurring tumor immunity.

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