Isolation of Human Antibodies against the Central DNA Binding Domain of p53 from an Individual with Colorectal Cancer Using Antibody Phage Display

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ABSTRACT

Purpose: Serum anti-p53 antibodies are detected in ~30% of patients with cancer, and most of these antibodies are directed against the NH2- and COOH-terminal domains of p53. The aim of this study was to identify individuals with serum reactivity against the central and COOH-terminal domains of p53 and to isolate and characterize these recombinant human antibodies.

Experimental Design: The serum of individuals with colorectal cancer was screened for the presence of anti-p53 antibodies. Antibody phage display libraries were constructed from four immunoreactive individuals, and the libraries were panned against the central and COOH-terminal domains of p53.

Results: An antibody fragment (1159.8) that was specific for the whole molecule as well as the central domain was isolated from one of the libraries. The serum from the original individual inhibited the binding of this Fab fragment to p53, thus indicating that the antibody reflected the specificity of the in vivo immune response. The VL and VH genes from Fab 1159.8 matched germ-line genes from the VH3 and VK2 families, respectively. Competition analysis with monoclonal antibodies showed that Fab binding could be inhibited most effectively with DO11 and, to a lesser extent, Pab240, indicating an epitope within or adjacent to residues 181–190 of p53.

Conclusions: The successful isolation of this human anticalcenal domain Fab provides additional insight into the nature and specificity of the tumor-specific immune response. This antibody could be used as a reagent for functional studies of p53, or it may be a candidate for use as an anticancer idiotypic vaccine.

INTRODUCTION

p53 is an important tumor suppressor protein that regulates the transcription of genes involved in cell growth arrest (1), apoptosis (2), and DNA repair (3). Mutations in the p53 gene are seen in one-half of all cancers and in up to 70% of colorectal cancers. The large majority of mutations are found in the central DNA binding domain of the molecule and are usually missense mutations (4, 5). They result in the expression of a structurally distinct and nonfunctional protein, which accumulates in the nucleus of the cell (6, 7).

Serum antibodies against p53 have been detected in up to 30% of individuals with cancer (8–12) and are usually associated with the accumulation of the mutant p53 protein in the tumor (10, 13–15). It has been demonstrated by Pepscan analysis that serum anti-p53 antibodies react primarily with the NH2-terminal (~90% of reactive patients) and COOH-terminal domains (~50% of reactive patients) of the molecule, and few individuals have antibodies to the central DNA binding domain (~5%; Ref. 16). Within the three domains, most antibody binding is restricted to distinct immunodominant regions including residues 10–25 and 40–50 in the NH2-terminal domain; 345–393 in the COOH-terminal domain; and 120–130, 200–210, and 285–295 in the sequence-specific DNA binding domain (16). Interestingly, the majority of murine anti-p53 antibodies also react with linear epitopes in the NH2- and COOH-terminal domains of p53, an epitope specificity that mirrors that of the human serum response (17).

The detection of serum IgG anti-p53 antibodies provides indirect evidence that the immune response to p53 is antigen driven and involves T-helper cells. The analysis of serum does not, however, provide detailed information on the epitope specificity, V gene usage, or affinity of these antitumor antibodies. The use of recombinant human antibody techniques has recently provided insight into the molecular basis of the humoral immune response to many diseases, including cancer. Human antibodies have been isolated against a number of tumor antigens, including p53 (18), and these human anti-p53 antibodies were shown to bind with high affinity to an epitope between residues 27 and 44 of the NH2 terminus of p53. Furthermore, the antibodies were derived from highly mutated V genes from a single heavy (VH41) and light chain (Vκ4) combination. To date, no other naturally occurring anti-p53 antibodies have been isolated, as such; the relationship between gene usage restriction and the specificity of the p53 immune response remains unknown.

Recent studies have also shown that murine anti-p53 antibodies can be used as idiotypic vaccines (19, 20). Specifically, the immunization of mice with the central domain reactive antibody Pab 240 was shown to induce idiotypic networks that reduced tumor growth in mice challenged with the tumor cell lines MethA and 3LL. Immunization with other murine antibodies specific to the wild-type conformation or NH2-terminal domain of p53 were less successful in protecting mice from the same tumor challenge. These studies have renewed interest in isolating novel antibodies specific to the central domain of p53, because these could be used as idiotypic vaccines. In this study,
we use phage display techniques to isolate such antibodies from the repertoire of human cancer patients, and specifically to isolate anti-p53 antibodies that have specificity for the central DNA binding domain.

MATERIALS AND METHODS

Sample Collection and Patient Details. After obtaining informed consent, blood and tissue samples were collected from individuals who were undergoing resection of colorectal cancer at St Vincent’s Hospital between 1993–1997. Individuals were included in this study where pericolic lymph nodes and matched serum were available. There were 93 individuals in the study; 38 were female and 55 were male. The ages ranged between 37 and 89 years, with a mean of 67 years. There were 11 individuals with tumors classified as Dukes A, 38 as Dukes B, 31 as Dukes C, and 13 as Dukes D.

Clotted blood was centrifuged at 2000 × g for 10 min, and serum was stored in aliquots at −70°C before use. The pericolic lymph nodes were stored in liquid nitrogen before RNA extraction by the method of Chomczynski (21). Serum from a panel of volunteers without cancer was collected under the same conditions and used as controls (22).

Immunohistochemical Detection of p53. Sections of paraffin-embedded tumor tissue from each individual were analyzed for p53 expression, as previously described (15). Tumor tissue was considered to have accumulated mutant p53 when the average of 10 high-powered fields showed >5% of tumor cells with nuclear staining in the absence of staining in the stromal cells and normal epithelium.

Construction of Central Domain and COOH-terminal Clones. Specific primers were used to amplify the central domain (amino acids 95–200: cenfor, 5′-GGCCCCCATATGCTTCTGTCCTTCCCAG; cenrev, 5′-AGTCATAGTGCTCACAGCTGTCGTCAGGCTC) and COOH-terminal region (amino acids 195–293: carboxfor, 5′-GAGACCATATGACAGGAAACAGAATCTC; carboxrev, 5′-AGTCATAGTGCTCACAGCTGTCGTCAGGCC) of p53 using wild-type p53 as the template. PCR product (1 μg) was digested with Ndel (1 u/μl) and cloned into the Ndel site of the pET 19b vector (Novagene, Madison, WI). The nucleotide sequence of the positive clones was sequenced, and recombinant protein was expressed in BL21(DE3). Each domain was purified by immobilized metal affinity chromatography, as previously described (15). SDS-PAGE and Western blot analysis, using the p53 monoclonal antibodies Pab 240 and Pab 421, were used to confirm the identity of the domains.

Serum ELISA. Wells of a microtiter plate (Polyisorb; Nunc, Roskilde, Denmark) were coated with purified recombinant p53 (5 μg/ml in PBS), central domain (2.5 μg/ml in PBS), or COOH-terminal domain (5 μg/ml in carbonate buffer) overnight at 4°C. Coated wells were washed three times each with 200 μl of PBS and then blocked with PBS/2% BSA for 1 h at room temperature. Patient serum samples (n = 93) were diluted 1:100 in PBS and then applied in duplicate to the coated plates for 1 h at room temperature. Bound antibodies were detected with an alkaline phosphatase conjugated goat antihuman IgG Fe-specific antibody (0.5 μg/ml in PBS/2% BSA; Jackson Immuno Research Laboratory, Inc., West Grove, PA). The forty serum samples with the highest reactivity in the primary screen against each antigen (whole p53, central domain, and COOH-terminal domain) were rescreened in a second ELISA and ranked according to reactivity. On the basis of cutoffs in previous studies (15, 18), a total of 19 patients (20%) were considered reactive to whole p53.

The reactivity of patient serum against central domain and COOH-terminal domain was ranked and reassessed by immunoprecipitation. Patients were considered reactive to these domains if they were reactive by immunoprecipitation and reactive by the domain-specific ELISA.

Immunoprecipitation of p53 and Central Domain Using Serum. Equimolar amounts of pure central domain (100 ng), whole p53 (200 ng), or COOH-terminal domain (80 ng) in 200 μl of 2% (w/v) BSA in PBS was added to 2 μl of serum, and the solution was mixed for 1 h at 4°C. Protein A-Sepharose (20 μl of packed Sepharose) was then added for an additional 1 h at 4°C, and the samples were washed four times with PBS and then resuspended in 20 μl of PBS. The Sepharose was reduced and denatured with a protein running buffer [50 mm Tris-HCl (pH 6.8), 5% β-mercaptoethanol (v/v), 2% SDS (w/v), 10% glycerol (v/v), and 0.1% bromphenol blue] at 95°C for 10 min, centrifuged at 13,000 × g for 30 s, and then run on 4–20% PAGE. The gel was electroblotted onto PVDF and then blocked with 10% (w/v) SMP/PBS (22). Electrophoretically transferred gels were immersed in immunoprecipitations of whole p53, central domain, or the COOH-terminal domain were probed with monoclonal antibody 1801 (0.1 μg/ml in 10% SMP/PBS), Pab 240 (0.1 μg/ml in 10% SMP/PBS), or Pab 421 (0.1 μg/ml in 10% SMP/PBS), respectively. After washing five times with 0.1% (v/v) Tween 20/PBS, HRP-conjugated goat antiserum (0.05 μg/ml in 10% SMP/PBS) was added for 1 h at room temperature before three to six washes with PBS. A chemiluminescent substrate (DuPont NEN, North Sydney, Australia) was used in the detection of immunoprecipitated antigen. The serum of an individual without cancer was used as a negative control in addition to that of an individual known to have p53 antibodies to the NH2-terminal region of the molecule but not to the central domain (18).

Library Construction and Biopanning. Antibody Fab libraries were constructed from lymph node RNA as described previously (18). Briefly, immunoglobulin genes were amplified by reverse transcription and PCR using primers specific for human κ, λ, and IgG immunoglobulin genes (18, 23, 24). The products were then cloned into the phage display phagemid vector, MCO1, to give the primary antibody phage library. The size of the library was calculated as the proportion of clones with unique heavy and light chain as identified by BstNI mapping and diagnostic PCR (18).

Panning was carried out as described previously with pure recombinant central (0.1–1.0 μg/ml) or COOH-terminal domain (0.1–10 μg/ml) coated onto the wells of a microtiter plate. Binding phage antibodies were eluted with 0.1 M glycine (pH 2.5) for 10 min.

Fab ELISA. Expression of Fab and the ELISA screening of Fabs after panning was performed as described previously.

2 The abbreviations used are: SMP/PBS, skim milk powder/PBS; HRP, horseradish peroxidase.
Human Antibody to the Central Domain of p53 was used to compare serum p53 antibody status with age. Clones identified as being positive in the primary screen were grown on L agar plates with 2% glucose and carbenicillin and retested as described above. The cross-reactivity of Fab's with other antigens was assessed by ELISA using the following antigens: insulin (5 μg/ml), ErbB2 extracellular domain (5 μg/ml), CEA (5 μg/ml), EPF (1 μg/ml; gift from Alice Cavanagh, Royal Brisbane Hospital, Brisbane), bovine submaxillary mucin (1 μg/ml; Sigma Chemical Co., Aldrich, Australia), and keyhole limpet hemocyanin (1 μg/ml; Sigma Chemical Co., Aldrich, Australia).

Competition analysis using excess soluble central domain, COOH-terminal domain, or whole p53 was also used for additional confirmation of the antibody specificity. In this ELISA, the Fab samples were preincubated with excess antigen (20 μg/ml) for 1 h before being added to the wells of the microtiter plate.

### RESULTS

#### The Detection of Serum Antibodies against p53.

Serum from 8.6% (9 of 93) of individuals were found to be reactive with the central domain of p53; 5.5% (5 of 93) had reactivity with the COOH-terminal domain, and 20% had reactivity with whole p53. Of note, not all individuals with central domain antibodies were reactive to whole p53 as assessed by ELISA and immunoprecipitation experiments. In fact, 3 of 9 individuals (2545, 2759, and 2989) with central domain antibodies were nonreactive to whole p53 by ELISA, and 2 of 9 (2545 and 2759) by immunoprecipitation (Fig. 1). The serum of all individuals reactive to the COOH-terminal domain was reactive with whole p53 by ELISA and immunoprecipitation (data not shown).

#### Clinicopathological Correlates.

There was no relationship between the presence of p53 antibodies (whole p53, central domain, or COOH-terminal region) and the patient age or sex, nor with the Dukes stage or degree of differentiation of the tumors. It was interesting to note, however, that 77% (7 of 9) of those with whole p53 antibodies had Dukes A and B tumors. Interestingly, there was no correlation between the presence of serum antibodies (whole p53, central domain, or COOH-terminal region-specific) and the overexpression of p53 in the primary tumor. Three of nine individuals with central-domain antibodies and five of nine people with whole p53 antibodies had no detectable accumulation of p53 in their tumor.

### Antibody Library Construction and Antibody Selection

Table 1 outlines the details of the libraries constructed for this study. Each library was panned over five rounds against purified central domain or the COOH-terminal region of p53. The antigen concentration was reduced in rounds four and five.
to minimize the number of clones with nonspecific reactivity and to select for those with a higher affinity. After each library was panned, a total of 384 clones were tested for reactivity to the original antigen as well as a range of other antigens. No Fabs with specific reactivity against the COOH-terminal domain were identified from the four libraries. Only one highly specific central domain Fab was isolated (1159.8), and this was derived from the library constructed from patient 1159. A large number of Fabs that bound to p53 as well as a range of other irrelevant antigens were not analyzed further.

The specificity of 1159.8 for the central domain and whole p53 is shown in Figs. 2 and 3. Its reactivity against the central domain was reduced by preincubating the Fab with either whole p53 or central domain, but not after incubation with the COOH-terminal region or BSA (Fig. 4). This provided additional evidence that the antibody was indeed reacting to the central domain, and that its epitope was also present in the whole p53 molecule.

The possibility that 1159.8 was a low-affinity polyreactive antibody was eliminated by demonstrating that it did not react with a panel of self-antigens, tumor-associated antigens, and hapten (Fig. 5).

**Sequence Analysis of 1159.8.** Sequencing of the clone 1159.8 showed that the heavy chain was an IgG1 and the sequence analysis of 1159.8 was performed by competition studies with known central domain-reactive monoclonals. Fig. 7B shows that DO-11 effectively reduced Fab binding (62%), although Pab240 also had some inhibitory effects (24%). DO-12 and the negative control COOH-terminal reactive antibody Pab421 had no effect on the binding of the Fab. Because the epitope of DO-11 extends from residues 181–190 (25), it is likely that the epitope of 1159.8 either overlaps that of DO-11 or is in close proximity to this region. The lower inhibition seen with Pab 240 may indicate that the 1159.8 epitope also includes residues 213–217, or D7–27/DHQ52 genes. The light chain V region was comprised of a V gene originating from the DPK15 gene (VK2 gene family) and a JK4, J gene. A comparison of the V genes of the 1159.8 Fab and the germ line genes indicated a nucleotide mutation frequency of 3.6% (11/303) and 8.6% (26/300) in the light and heavy chain V genes, respectively.

**Epitope Mapping.** The binding of 1159.8 to whole p53 was reduced in the presence of serum from the donor individual (Fig. 7A). Control sera from an individual without p53 reactivity did not effect the binding of the Fab. These results demonstrated that the binding specificity of Fab 1159.8 reflected that of the in vivo immune response of patient 1159. The epitope mapping of Fab 1159.8 was performed by competition studies with known central domain-reactive monoclonals. Fig. 7B shows that DO-11 effectively reduced Fab binding (62%), although Pab240 also had some inhibitory effects (24%). DO-12 and the negative control COOH-terminal reactive antibody Pab421 had no effect on the binding of the Fab. Because the epitope of DO-11 extends from residues 181–190 (25), it is likely that the epitope of 1159.8 either overlaps that of DO-11 or is in close proximity to this region. The lower inhibition seen with Pab 240 may indicate that the 1159.8 epitope also includes residues 213–217, or, alternatively, this inhibition may simply reflect steric hindrance between two antibodies with adjacent epitopes.
DISCUSSION

In this study, pericolic lymph nodes from individuals with an observed immune response to the central domain of p53 were used to construct recombinant antibody libraries. From these libraries, we were able to isolate a Fab with specificity for the central domain of p53. We subsequently showed that this antibody bound the denatured form of whole p53 and also recognized an epitope that was close to, or perhaps overlapped, the binding site of the murine antibody DO11 (181–190). This is the first report of the isolation of a human antibody to the central domain of p53, and highlights the ability of antibody phage display to isolate antibodies with novel reactivity to p53.

Approximately 10% of individuals in this study had serum antibodies to the central domain of p53, a frequency that is almost twice that found in previous studies of cancer patients (12, 16, 26). There are a number of methodological differences in published studies that may account for this discrepancy. These include the type of antigen used in the ELISA, the assay format, and the type of underlying malignancy. The influence of antigen presentation is illustrated in the current study where the serum from three individuals demonstrated clear reactivity with the central domain, yet surprisingly had negligible reactivity with whole p53. The simplest explanation for this finding relates to the structure of the p53 domains. The central domain is largely hydrophobic, reflecting the complex structure required for sequence-specific DNA binding (27). Furthermore, in its native state, the central domain is flanked by the acidic transactivation domain and the basic COOH-terminal region (28). Considering these structural constraints, it is likely that the ability to detect central reactive serum antibodies will depend on whether the serum is tested against whole p53 or only the truncated central domain. The assessment of antibody binding by Western analysis provided additional evidence for the importance of antigen source and epitope presentation in the assessment of the p53 immune response. By Western analysis, there was clearly less binding of either serum, Fab 1159.8 or Fab 240, to whole p53 as compared with their reactivity to the central domain. An additional factor influencing the frequency of serum immunoreactivity is the underlying malignancy, e.g., Lubin et al. (16) demonstrated that the frequency of serum reactivity to whole p53 varied from 2.4% in prostate cancer to 24% in lung cancer. Taken together, these findings indicate that there are a number of clinical and methodological variations that may influence the identification of antibodies against the central domain anti-p53, and that these variations may have resulted in a significant underestimate of the frequency of these antibodies in patient serum.

To date, it has proven difficult to isolate central domain antibodies using traditional hybridoma methods, a fact reflected in the small number of central domain reactive antibodies currently available (25, 26). Many studies have demonstrated that phage display offers the opportunity to isolate and characterize antibodies that have been difficult to identify using hybridoma techniques. In isolating antibody 1159.8 from a patient with an observed serum anti-p53 central-domain response, we have demonstrated that such antibodies are indeed produced by at least some patients with cancer, and that these antibodies can be identified using phage display. Although we were unable to isolate antibodies from the other patients with central domain reactivity, it may be possible to identify such antibodies by varying the panning strategy. Critics of the phage display have suggested that the combinatorial generation of antibody libraries makes it possible to generate Fabs with specificities that were not present in the original source tissue. However, many recent studies have provided support for the premise that the antibody phage display process retrieves antibodies that are representative of the in vivo immune response (18, 29, 30). Our observation that the binding of 1159.8 to p53 was inhibited by donor serum but not by control serum provides additional, albeit indirect, evidence in support of this conclusion.

Most studies of the humoral immune response to cancer have focused on the antigenic specificity of serum antibodies. However, it is apparent that a knowledge of V gene usage within an immune response can translate into a better understanding of the molecular
forces that influence its development and the relationship of the immune repertoire to disease. Over the last few years, the use of particular V genes has been favored in a range of diseases, including autoimmunity (31). For example, the production of polyreactive antibodies in autoimmune disease is linked to the preferential use of VH6 genes by B cells from the fetal antibody repertoire. Because the antibody 1159.8 used germ-line genes from the VH3 family, and the previously reported NH2-terminal reactive p53 antibodies were derived from VH1 (18), it appears that the V genes used in the p53 immune response are those used commonly in the adult repertoire rather than the rare genes used in autoimmune disease (32). We have also shown that the constant region of the central domain antibody used the γ1 subclass, thus providing additional evidence that the humoral response to p53 occurs as a specific response to this antigen rather than as a result of the reactivation of polyreactive fetal B cells.

Serum anti-p53 antibodies and murine monoclonal antibodies previously have been thought to react to specific immunodominant regions within the p53 molecule (16). However, the epitope of Fab 1159.8, which includes amino residues 181–190, does not lie within one of these immunodominant regions of p53 (16). In a recent study, a panel of recombinant human anti-p53 antibodies reactive with the NH2-terminus of the molecule also failed to show reactivity with one of these putative immunodominant regions. Taken together, these observations suggest that anti-p53 antibodies arising in humans in vivo may actually show a broader range of epitope specificities than has to date been suggested by serum studies and animal immunization.

Antibodies against p53 can serve as valuable reagents in better understanding the relationship between the structure and function of this molecule. Interestingly, some antibodies have been shown to stabilize and even restore the function of mutant p53 (33, 34). Nevertheless, the use of anti-p53 antibodies as therapeutic agents has been limited by the need for intracellular localization of the antibody (35, 36). More recently it has been shown that anti-p53 antibodies can be used as idiotypic vaccines to induce tumor-specific immune responses (19, 20). In particular, immunization with the central domain reactive antibody Pab 240 was able to prevent tumor growth and metastasis. Unfortunately, murine antibodies such as Pab 240 would generate a human antimouse immune response upon immunization, thus reducing their effectiveness as a vaccine (37). This study shows that antibody 1159.8 binds to an epitope in close proximity to Pab 240, and as such may prove useful as an antitumor idiotypic vaccine while avoiding the undesirable features of the murine antibody.

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Human Antibody to the Central Domain of p53


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