Microinjection of Monoclonal Antibody PAb421 into Human SW480 Colorectal Carcinoma Cells Restores the Transcription Activation Function to Mutant p53

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Abstract

The p53 tumor suppressor is a transcription factor frequently mutated in human malignancies. Tumor-derived p53 missense mutants are defective in sequence-specific DNA binding and fail to activate p53 target genes. mAb PAb421 was shown previously to restore DNA binding to selected p53 mutants in vitro. Here we show that mAb PAb421 when microinjected into human SW480 colorectal carcinoma cells restores the transcription activation function to the resident mutant p53 (arg to his 273, pro to ser 309). Codon 273 is the second most frequent p53 missense mutant found in human tumors. Our results lend support to the concept of restoring wild-type function to mutant p53 as a strategy for cancer therapy.

Introduction

The most common genetic alterations in human malignancies are missense mutations of the p53 gene (1, 2). p53 is a nuclear phosphoprotein that acts as a tumor suppressor. It inhibits oncogene-mediated cell transformation and suppresses proliferation of tumor-derived cell lines in culture (3–5). In certain cell types, p53 induces programmed cell death or apoptosis (6, 7). Oncogene-transformed cells undergo p53-mediated apoptosis in response to conflicting growth signals (8, 9). p53 also mediates apoptosis caused by conventional DNA-damaging cytotoxic agents (10). Recently, tumor development and progression in vivo has been shown to be restricted by p53-dependent apoptosis (11). By contrast, tumor-derived p53 mutants lack tumor suppressor activity, and they do not exhibit any of the biological activities associated with the wild-type protein (3–5). Moreover, p53 mutants are able to cooperate with ras in the malignant transformation of primary rat embryo fibroblasts in culture, a property shared with nuclear and viral oncoproteins (3, 4, 12).

Biochemical studies have shown that wild-type p53 is a sequence-specific DNA-binding protein that recognizes a 20-bp motif consisting of two copies of the consensus sequence 5′-PuPuPuC(T/A) (T/A)GPyPyPyPyPy-C3′ (13–15). Both copies of the consensus are needed for efficient binding by p53 (14). A strong acidic transcription activation domain has been located near the amino terminus of p53, which can stimulate the expression of genes downstream of p53 binding sites both in vitro and in vivo (15–20). The sequence-specific transcriptional activation activity associated with p53 suggested that p53-regulated genes mediate its biological function as a tumor suppressor. Several genes have been identified that contain p53-binding sites with high homology to the p53 20-bp consensus motif. These include the muscle creatine kinase, GADD45, MDM2, epidermal growth factor, cyclin G, and WAF1/Cip1 genes (20–26). Interestingly, the recently discovered WAF1 gene, a strong cyclin-dependent kinase inhibitor, was shown to inhibit cell growth when expressed constitutively in tumor cells, thus supporting its role as an important mediator of p53 tumor suppression (24).

The transcription activation function of p53, which is defective in naturally occurring p53 mutants (17–20), has been shown to be essential for its biological activity as a suppressor of tumor cell growth (27). In most tumors, mutant p53 is expressed at high levels as a result of an increased stability. Thus, restoring DNA-binding and transactivation function to mutant p53 is an appealing strategy for the development of therapeutic agents that are capable of restoring p53 function and tumor suppressor activity. Investigating the mechanism of p53 DNA binding and its regulation should lead to a better understanding of why mutant proteins exhibit a reduced affinity for DNA and to possible ways of reversing this defect.

The specific DNA-binding function of p53 seems to be negatively regulated by the COOH-terminal basic region located between amino acids 363 and 393. Deletion or protease digestion of this region from Escherichia coli-produced wild-type p53 was shown to activate the intrinsic DNA-binding ability of the protein (28). Similarly, mAb PAb421, the epitope of which maps to amino acids 370–378, upon binding to p53 also activates DNA binding. This effect was specific since mAbs binding to the NH2-terminal region of p53 did not elicit the same response (28). It was also found that binding of mAb PAb421 to certain missense p53 mutants restored DNA binding (29, 30), suggesting that some p53 mutants possess a reduced affinity to interact with DNA that can be corrected by alterations of the COOH-terminal.

The demonstrated ability of mAb PAb421 to restore DNA-binding activity to some p53 mutants raises the possibility that molecules that mimic the antibody could potentially restore tumor suppressor activity to mutant protein. However, all these studies have been done in vitro; therefore, it is not clear whether restoring DNA binding is sufficient for transcription activation and tumor suppression in vivo. Here we report that mAb PAb421 when microinjected into SW480 colorectal carcinoma cells restores the transcription activation function to the resident p53 mutant, thus demonstrating the feasibility of restoring wild-type function to certain p53 mutants in vivo.

Materials and Methods

Plasmids. The wild-type p53 expression vector (pC53-SN3) was a gift of B. Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD; Ref. 5). In this plasmid, p53 is expressed from the CMV2 immediate early promoter. To construct βgal reporter plasmids, the bacterial βgal gene and the SV40 polyadenylation site were isolated as a HindIII fragment from pCH110 and cloned into Bluescript KS (BS/βgal). The PG13/βgal reporter was constructed by cloning a HindIII fragment from plasmid PG13/Luc, which contains 13 copies of oligo PG and the polyoma promoter (18) into the HindIII site of BS/βgal. The MG15/βgal reporter was constructed in a similar fashion and contains 15 copies of oligo MG and the polyoma promoter (18). The WAF1 promoter was isolated as a 2.4-kb HindIII fragment from plasmid WAF1-Luc (24) and cloned into BS/βgal to make reporter WAF1/βgal.

Preparation of Cell Extracts and DNA-binding Assay. SW480 cells were plated on 100-mm dishes and grown to near confluency at 37°C in...
DMEM supplemented with 10% FBS. Whole-cell extracts were prepared by three cycles of freezing and thawing in 1 ml of buffer containing 20 mM Hepes (pH 7.9), 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.4 mM KC1, 1 mM PMSF, 1 μg/ml each of leupeptin, aprotinin, and pepstatin, and 20% glycerol. Extracts were clarified by centrifugation at 14,000 x g for 10 min and stored at -75°C. Protein concentration was determined by the Bradford procedure.

Protein extracts were preincubated with 1 μg poly(dI-dC) and 0.5 μg mAb for 30 min at room temperature in 20 μl of buffer containing 20 mM Hepes (pH 7.9), 1 mM DTT, 50 mM KC1, 5 mM MgCl2, 10 μM ZnSO4, 0.1 mg/ml BSA, 0.1% NP40, and 5% glycerol. A 32P-labeled double-stranded oligonucleotide probe containing a nonpalindromic self-annealing consensus p53 binding site (5'-aggaagatct-GGACA-TGCCC-GGCCGATGCT-3') or the WAF1 p53 binding site (top strand, 5'-aggaagatct-GAACA-TGTGc-GGACA-TGGTc-3'; bottom strand, 5'-aggaagatct-cAACA-TGTTg-GGACA-TGTTC-3') was then added, and incubation continued for 30 min at room temperature. Competitor oligonucleotides were added during the preincubation step. The nonspecific oligonucleotide competitor contains a vitamin D responsive element from the human osteocalcin gene (top strand, 5'-aggaagatct-TGTTGACCTACCGGTTGAAAGGGGCTT-3'; bottom strand, 5'-aggaagatct-CAATGC-GGACA-TGCCC-GGGCA-TGTCC-3'). Oligonucleotides were annealed, and the 5' overhangs were filled in with the Klenow fragment of E. coli DNA polymerase I and dATP, dGTP, dTTP, and either dCTP (competitors) or [a32P]dCTP (radiolabeled probes). Reaction mixtures were loaded onto a 5% polyacrylamide gel (80:1, acrylamide: bisacrylamide). The gel was run at 200 V in 0.5 X TBE buffer [50 mM Tris-Borate (pH 8.3)-0.5 mM EDTA] at room temperature for 90 min. The gel was dried under vacuum and exposed to X-ray film.

Microinjection of SW480 Cells and βGal Staining. Microinjections were performed under a Nikon Diaphot phase contrast inverted microscope equipped with a Narishige four-dimensional hydraulic micromanipulator and Nikon pico-injector. Needles were fabricated from 1.0 mm outer diameter, 90-μm bore silica capillary tubes, using a Flaming/Brown type micropipette puller (Sutter Instrument Co., Novato, CA). Capillary tubes were pulled with parameters resulting in needles with a 0.8–1.0-μm opening. Plasmids and antibiotics were diluted in Ca2+/Mg2+-free PBS to a final concentration of 10–200 μg/ml and 2–4 mg/ml, respectively, and passed through a 0.22 μm filter before being back-loaded into the needle. SW480 cells (5 X 105) were overlaid with X-gal staining solution [100 mM NaPO4 (pH 7.3), 1.3 mM MgCl2, 3 mM K3Fe(CN)6, 3 mM H4Fe(CN)6, and 1 mg/ml X-gal]. Color was allowed to develop at 37°C, and stained cells were counted visually by low power light microscopy.

**Results and Discussion**

We first sought to determine whether mAb PAB421 could restore DNA binding to a mutant p53 that was selected in vivo and expressed in its natural cellular environment. Previous studies have suggested that different p53 mutants exhibit distinct biological activities and could be selected in vivo in a tissue- or cell type-specific manner. We chose the SW480 colorectal carcinoma cell line since it met both criteria and also because it expresses a mutant p53 (Arg to His 273) that could be selected in vivo in a tissue- or cell type-specific manner. We chose the SW480 colorectal carcinoma cell line since it met both criteria and also because it expresses a mutant p53 (Arg to His 273) that was shown previously to be activated in vitro for DNA binding by PAB421 and is the second most common p53 missense mutant found in human cancers (29, 30). In addition, p53 from SW480 cells contains a second point mutation at amino acid residue 309 (pro to ser; Ref. 31); therefore, it was important to determine whether this second amino acid change would preclude restoration of DNA binding by mAb PAB421.

Whole cell extracts from exponentially growing SW480 cells were prepared and tested for p53-binding activity by electrophoretic mobility shift assay. We used two different double-stranded oligonucleotide probes. The first probe contains a nonpalindromic consensus p53-binding site (14), whereas the second contains the natural p53-binding site from the WAF1 gene, which differs from the consensus at two nucleotide positions (24). When extracts from SW480 cells were incubated with these probes, no specific band was observed (Fig. 1, Lane 1). The addition of mAb PAB421 induced the appearance of a strong retarded band with the consensus probe (Fig. 1A, Lane 4) and a weaker band with the WAF1 probe (Fig. 1B, Lane 4). This band was not induced by the p53-specific mAb PAB1801, which binds to the NH2-terminal region of p53, nor by the SV40 T antigen-specific mAb PAB419 (Fig. 1, Lanes 2 and 3). The presence of p53 in the PAB421-induced complex was further confirmed by shifting the band with mAb PAB1801 (Fig. 1, Lane 6). In addition, the complex was also shifted by an antimouse IgG (Fig. 1, Lane 7). These results demonstrate that both p53 and PAB421 are present in this protein/DNA complex. The specificity of the PAB421-induced complex was examined by competition experiments (Fig. 1A, Lanes 9–12; Fig. 1B, Lanes 9–14). Only oligonucleotides containing p53-binding sites competed effectively in the formation of this retarded band. Therefore, PAB421 can restore the sequence-specific DNA-binding function to a p53 mutant that was naturally selected and expressed in human colorectal carcinoma cells. Our results also suggest that the mutation at codon 309 of p53, which is located outside the DNA-binding core domain (32), does not further impair sequence-specific DNA binding significantly.

To demonstrate that activation of DNA binding by PAB421 leads to restoration of the transcriptional activation function of mutant p53, we microinjected βgal reporter plasmids and antibodies into SW480 cells, followed by cytochemical staining. For the experiments to be successful, we needed to identify a reporter plasmid that readily responds to p53 but which has low basal activity. We examined two sets of plasmids by transient transfection assays in SW480 cells (data not shown). The first plasmid encodes the βgal gene driven by the native p53-responsive WAF1 promoter, whereas the second plasmid contains the same reporter gene driven by the mouse polyoma promoter and a multimer of a genomic p53-binding site from the ribosome gene cluster (PG13/βgal; Refs. 14 and 18). Transfection of the WAF1/βgal reporter resulted in a small but significant number of cells staining positive for βgal. By contrast, we failed to detect any stained cells that were transfected with the PG13/βgal plasmid. Transfection of SW480 cells of both plasmids with a wild-type p53 expression vector resulted in a dramatic increase in the number of cells staining positive for βgal. Thus, PG13/βgal was chosen initially for the microinjection experiments since the basal activity in transient transfection assays was undetectable by cytochemical staining but responded strongly to the presence of wild-type p53. As a negative control, we used the MG15/βgal plasmid, which contains a multimer of a mutant p53 binding site and the polyoma promoter (18).

Exponentially growing SW480 cells were microinjected directly into the nucleus with reporter plasmid DNA together with an expression vector for wild-type p53 or mAb. Twenty-four h later the cells were fixed and stained for βgal activity. Results of two representative experiments are summarized in Table 1. Data have been normalized for microinjection efficiency and cell viability by measuring the fraction of stained cells following microinjection of plasmids CMV/βgal or RSV/βgal. As expected, microinjection of a wild-type p53 expression vector induced βgal activity from PG13/βgal but not from MG15/βgal. Similarly, microinjection of mAb PAB421 resulted in a

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Fig. 1. mAb PAb421 restores sequence-specific DNA binding to mutant p53 expressed in SW480 cells. Whole-cell extracts from SW480 cells were prepared as described in "Materials and Methods" and assayed for DNA binding by electrophoretic mobility shift assay using an oligonucleotide probe containing a consensus p53-binding site (A) or the WAF1 p53-binding site (B). mAbs were added as indicated, and the protein/DNA complexes were resolved in a native 5% polyacrylamide gel. Competitor oligonucleotides were added in molar excess as indicated (CON, consensus p53-binding site; WAF1, p53-binding site from WAF1 gene; VDRE, vitamin D responsive element from human osteocalcin gene). Arrows, the migration of unbound and protein-bound DNA. *, nonspecific bands.

A.

B.

significant fraction of cells expressing βgal from PG13/βgal but not from MG15/βgal. In contrast, p53-specific mAb PAb1801 and SV40 T antigen-specific mAb PAb419 failed to elicit a similar response. These results indicate that microinjection of PAb421 restored the sequence-specific transcriptional activation function to the resident p53 mutant his 273 in SW480 cells.

To confirm the above results using a natural promoter, we repeated these experiments using the WAF1/βgal reporter. Results are shown
in Table 2. Microinjection of a wild-type p53 expression plasmid induced βgal activity from the WAF1 promoter, confirming the p53-dependent activation of this promoter. At the plasmid concentrations used in these microinjection experiments (pWAF1/βgal, 50 μg/ml; pCMV/p53WT, 10 μg/ml), we did not observe any stained cells in the absence of wild-type p53. Similar to the results we obtained with the PG13/βgal reporter, microinjection of Mab PAB421, but not PAB1801 or PAB419, induced βgal activity in a significant fraction of microinjected cells. Furthermore, microinjection of PAB1801 together with PAB421 abolished this response. This provides further evidence that endogenous mutant p53 is indeed responsible for the observed βgal activation from the WAF1 promoter.

Our results thus far strongly suggest that Mab PAB 421, when microinjected into SW480 cells, can restore the transcription activation function to the resident mutant p53. This was further supported by the observation that Mab PAB1801, which binds to the NH₂ terminus of p53 where the acidic transcription activation domain is located, blocked PAB421-induced transcription of the WAF1 promoter. To rule out the possibility that PAB421 by itself can induce transcription of a p53-responsive promoter, we performed microinjection experiments with the lung carcinoma H1299 cell line that has both p53 alleles deleted (33). Results are summarized in Table 3. Microinjection of a wild-type p53 expression vector led to transactivation of the PG13/βgal reporter, but not MG15/βgal, indicating that this cell line supports p53-mediated transactivation. However, microinjection of mAb PAB421 failed to induce βgal activity, demonstrating that this mAb only induces transactivation of this reporter gene in a p53-mediated manner. Additionally, microinjection of Mab PAB421 into H1299 cells stably transfected with p53 mutant his 273 results in transcription of the PG13/βgal reporter.³

The possibility of restoring suppressor function to mutant p53 in human tumors has been suggested previously in vitro studies (29, 30). The vast clinical potential of this approach for cancer therapy is enormous since p53 is mutated in about 50% of all human malignancies. Most, if not all, tumor-derived p53 mutants are defective in DNA binding and transcriptional activation, suggesting that reactivation of p53 target genes should lead to suppression of cell proliferation and possibly induction of apoptosis. We have shown here that microinjection of mAb PAB421, the prototype molecule for in vitro activation of mutant p53 DNA binding, results in the specific transcription activation of two different p53-responsive promoters in SW480 colorectal carcinoma cells expressing a p53 with a missense mutation at residue 273. This is the second most frequent mutant in human tumors. We have observed that approximately 50% of the most common p53 mutants can be induced to bind DNA by PAB421.⁴ Our results suggest that the goal of restoring wild-type function to a subset of p53 mutants in vivo is achievable. Identification of a small molecule or peptide that mimics the action of antibody PAB421 and is permeable to cells should provide an answer to the critical question of whether restoring p53 transcription activation will lead to tumor suppression.

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References

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