A Mutated β-Catenin Gene Encodes a Melanoma-specific Antigen Recognized by Tumor Infiltrating Lymphocytes

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Summary

A number of antigens recognized by tumor-reactive T cells have recently been identified. The antigens identified in mouse model systems appear, with one exception, to represent the products of mutated genes. In contrast, most of the antigens recognized by human tumor-reactive T cells reported to date appear to represent the products of non-mutated genes. Here we report the isolation of a cDNA clone encoding β-catenin, which was shown to be recognized by the tumor-infiltrating lymphocyte (TIL) 1290, a HLA-A24 restricted melanoma-specific CTL line from patient 888. The cDNA clone, which was isolated from the autologous melanoma cDNA library, differed by a single base pair from the published β-catenin sequence, resulting in a change from a serine to a phenylalanine residue at position 37. Normal tissues from this patient did not express the altered sequence, nor did 12 allogeneic melanomas, indicating that this represented a unique mutation in this patient’s melanoma. A peptide corresponding to the sequence between amino acids 29 and 37 of the mutant gene product was identified as the T cell epitope recognized by TIL 1290. The observation that HLA-A24 binding peptides contain an aromatic or hydrophobic residue at position 9 suggested that the change at position 37 may have generated a peptide (SYLDSGIF) which was capable of binding to HLA-A24, and a competitive binding assay confirmed this hypothesis. The β-catenin protein has been shown previously to be involved in cell adhesion mediated through the cadherin family of cell surface adhesion molecules. The high frequency of mutations found in members of cellular adhesion complexes in a variety of cancers suggests that these molecules may play a role in development of the malignant phenotype.

The majority of murine antigens recognized by tumor-reactive T cells represent the products of mutated genes (1, 2). The only reported example of a non-mutated gene product recognized by tumor reactive T cells in the mouse is the P1A antigen of the P815 mastocytoma, which is also expressed on some normal mast cell lines (3). A number of genes encoding antigens recognized by human melanoma-reactive T cells have recently been isolated. The MAGE (4, 5), BAGE (6), and GAGE (7) gene products are non-mutated proteins and are not expressed in normal tissues with the exception of testes, and thus appear to represent tissue-specific antigens with a restricted pattern of expression. In addition, melanoma-reactive T cells have been shown to recognize tyrosinase (8, 9), MART-1 (10), gp100 (11), and TRP-1 (12). Non-mutated epitopes were identified in these proteins, whose expression in normal tissues was limited to melanocytes and retina. Two reports have recently appeared, however, demonstrating that human melanoma-reactive T cells recognize mutant gene products (13, 14).

The HLA-A24 restricted melanoma-reactive tumor-infiltrating lymphocyte (TIL) 1290 cell line established from patient 888 was recently shown to recognize the product of a newly described gene termed p15 (15). This product appeared to be expressed in a wide variety of normal tissues, and a non-mutated peptide from this protein was recognized by TIL 1290. This report presents the isolation of a second gene encoding an antigen recognized by TIL 1290. Two clones which were isolated by screening a cDNA library with TIL 1290 represented partial cDNAs derived from the β-catenin gene. The T cell epitope identified in

Abbreviations used in this paper: mel, melanoma; TIL, tumor-infiltrating lymphocytes.
this product was encoded by a region containing a single point mutation which gave rise to a peptide with significantly enhanced binding to the HLA-A24 class I molecule.  

β-catenin has been shown to be involved in the binding of members of the cadherin family of cell surface adhesion molecules to cytoplasmic actin microfilaments (reviewed in 16), and the mutation described in this report may have functional significance for the development of the malignant phenotype of this tumor.

Materials and Methods

**Cell Lines.** The derivation of the 293-A24 stable transfectant of the 293 human kidney cell line expressing the HLA-A24 class I gene was previously described (9). The 293-A24 cells were maintained in DMEM containing 7.5% FBS, and melanoma (mel) cell lines maintained in RPMI containing 5% FBS. The melanoma-specific CTL were grown from TIL in AIM V (Life Technologies, Gaithersburg, MD) media containing 5% human serum plus 6,000 IU/ml IL2 for 30–60 d, as previously described (17). TIL 888 was grown from a tumor derived in 1989 from patient 888, and when administered to the autologous patient with IL2 resulted in the complete regression of multiple metastases. TIL 1290 was grown from a recurrent pelvic mass in patient 888 found three years later, and was administered in combination with TIL 888, resulting in regression of an additional pelvic tumor mass. TIL 1541 was grown from a small subcutaneous nodule isolated from patient 888 in 1995.

**cDNA Library Construction and Screening.** The cDNA library was constructed using 10 µg of poly (A)+ mRNA from the 1290 melanoma which had been doubly selected using the FastTrack poly A isolation kit (Invitrogen, San Diego, CA). The cDNA was synthesized using a random primer cDNA synthesis kit (Promega, Madison, WI), BstXI adaptors (Invitrogen) were ligated to the cDNA, and the cDNA was then cloned into pcDNA3 (Invitrogen) which had been digested with BstXI. Following the transformation of DH5α cells, the volume needed to obtain 50 bacterial transformants was calculated and used to inoculate 1 ml of Super Broth (Biofluids, Gaithersburg, MD) in a 96-well culture block (AGCT, Gaithersburg, MD) for 48 h. Plasmid DNA was then prepared using the 9600 Wizard system (Promega Corp., Madison, WI). Transfection of 293-A24 was carried out using 200 ng of plasmid DNA as previously described (15), 18–24 h later TIL were added, and 18–24 h later supernatants assayed for GM-CSF using a GM-CSF ELISA (R&D Sys., Inc., Minneapolis, MN).

**Sequencing and PCR Analysis.** DNA sequencing was conducted using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). A search of the GenBank database was carried out using the Blast sequence alignment algorithm (18). To carry out the reverse transcriptase (RT) PCR analysis, 5 µg total cellular RNA was reverse transcribed using oligo-dT, and 1/10 of this reaction then used to carry a PCR. The PCR was carried out for 30 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. The PCR was carried out using the primers 60F (5'-AAAG-GTCTGAGGAGGAGCCTTC-3') and 7271< (5'-TGGACCAT-AACTGCAACCTT-3').

**Peptide Synthesis and Purification.** The peptides were synthesized on the Gilson AMS222 multiple peptide synthesizer using standard F-moc chemistry. Peptides were purified on a R2 reverse-phase HPLC column (PerSeptive Biosystems, Framingham, MA) as well as a C8 column (VYDAC, Hesperia, CA) using an aceto-
Figure 1. Sequence of the two β-catenin cDNA clones. The sequence of the two cDNA clones were aligned with the published β-catenin sequence, GenBank accession number Z19054 (29). The translated sequence is listed above the nucleotide sequence, with dashes indicating identity with the published sequence and periods indicating a gap in the 5' untranslated region of clone 12g2. The sequence of the peptide which represents the T cell epitope is underlined.
previously been shown to stimulate TIL 1290, but no response was seen with transfectants expressing tyrosinase. In contrast, TIL 888 responded to transfectants of tyrosinase but failed to respond to 293-A24 cells transfected with either the newly isolated clones or the p15 gene.

**Sequence Determination.** The sequences of the two positive clones were determined and a search of the GenBank database indicated that the sequences of both clones were nearly identical to that of the β-catenin gene (Fig. 1). The DNA sequence obtained for one of the cDNA clones, 12g2, corresponded to that found between residues 15 and 890 of the published human β-catenin sequence, but contained a deletion of 49 nucleotides within the 5’ untranslated region. When RNA from the 1290 melanoma was reverse transcribed using oligo-dT and a PCR carried out using primers which flanked this region the deletion was not observed, however, indicating that the deletion may represent a cloning artifact (data not shown). The sequence of the second clone, 11A1, corresponded to that of the region between residues 55 and 582 of the published β-catenin sequence. The 5’ end of clone 11A1 contained 24 base pairs of unidentified sequence, which could either reflect alternative processing of this gene product or may again reflect a cloning artifact.

A comparison of the coding regions of the two cDNA clones with the published sequence of β-catenin demonstrated that a single nucleotide substitution of a T for a C residue was found in both of the cDNA clones at residue 310, resulting in a change of serine at position 37 to phenylalanine. To determine if this represented a mutation or an allelic polymorphism, RNA from autologous normal tissues as well as from the 888 and 1290 melanomas was reverse transcribed using oligo-dT, and a PCR carried out using two primers which flanked residue 310. The resulting 668 base pair product was then digested with XmnI, which should give rise to two products of 246 and 422 base pairs, in the case of the previously published sequence. The substitution found at residue 310 in the cDNA clones that were isolated, however, eliminated the XmnI restriction enzyme site, and as a result the size of this product should not be altered following digestion with this enzyme. Digestion of the PCR products obtained using RNA from autologous fibroblasts, TIL or EBV cells with XmnI resulted in two bands of the expected size based on the published β-catenin sequence. Digestion of the PCR products derived from the 1290 melanoma RNA with XmnI resulted in a product of approximately 700 base pairs, corresponding to the undigested product, as well as two bands of about 300 and 400 base pairs (Fig. 2). The observation that the undigested product was found in the samples derived from the melanoma but not the normal tissues from patient 888 indicated that this represented a mutation and not an allelic polymorphism. In addition, the 1290 melanoma appeared to express the allelic β-catenin gene product which did not contain this mutation. The same pattern was observed for the 888 melanoma that had been isolated from the same patient three years earlier than the 1290 melanoma. When DNA sequencing reactions were carried out on the PCR products obtained from the 1290 and 888 melanomas, both a T and a C residue were observed at this position, confirming the results of the restriction enzyme digestion (data not shown). In addition, 12 allogeneic melanomas that were examined did not exhibit this mutation, indicating that this represented a relatively rare mutational event (Fig. 2).

**Recognition of Mutated β-Catenin Sequence.** An attempt was then made to identify the T cell epitope in β-catenin recognized by TIL 1290. The HLA-A24 binding motif was previously shown to consist of either a phenylalanine, leucine, isoleucine or tryptophan residue at the last position, and a tyrosine, phenylalanine, or methionine residue at position two (21). A peptide containing the amino acid 37 phenylalanine at position 9 would also contain a tyrosine at position two, which would conform to this motif. Therefore, the peptide corresponding to residues 29 to 37 of the mutant sequence, SYLDSGIHF (β-cat29_37 mut), as well as the peptide corresponding to the normal allele, SYLDSGIHS, (β-cat29_37) were synthesized. When these peptides were tested for their ability to sensitize targets for lysis, the β-cat29_37 mut peptide was found to sensitize target cells at a concentration of 1 μg/ml or higher, whereas concentrations of 1 μg/ml or greater were required for sensitization of target cells with the normal β-cat29_37 peptide (Fig. 3). Thus, TIL 1290 demonstrated 106-fold better recognition of the mutant peptide than the normal peptide.

The effect of the mutation at position 37 on the affinity of this peptide for the HLA-A24 class I allele was then assed...
Figure 3. Titration of β-catenin peptides for recognition by TIL. 888 EBV B cells were incubated with the indicated concentrations of peptides and lysis by TIL1290 was measured in a 4-h 35Cr release assay at an E/T ratio of 40:1.

Figure 4. Competitive binding assay. The indicated peptides were diluted and tested for their ability to inhibit the binding of the labeled standard peptide AYIDNYNKF to 888 EBV B cells as described in the Materials and Methods. The average total and non-specifically bound cpm were 3,738 and 75 cpm, respectively. Non-specific binding was determined by incubating cells with the labeled peptide in the presence of 100 μg/ml of the cold standard peptide.

Discussion

This report demonstrates that the mutated product of the β-catenin gene was recognized by the melanoma-specific TIL 1290. The mutation described in the β-catenin gene appeared to convert a peptide from a very low affinity binder to a very high affinity binder of the HLA-A24 class I gene product. Extremely high levels of the normal β-catenin peptide were shown to be required to sensitize target cells whereas transfectants of the β-catenin gene product were only weakly recognized. This assay was carried out using a 51-d-old culture of TIL 1290, whereas the previous assay (Table 1) was carried out using a 38-d-old culture of TIL. The result of this as well as additional assays have demonstrated loss of reactivity with β-catenin between seven and eight weeks of culture of TIL 1290, whereas reactivity with p15 was maintained for several additional weeks (data not shown). The loss of reactivity may have resulted from the slower growth or more limited lifespan of β-catenin-reactive T cells relative to other T cells present in this culture.

The fresh un-cultured 1541 tumor was also analyzed for expression of the normal and mutated β-catenin sequence, in order to determine whether or not immunoselection of an antigen loss variant had occurred in vivo following treatment of the patient three years earlier with TIL 1290. The result of this analysis indicated that the 1541 tumor expressed the normal as well as the mutated β-catenin gene product (Fig. 5).
Dramatic difference was seen in the ability of the nor-

The mutation present in this gene product gave rise to a
tated cyclin-dependent kinase 4 (CDK4) gene product (14).

The demonstration in this report, as well as two others,

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The cytokine release from TIL stimulated by 293-A24 transfectants fol-

Table 2. Antigen Recognition by TIL 888, 1290, and 1541

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| Stimulator cell line | Transfected gene | HLA-A24 | TIL 888 | TIL 1290 | TIL 1541 |
|---------------------|-----------------|---------|---------|----------|----------|
| 293-A24             | p15             | +       | <8      | 1,360    | <8       |
| 293-A24             | β-catenin       | +       | <8      | 120      | 1,850    |
| 293-A24             | Tyrosinase      | +       | 2,400   | 11       | <8       |
| 293-A24             | None            | +       | <8      | 9        | <8       |
| 888 mel             | None            | +       | 5,000   | 930      | 3,100    |
| 397                 | None            | −       | <8      | <8 <8    | <8       |

The cytokine release from TIL stimulated by 293-A24 transfectants fol-

none None <8

13-catenin cDNA construct was transfected in this assay.

none None <8

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topes, they could potentially lead to the development of useful therapeutic reagents. These epitopes should also be truly tumor specific, which may help to avoid the development of potentially destructive auto-immune responses which could occur when patients are immunized with antigens expressed on normal tissues such as melanocytes and retina. In addition, the high frequency of mutations found in tumors of many different histologies raises the possibility that T cells may be present in many of these patients which recognize mutated products, and that some relatively common mutations could serve as the targets of additional anti-tumor vaccines.

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