CD4+ T-Cell Immunity to Mutated ras Protein in Pancreatic and Colon Cancer Patients

Huillian Qin, Wei Chen, Masazumi Takahashi, Mary L. Disis, David R. Byrd, Larry McCahill, Kenneth A. Bertram, Robert G. Fenton, David J. Peace, and Martin A. Cheever

Abstract
Mutated p21 ras proteins contain single substituted amino acid residues and represent cancer-specific proteins. The current study examined whether primed T cell immunity to mutant p21 ras proteins and/or peptides can be detected in patients with pancreatic or colon cancer. Studies focused on the aspartic acid substitution in amino acid position 12 (denoted D12) as the commonest mutation in gastrointestinal malignancy. Peripheral blood lymphocytes from patients or normal individuals were tested for the ability to proliferate in response to normal or mutated ras peptides or proteins. T-cell responses were defined as a stimulation index of >2.0. Results showed that 7 of 16 (44%) pancreatic cancer patients responded to ras-D12 peptide. Responses to ras-D12 protein were studied in only the last four patients that responded to D12 peptides. Three of the 4 patients that responded to ras-D12 peptide showed a substantial response to p21 ras-D12 protein (stimulation indices of 12, 8, and 24). Specifivity was validated by examining responses to normal and alternate ras peptides and proteins. T-cell responses to ras-D12 peptides were detected in only 2 of 25 (8%) colon cancer patients. None of 11 normal individuals tested had positive responses to normal or mutant ras p21 proteins and/or peptides. Thus, CD4+ T-cell immunity to the mutated segment of ras protein is present in some patients with gastrointestinal cancer.

Introduction
Somatic point mutations of ras oncogenes occur in approximately 90% of pancreatic adenocarcinomas and 45% of colon adenocarcinomas. The activated ras oncogenes encode a highly conserved group of MT 21,000 proteins, denoted as p21 ras, with oncogenic activity involved in the pathogenesis of cancer (1-4). Activation of the ras oncogene occurs most commonly at codon 12 or codon 61 and results in corresponding single amino acid substitutions within the p21 protein. In pancreatic cancer, the most common resultant amino acid substitution is aspartic acid, replacing glycine at amino acid position 12. The D12 substitution occurs in approximately 34% of patients. In colorectal cancer, the most common substitution is also D12 (5). Mutated p21 ras proteins are not expressed by normal tissue and thus represent cancer-specific proteins. In animal models, it has been shown that mutated ras proteins can serve as cancer-specific antigens that are recognizable by the T cell of the immune system. In defined circumstances, mice immunized to intact mutated protein (7, 8).

Materials and Methods
Subjects. Twenty-one patients with pancreatic cancer, 35 patients with colon cancer, and 17 healthy normal individuals from the University of Washington Medical Center, Madigan Army Medical Center, and Loyola University Medical Center were studied.

Peptides. The amino acid sequences of the normal and mutated ras peptides are shown in Table 1. All peptides were synthesized by Dr. Patrick S. H. Chou (Biopolymer Facility, Department of Immunology, University of Washington) using FMOC Chemistry in an automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA).

Ras Proteins. Recombinant wild-type and mutated p21 ras proteins were expressed and purified at the University of Washington and at the Biological Response Modifiers Program, National Cancer Institute. For preparation of p21 K-ras (C12, S12, and V13) proteins, Escherichia coli were transfected with plasmids containing coding sequences for one of the three activated p21 ras proteins (11). Lysates of transfected bacteria were purified by Sephadex G75 column. Fractions containing M, 21,000 ras protein were concentrated by filtration and further by HPLC on a DEA E ion exchange column. Fractions containing ras protein were analyzed by SDS-PAGE and Western blotting. The fractions containing p21 ras protein were dialyzed, sterile filtered, and dried; then the endotoxin was removed. Protein concentrations were determined by spectrophotometry. For preparation of p21 K-ras (G12, D12, and D13) proteins, transfected bacteria were grown to an A600 of 1.0, at which time isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of

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2984
Fig. 1. CD4⁺ T-cell immunity to mutated ras-D12 peptides can be detected in patients with pancreatic cancer. Peripheral blood mononuclear cells from 16 pancreatic cancer patients (A) and 11 normal individuals (B) were tested for proliferative responses to a panel of 18-mer mutated or normal ras peptides (ras-D12, G12, S12, V12, D12, and D13). Cells (2 × 10⁵) were incubated without antigen or with varying ras peptides (50 μg/ml) in wells of 96-well microtiter plates at 37°C for 96 h. One μCi (37 MBq) of [³H]thymidine was added into each well 8 h before harvesting. Thymidine incorporation and S.I. were calculated.

Ras Peptides (50 μg/ml)

Table 1 Normal and mutated ras peptides used

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Length</th>
<th>Amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>Ras G12 (p4-21)</td>
<td>18 mer.</td>
<td>Y K L V V G A G V G K S A L T I</td>
</tr>
<tr>
<td>Ras C12 (p4-21)</td>
<td>18 mer.</td>
<td>Y K L V V G A G V G K S A L T I</td>
</tr>
<tr>
<td>Ras S12 (p4-21)</td>
<td>18 mer.</td>
<td>Y K L V V G A G V G K S A L T I</td>
</tr>
<tr>
<td>Ras V12 (p4-21)</td>
<td>18 mer.</td>
<td>Y K L V V G A G V G K S A L T I</td>
</tr>
<tr>
<td>Ras D12 (p4-21)</td>
<td>18 mer.</td>
<td>Y K L V V G A G V G K S A L T I</td>
</tr>
<tr>
<td>Ras D13 (p4-21)</td>
<td>18 mer.</td>
<td>Y K L V V G A G V G K S A L T I</td>
</tr>
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* Normal (wild-type) p21 ras protein contains Gly at amino acid position 12.

Results

CD4⁺ T-Cell Immunity to Mutated ras-D12 Peptides Can Be Detected in Patients with Pancreatic Cancer. Patients with far advanced cancer often develop general immune incompetence and an inability to respond to T-cell antigens. To exclude such patients from the study, only the patients and normal donors who showed positive proliferative responses to the standard recall antigen tetanus toxoid were selected for studies. PBL from each pancreatic cancer patient or normal individual were first tested for the ability to respond to tetanus toxoid. Proliferative responses to tetanus toxoid were measured by incubating PBL from each patient or normal individual with 5 μg/ml tetanus toxoid in a 96-h [³H]thymidine incorporation assay. Sixteen of 21 (76%) pancreatic cancer patients showed positive proliferative response to tetanus toxoid, with S.I. greater than 2. The 16 patients were studied further.

Studies to examine immunity to mutated ras peptides focused on the aspartic acid substitution (denoted D12). PBL from patients or normal individuals were tested in proliferative assays against an 18-mer ras-D12 peptide that spanned the mutated segment (Table 1). Control peptides used were a panel of 18-mer ras peptides including normal ras-G12 peptide or mutated ras-S12, C12, V12, and D13 peptides that span the same mutated segment. The results showed that 7 of 16 pancreatic cancer patients (44%) had positive responses to the mutated ras D12 peptide, with S.I. equal or greater than 2 (Fig. 1A).

Four positive patients and three negative patients were retested for proliferative responses to the mutated ras D12 peptide at least twice. The results were reproducible in the repeated examination (data not shown). Of the seven patients that responded to D12 peptides, three responded to only D12 peptide, three cross-responded to D13 and no other peptide, and one patient responded to D12 and S12. None of the 11 normal individuals tested had positive responses to mutant ras p21 peptides (Fig. 1B).

Pancreatic Cancer Patients Responding to ras-D12 Peptides Can Also Respond Specifically to p21 ras-D12 Protein. Subsequent studies tested whether PBL from pancreatic cancer patients that
responded to ras-D12 peptides can respond to p21 ras protein containing the same mutation. Four patients that responded positively to ras-D12 peptides were tested for response to p21 ras-D12 protein in a proliferation assay. Results (Fig. 2) showed that three of the four patients responded to ras-D12 peptide exhibited substantial responses to p21 ras-D12 protein. One patient responded to ras-D12 protein but also to the normal or other alternate p21 ras proteins. One patients responded to neither. None of 11 normal individuals tested had positive responses to mutant p21 ras-D12 proteins (data not shown).

**Discussion**

Extensive studies in animal models have shown that T cells specifically immune to malignant cells can cure advanced malignancy (12). Both CD8+ and CD4+ T-cell subsets alone are capable of eradicating established tumor, but both subsets collaborate in most normal immune responses, and optimal T-cell therapy requires both.

**CD4+ T-Cell Immunity to Mutated ras-D12 Peptides Was Detected in Much Lower Frequency in Patients with Colon Cancer.** Mutated ras proteins are present in approximately 45% of colon adenocarcinomas. To evaluate CD4+ T-cell immunity to mutated ras-D12 peptides, 25 of 35 colon cancer patients (71%) who retained positive proliferative response to tetanus toxoid were selected for studies. A proliferative assay was performed by testing PBL from each patient to normal ras-G12 peptide or mutated ras peptides (ras-S12, V12, D12, and D13). The results showed that T-cell responses to ras-D12 peptides were detected in 2 of 25 (8%) colon cancer patients (Fig. 3). Thus, CD4+ T-cell immunity to mutated ras-D12 peptides was found in colon cancer patients but in much lower frequency than in patients with pancreatic cancer. Whether the D12 peptide-reactive T cells derived from colon cancer patients can proliferate to ras D12 protein was not tested.
mutations of ras oncogenes occur in approximately 90% of pancreatic adenocarcinomas and 45% of colon adenocarcinomas (1–3). Our previous studies examined sera from patients with pancreatic and colon cancer for the presence of antibody to ras protein using ELISA. A panel of purified p21 ras proteins with several known single amino acid substitutions was generated by prokaryotic expression of a mutated synthetic H-ras genes. In a study examining sera from 161 patients with colon cancer and 60 normal volunteers, ras-reactive IgA antibodies were detected in 38% of patients but only 3% of normals. The greater incidence of antibody in cancer patients provides strong evidence that immunization to the protein occurred as a result of the malignancy. The formation of antibodies directed against other oncogenic proteins has been described (15–18), but the relationship between the formation of human antibodies and the generation of a T-cell response has not been elucidated. Detection of existing antibody responses to ras protein implies that these patients may have been immunized to ras protein by virtue of the existence of a positive tumor. Often, helper/inducer T cells and antibodies respond to the same protein. Antibody responses to small globular proteins such as ras generally require cognate T-cell help. Immunoglobin class switching from IgM to IgA or IgG is considered to require T-cell help. These responses are usually directed against different epitopes of the same protein. The antibody responses to p21 ras detected in colon cancer patients was an IgA response; therefore, T-cell help was most probably involved. It is possible that help for serum CD4+ T-cell responses to p21 ras was provided by T-cell responses to the mutated segment. The observation that patients with pancreatic or colon cancer have antibodies directed against ras protein indicates that the protein is recognizable by the immune system and can be processed in the class II pathway for interaction with CD4+ T cells.

The current studies focused on the aspartic acid substitution and demonstrated that T-cell immunity to the mutated segment of ras protein is present in some patients with gastrointestinal cancer. These studies validate that mutant ras protein sequences are within the realm of the human T-cell repertoire. Other investigators have demonstrated that the human T cell can recognize peptides that span the mutated segment of the ras proteins (9, 10, 19, 20). A critical demonstration of the current studies is that the ras peptide-specific T cells in cancer patients respond to ras protein containing the same substitution. Peptide-specific T cells need not necessarily respond to the parental protein containing the peptide. The ability of any cancer-specific protein to serve as a tumor antigen for CD4+ T cells in any particular host depends upon whether the targeted segment of the protein has the proper molecular configuration to be presented by host class II MHC molecules and whether the resultant peptide/MHC complex is within the host T-cell repertoire. Not every protein expressed by mutated tumor-related DNA is antigenic and can serve as a T-cell target. Some segments of protein will not have an amino acid motif appropriate for binding in the cleft of particular MHC molecules, and many potential epitopes may be destroyed by the degradative aspects of antigen processing, which occurs via the action of specific proteases. Moreover, the mutated epitope may be present in the cleft of MHC antigens but in too low of a concentration to be recognized by T cells.

Specificity was validated by examining responses to alternate ras peptides and proteins. The results described here indicate that individual do develop CD4+ T-cell responses against ras proteins. Some patients had a higher response to the mutated proteins as compared to wild-type ras protein, but some responses may be to the epitopes of both the normal or mutated protein. The number of patients evaluated here is too small to attempt correlation of CD4+ T cell response formation to prognosis and to determine the actual frequency of anti-ras CD4+ T-cell response cancer patients. A large number of patients and normal individuals need to be evaluated over time. Although the results demonstrated bulk-cultured CD4+ T-cell responses against epitopes located in the mutated sequence of the p21 ras protein, studies need to be performed to determine if mutation-specific T-cell clones are present. The observation that immunity to ras proteins is present in some patients begs the issue of when in the course of tumor evolution immunity develops, whether the development of immunity influences the molecular phenotype of the malignancy, whether immune responses play any role in slowing cancer progression, and whether boosting of immune responses by vaccination can offer any therapeutic benefit. In the past, there was much speculation as to the existence of tumor antigens because the molecular etiology of cancer was a mystery, and there was little firm evidence as to the molecular identity of the cancer antigens. Few studies have evaluated immunity to known and defined antigens. Such studies are now possible in the ras system.

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References

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