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

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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 peptide. 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). Specificity 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 oncoproteins encode a highly conserved group of proteins that are recognizable by the T cell of the immune system. In defined circumstances, mice immunized to whole mutared ras proteins are able to mount a T-cell response that is specific for the mutated segment of the molecule (6). Conversely, immunizing animals with peptides that span the mutated segment can result in the generation of both helper/inducer T-cell and cytolytic T-cell responses that recognize intact mutated protein (7, 8).

The observation that p21 ras protein can be immunogenic in mice begs the issue of whether primed responses to p21 ras protein are present in patients with ras-positive cancers. In previous studies, we have been able to detect antibody to ras protein in 36% of patients with colon cancer compared to 3% of normal individuals. The greater incidence of antibody in cancer patients provides strong evidence that immunization to the protein occurs as a result of malignancy.3 Helper T cells and antibodies often respond to the same protein. Others have shown that T cells from patients with follicular thyroid carcinoma and colorectal cancer can recognize peptides that span the mutated segment of mutated ras protein (9, 10). The current study extended those findings by examining whether ras peptide-specific CD4+ T cells can be detected in pancreatic cancer patients in whom the incidence of ras mutations is higher and, furthermore, whether ras peptide-specific T cells can specifically recognize ras protein with the same mutation. The demonstration of existent peptide-specific T-cell responses to mutant ras proteins and proteins strongly imply that the patient’s own tumor can act as a source of immunizing protein and that mutated ras proteins can elicit an immune response in humans.

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 DEAE ion exchange column. Fractions containing ras protein were analyzed by SDS-PAGE and Western blotting. The fractions containing p21 ras were pooled, 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-ß-D-galactopyranoside was added to a final concentration of

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2 To whom requests for reprints should be addressed, at Division of Oncology, BBB321 Health Sciences Building, University of Washington, Box 356527, Seattle, WA 98195.

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 × 10^5) 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. T-cell Proliferative Assay. Fifty to 100 ml blood from each cancer patient or normal individual were obtained following informed consent. PBL were incubated with no antigens, or 100 μg/ml ras peptide (20 μg/ml), or tetanus toxoid (10 μg/ml), or phytohemaglutinin (5 μg/ml). The plates were incubated in a humidified atmosphere under 5% CO2 tension at 37°C for 96 h and then incubated for 8 h with 1 μCi (37 MBq) of [3H]thymidine/well. Plates were then harvested, and thymidine incorporation was determined.

All determinations of proliferation were carried out in at least five replicated wells. The S.I. was calculated by dividing cpm (mean) obtained from each group with cpm (mean) from autologous PBL incubated with no antigen. Basal proliferation levels varied from patient to patient but were less than 2000 (cpm) in all individuals. Positive proliferative responses were defined as a S.I. greater than 2. 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 (Fig. 1B).

### 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.

### Table 1 Normal and mutated ras peptides used

<table>
<thead>
<tr>
<th>Peptide name</th>
<th>Length</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras G12 (p4-21)</td>
<td>18 mer</td>
<td>YKLVVAGGVGKSLTI</td>
</tr>
<tr>
<td>Ras C12 (p4-21)</td>
<td>18 mer</td>
<td>YKLVVAGGVGKSLTI</td>
</tr>
<tr>
<td>Ras S12 (p4-21)</td>
<td>18 mer</td>
<td>YKLVVAGGVGKSLTI</td>
</tr>
<tr>
<td>Ras V12 (p4-21)</td>
<td>18 mer</td>
<td>YKLVVAGGVGKSLTI</td>
</tr>
<tr>
<td>Ras D12 (p4-21)</td>
<td>18 mer</td>
<td>YKLVVAGGVGKSLTI</td>
</tr>
<tr>
<td>Ras D13 (p4-21)</td>
<td>18 mer</td>
<td>YKLVVAGGVGKSLTI</td>
</tr>
</tbody>
</table>

* The abbreviations used are: PBL, peripheral blood lymphocytes; S. I., stimulation index.

### Table 2 Normal and mutated ras peptides used

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* Normal (wild-type) p21 ras protein contains Gly at amino acid position 12.

[Diagram showing T-cell immunity to ras proteins]
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).

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.

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 subsets. Extrapolating the results to humans requires a better definition of which proteins expressed by malignant human cells can serve as antigens for T-cell attack. The search for tumor antigens has become more sharply focused by the realization that T-cells recognize peptides in the cleft of MHC molecules and by the realization that malignant transformation is the end result of a series of DNA mutations in genes related to normal cell growth control and differentiation. A major potential source of tumor antigens might then be peptide fragments of proteins expressed uniquely by dominant oncogenes or by the genes turned on down-stream of the oncogene (13). The Ras protein has been studied by our group as a prototype of an oncogenic protein that is a result of a somatic DNA point mutation. ras oncogenes are activated by point mutations that encode proteins with single substituted amino acids. The activating amino acid substitutions impair intrinsic GTPase activity of the ras protein and generate constitutively activated signaling complexes with transforming activity (4, 14). Ras protein was chosen for study because ras mutations are common in human malignancy, and the common mutations are limited in number and location in the molecule. Importantly, the common mutations present in human malignancy are also commonly observed in murine malignancies. Therefore, the principles developed in animal models should be extrapolatable to humans. Previous studies have been shown that mutated ras proteins are able to serve as tumor-specific antigens in animal models (13). Under well-defined circumstances, CD8+ CTLs and CD4+ helper T lymphocytes specific for the mutated segment of p21 ras protein can be generated (7, 8). T cells immune to mutated ras peptides can lyse cells transformed by the ras protein with the same mutation (8).

The observation that p21 ras protein can be immunogenic in mice increases the likelihood that primed T- and B-cell responses might be present in patients with ras-positive malignancies and begs the issue as to whether detection of immune responses to p21 ras can be used to indicate the presence of small foci of cancer in vivo. Somatic
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 with an 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.2 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 have 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 ras-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. Immunoglobulin 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 can 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 T cell 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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