Mutation-Selective Tumor Remission with Ras-Targeted, Whole Yeast-Based Immunotherapy

Yingnian Lu, Donald Bellgrau, Lori D. Dwyer-Nield, Alvin M. Malkinson, Richard C. Duke, Timothy C. Rodell, and Alex Franzusoff

Abstract

Activating mutations in Ras oncoproteins represent attractive targets for cancer immunotherapy, but few vectors capable of generating immune responses required for tumor killing without vector neutralization have been described. Whole recombinant yeast heterologously expressing mammalian mutant Ras proteins were used to immunize mice in a carcinogen-induced lung tumor model. Therapeutic immunization with the whole recombinant yeast caused complete regression of established Ras mutation-bearing lung tumors in a dose-dependent, antigen-specific manner. In combination with the genomic sequencing of tumors in patients, the yeast-based immunotherapeutic approach could be applied to treat Ras mutation-bearing human cancers.

Introduction

Activating mutations in the K-, H- or N-Ras proto-oncogene family occur in a high percentage of human epithelial cancers, including pancreatic, colorectal, and non-small cell lung cancer. It is estimated that mutated Ras proteins are involved in 20% of all human cancers (1, 2). Ras gene products are converted from inactive (GDP-bound) to active (GTP-bound) states for signaling in cell proliferation pathways downstream of receptor tyrosine kinases, such as the epidermal growth factor receptor family (2, 3). Deregulated Ras activation in tumors is accounted for by mutations in codons 12, 13, and 61 that cause Ras to remain in the constitutive GTP-bound, activated state. Because of the central role for Ras activation in tumor proliferation, targeted destruction of cells harboring mutant Ras proteins could result in remission of a broad range of human cancers. Immune-mediated destruction of mutant Ras-containing cells represents a desirable and highly selective approach to tumor control.

We reported previously the development of a novel approach to antigen presentation and immune activation through the use of whole yeast-based immunotherapeutics (4). Administration of intact baker’s yeast, Saccharomyces cerevisiae, engineered to produce recombinant antigens present in tumor targets provoked cell-mediated immune responses in vivo. Previous work has shown that yeast is avidly taken up by dendritic cells and macrophages (4–7). The phagocytosis and degradation of the internalized yeast by dendritic cells is driven by pattern recognition receptors, such as Toll-like receptors and mannose receptors (8–10). Because yeast exhibits molecular patterns typically associated with pathogens, dendritic cells mature and become activated after receptor-mediated yeast phagocytosis (4). The heterologous tumor antigens in yeast are digested into peptides for presentation via class II and class I MHC receptors that trigger the activation of antigen-specific cytotoxic T lymphocytes (CTL) and helper T cells (4, 6, 7, 11). Prophylactic immunization of mice with ovalbumin-expressing yeast elicited immune-mediated rejection of transplanted ovalbumin-transfected lymphomas or melanomas (Ref. 4; data not shown). Although tumor transplant models effectively establish that yeast invoke a productive immune response, transfected antigens (in this case ovalbumin) are not naturally expressed by tumor targets. In addition, because grafted tumor cell lines grow rapidly, it is difficult to apply meaningful post-graft immunotherapy in these transplant tumor models. Therefore, recombinant yeast strains were developed for immunotherapeutic use in more clinically relevant animal tumor models. In the present study, we report that administration of whole yeast expressing mutated Ras proteins triggered the selective ablation of established, carcinogen-induced, mutant Ras-expressing lung tumors.

Materials and Methods

Yeast Engineering. GI-4001 yeast was engineered to express K-Ras mutant Q61R-epitope containing gene product by cloning the mutant gene from E9 mouse lung adenocarcinoma cell line. Total RNA extracted using Trizol reagent (Invitrogen Life Technologies, Inc.). The SuperScript reverse transcriptase kit (Invitrogen life Technologies) was used for cDNA synthesis with a K-Ras-specific reverse primer (5′-GCTCGGCTGCGGCCGCTACA-TACAACGTGACACCTTGTCCT-3′, all primers from Qiagen) followed by PCR using high fidelity TaqDNA polymerase (Invitrogen) with the same reverse primer plus the K-Ras-specific forward primer (5′-GAATTCCTAC-GATGGCAGCAGTGATATAACCTTGTTG-3′). The amplified DNA fragment encoding K-Ras Q61R protein was ligated to pYEX-BX (Amrad, Richmond, Victoria, Australia), transfected into W303α yeast (American Type Culture Collection) using the standard lithium acetate protocol. Ras protein expression in yeast is under control of the copper inducible Cup1 promoter. Copper sulfate is added to yeast cultures during log phase growth. Cells are harvested, washed in PBS, then heat-killed by incubation at 56°C for 1 h, followed by washing and resuspension in PBS. Heat-killed yeast suspensions were stored at 4°C until use. Positive clones were screened by immunoblot, using anti-e-k-ras antibody (Ab-1; Oncogene) to detect the Ras protein, and goat antimouse IgG-HRP (Jackson Immunolaboratory) plus Western Lightning Chemiluminescense reagent (Perkin-Elmer Life Sciences) to visualize the primary antibody. The GI-4014 yeast was engineered for expression of mammalian Ras protein expressing Q61L, G12V, and Q61R epitopes in two steps by PCR-mediated cloning of a truncated K-Ras Q61R template from GI-4001 yeast. The forward primer 5′-CGGAATTCACCATGGCAGCAGTGATATAACCTTGTTGTTGGAGCTTGC-3′ and the reverse primer 5′-GCTCGGCTGCGGCCGCTACAATTTCTCGGACTAATGT-3′ were used with the same reverse primer as above to generate the Ras construct harboring the G12V mutant epitope. The G12V-encoding DNA was then used as a template for appending the Q61L epitope and truncating the K-Ras-specific sequences from the 3′ end of the gene. The forward primer 5′-GGAATTCACCATGGCAGCAGTGATATAACCTTGTTGTTGGAGCTTGC-3′ and the reverse primer 5′-GCTCGGCTGCGGCCGCTACAATTTCTCGGACTAATGT-3′ were used to generate the PCR fragment with the truncated Ras gene...
construct harboring the Q61L, G12V, and Q61R mutant epitopes. The DNA fragment was ligated into pYEX-BX vector, transfected into yeast, and screened for positive clones, as described above.

**K-Ras Protein Quantification.** Yeast were lysed by vortexing with glass beads in 2X SDS gel sample buffer containing protease inhibitors (Roche Molecular Biochemicals). Ras protein quantification was performed by immunoblot, as above, using recombinant Ras protein (BioMol) as the standard. Chemiluminescent signals were captured with the ChemiDoc XRS system (Bio-Rad) and quantified using QuantityOne software (Bio-Rad).

**Urethane-Induced Tumor Model.** Urethane induction was performed with A/J mice (males, 4–6 weeks) purchased from The Jackson Laboratory. All animals were housed under standard laboratory conditions in the Center of Laboratory Animal Care in University of Colorado Health Sciences Center. The animals were treated with freshly prepared urethane (ethanol carboxamide; Sigma) administered as a single i.p. injection of 1 mg of urethane/g body weight dissolved in 0.9% NaCl (saline). For immunotherapy, PBS (mock-treated) or yeast suspended in PBS were administered s.c. to animals at the intervals and doses described in the text. Animals were sacrificed 100 to 120 days post-urethane exposure, and the lungs were dissected to harvest the adenomas. Individual tumors were measured with digital calipers under the dissection microscope.

**Tumor DNA Isolation and Sequencing.** Genomic K-Ras exon-2 mutation analysis was performed after proteinase K (Invitrogen, Inc.) digestion of tumor suspended in TNES lysis buffer (50 mM Tris-Cl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS) at 55°C overnight. Genomic DNA was precipitated by isopropanol alcohol and dissolved in 20 μl of distilled water. The K-Ras exon-2 DNA was PCR amplified from genomic DNA with two primers that hybridize to introns flanking exon 2 sequences, with the forward primer being 5'-GTTCTTCTATTGTTGAGCTG-3' and the reverse primer being 5'-ACAGGAAATCTGCTATCTG-3'. Thermal cycling conditions were as follows: 94°C, 5 min followed by 36 cycles of 94°C, 30 s; 55°C, 25 s; 68°C, 30 s; and 68°C, 5 min in GeneAmp PCR System 2700 (Applied Biosystems). The PCR fragment was isolated from 1.5% agarose gels, then subjected to double-stranded sequencing at the DNA-Sequencing facility of the University of Colorado Cancer Center using the same primers used for PCR amplification.

**Results and Discussion**

Urethane-induced lung tumors in A/J mice harbor Ras codon 61 mutations (12, 13). In this model, hyperplasias appear in the lung 2–3 weeks after urethane exposure (Fig. 1A), whereas microscopically visible adenomas are evident after 5 weeks (Fig. 1B). By 14 weeks after urethane exposure, 21 to 50 macroscopic adenomas are observed in the lungs of A/J mice (Fig. 1C). By 10 months, adenocarcinomas occupy a whole lung lobe, and by 12 months, the mice die from respiratory distress (14–16). Virtually all malignant mutant Ras-containing tumors after urethane exposure have been reported to encode arginine in place of wild-type glutamine (Q61R) at codon 61 (12). GI-4001, which is composed of whole yeast expressing the Q61R mutant K-Ras protein as the tumor antigen (Fig. 2A) was administered s.c. in 4 doses (2 × 10⁷ yeast/dose) to A/J mice beginning at, or solely after urethane treatment (one i.p. injection). Sixteen weeks after urethane exposure, adenomas in the lungs were excised, counted, and the tumor volume was measured. The results of this study demonstrated that animals receiving 4 post-urethane doses of GI-4001 yeast showed a statistically significant reduction in the average total tumor volume per mouse, accompanied by a 10% reduction in average total tumor number, when compared with mock-immunized mice (Table 1).

Despite reports that activating K-Ras Q61R mutations dominate the genotype of urethane-induced tumors in mice (12, 17, 18), we hypothesized that the presence of residual tumors after treatment with GI-4001 yeast could be attributable to mutations unrelated to K-Ras Q61R. Genotype analysis of tumors showed that after 4 doses with GI-4001 yeast, the percentage of K-Ras Q61R mutant-containing tumors was reduced by 38% compared with mock-treated animals (Table 2). Nevertheless, the other tumors post-urethane exposure...
and the results are presented as the % reduction in average tumor volume per mouse in the GI-4001-treatment group compared to mock-treated animals. ANOVA-Student

solely post-urethane exposure (days

H11001

A

C

, the tumors were excised, counted, and measured, shown in Fig. 1

challenge in mice (data not shown). Hence, in the urethane-induced

doses was required to achieve protective immunity against tumor

yeast-based immunotherapy showed that the administration of at least

mutations and those exhibiting no mutations in

tumors, the percentage of tumors expressing wild-type K-Ras protein

with GI-4014 yeast alone or in the GI-4001 plus GI-4014 treatment

with GI-4001 yeast (Fig. 3

versus

mock treatment); however, these doses had no impact on the

with GI-4014 yeast (Fig. 3

or K-Ras with leucine mutations at codon 61 was not affected by

exon-2 genotype of tumors from each treatment group

was analyzed by PCR amplification and DNA sequencing of isolated

genic DNA. In the mock-treatment group, 56% of the tumors

K-Ras Q61R mutations, whereas the remaining 44% of

tumors were evenly divided among those that harbored K-Ras Q61L

and those exhibiting no mutations in K-Ras exon-2 (Fig. 3C).

Dosing with the GI-4001 series yeast strains resulted in the

immune-mediated remission of Q61R-containing adenomas in a dose-

dependent manner. Six doses of GI-4001 yeast decreased the percent-

age of K-Ras Q61R-expressing tumors to 22% of those sequenced.

Ten doses of GI-4014 yeast further decreased the percentage of K-Ras

Q61R-expressing tumors to 11% (Fig. 3C). Six doses of GI-4014 plus

GI-4001 yeast elicited complete remission of tumors harboring K-Ras

Q61R mutations in a statistically significant manner (\(P = 0.024\) versus mock treatment); however, these doses had no impact on the

percentage of tumors harboring K-Ras Q61L mutations or tumors

without mutations in the K-Ras exon-2 gene (Fig. 3C).

In contrast to the immunotherapeutic ablation of Q61R-containing

tumors, the percentage of tumors expressing wild-type K-Ras protein

or K-Ras with leucine mutations at codon 61 was not affected by

treatment with GI-4014 yeast (Fig. 3C). Interestingly, the size of

mutant Q61L-expressing tumors decreased by 28% in mice treated

with GI-4014 yeast alone or in the GI-4001 plus GI-4014 treatment

group, compared with a 4% decrease in the size of tumors expressing

wild-type K-Ras. These results confirm other studies that tumor-

protective immune responses were antigen-specific and could not be

provided by mock treatment of administering yeast lacking the target

antigen (Ref. 4; data not shown). The partial diminution in Q61L-

expressing tumors suggests that the Q61L-containing epitope in GI-

4014 yeast may be eliciting incomplete antigen-specific responses in

immunized animals. The weaker Q61L-directed response may be

attributable to three factors: the restricted size of the Q61L epitope

encoded a leucine mutation at codon 61 (Q61L) or expressed only

wild-type K-Ras protein. Thus, a significant percentage of urethane-

induced tumors expressed mutations not targeted by GI-4001 yeast

immunotherapy. The GI-4014 yeast strain (Fig. 2B) was engineered to

express a mutant Ras protein that would target tumors bearing Q61R,

Q61L, and a codon 12 mutation more frequently found in human cancers. As all three (K-, H-, and N-) Ras family members in humans

and rodents have identical polypeptide sequences in the domains

where the tumor activating mutations occur, the yeast-expressed Ras gene was truncated to encode only a sequence common to all members of rodent and human Ras proteins.

The administration of GI-4014 yeast was compared to dosing with

GI-4001 yeast in the A/J mouse urethane induction tumor model. The
dosing regimen was altered to achieve a greater therapeutic effect.

Dosing was initiated 2 weeks after urethane exposure, where hyper-

plasias were already established (Fig. 1A). Dosing in this study was bi-weekly (6 doses) or weekly (10 doses) with 5 \(\times\) 10^7 yeast/dose, compared with mock treatment with saline. Previous experience with yeast-based immunotherapy showed that the administration of at least 2 doses was required to achieve protective immunity against tumor challenge in mice (data not shown). Hence, in the urethane-induced tumor model, yeast-triggered immune responses would begin to impact the tumors only after they had reached the size of microadenomas at \(\sim\)5 weeks post-urethane exposure (Fig. 1B). At 14 weeks post-

urethane exposure, where untreated tumors would reach the size shown in Fig. 1C, the tumors were excised, counted, and measured, and the average total tumor volume per mouse is shown in Fig. 3A.

The administration of 6 doses of GI-4001 yeast resulted in a statistically significant 39% reduction in average tumor volume per mouse (Fig. 3A, cf. Lanes 2 and I, with 18% decrease in average total tumor number per mouse) compared with the previously observed 28% tumor volume reduction per mouse with four GI-4001 doses (Table 1). Six doses of GI-4014 yeast caused a 28% reduction in average total tumor volume per mouse (Fig. 3A, cf. Lanes 3 and I). Increasing the GI-4014 yeast administration to 10 (weekly) doses elicited a statistically significant 55% reduction in average tumor volume per mouse (Fig. 3A, cf. Lanes 4 and I, with 24% reduction in tumor number). Dosing six times with both GI-4001 and GI-4014 yeast (administered at distinct s.c. sites) yielded a statistically significant 50% reduction in average tumor volume (Fig. 3A, cf. Lanes 5 and I, with 25% reduction in tumor number). Although the accuracy of determining changes in tumor volume may be limited, Zhang et al. (19) demonstrated recently a nearly perfect correlation between tumor burden and the circulating titer of surfactant protein D, a collectin involved in inflammation. This linearity supports the validity of this method for evaluating tumor burden, which is identical to the methods used herein.

The results suggested that eliciting immune-mediated control of urethane-induced tumor burden was dose-dependent. This hypothesis was supported by analysis of the Ras antigen content in each of the immunotherapeutic yeast strains. GI-4001 yeast expressed the highest level of Ras antigen at 1,300 ng of Ras protein/10^7 yeast compared with 600 ng of Ras protein/10^7 GI-4014 yeast cells (Fig. 3B). Hence, 6 doses of GI-4001 provided the immune system with approximately 2.2-fold more Ras antigen content than 6 doses of GI-4014 yeast.

Combining GI-4001 and GI-4014 for 6 doses provided the immune system with 50% more Ras antigen content than dosing with GI-4001 alone and 3-fold more Ras antigen than GI-4014 yeast administered alone. Therefore, the improved control of tumor burden correlated with Ras antigen content in GI-4001 and GI-4014 yeast.

The K-Ras exon-2 genotype of tumors from each treatment group

was analyzed by PCR amplification and DNA sequencing of isolated

genic DNA. In the mock-treatment group, 56% of the tumors

expressed K-Ras Q61R mutations, whereas the remaining 44% of

tumors were evenly divided among those that harbored K-Ras Q61L

mutations and those exhibiting no mutations in K-Ras exon-2 (Fig. 3C).

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Ten doses of GI-4014 yeast further decreased the percentage of K-Ras

Q61R-expressing tumors to 11% (Fig. 3C). Six doses of GI-4014 plus

GI-4001 yeast elicited complete remission of tumors harboring K-Ras

Q61R mutations in a statistically significant manner (\(P = 0.024\) versus mock treatment); however, these doses had no impact on the

percentage of tumors harboring K-Ras Q61L mutations or tumors

without mutations in the K-Ras exon-2 gene (Fig. 3C).

Table 2 Genotype analysis of urethane-induced tumors

<table>
<thead>
<tr>
<th>Immunotherapy</th>
<th>K-Ras</th>
<th>K-Ras</th>
<th>K-Ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-Ras</td>
<td>Q61L-Arg</td>
<td>Q61L-Leu</td>
<td>Q61L (wt)</td>
</tr>
<tr>
<td>GI-4001</td>
<td>20%</td>
<td>67%</td>
<td>13%</td>
</tr>
</tbody>
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Abbreviations: wt, wild type.

The percent of tumors harboring mutations in the K-Ras exon 2 gene at codon 61 is shown.

10% (\(P < 0.05\))

Mock 32% 50% 18%

GI-4001 20% 67% 13%

GI-4001 (4 doses) initiated post-urethane

GI-4001 yeast elicited complete remission of tumors harboring K-Ras Q61L mutations in a statistically significant manner (\(P = 0.024\) versus mock treatment); however, these doses had no impact on the percentage of tumors harboring K-Ras Q61L mutations or tumors without mutations in the K-Ras exon-2 gene (Fig. 3C).

In contrast to the immunotherapeutic ablation of Q61R-containing tumors, the percentage of tumors expressing wild-type K-Ras protein or K-Ras with leucine mutations at codon 61 was not affected by treatment with GI-4014 yeast (Fig. 3C). Interestingly, the size of mutant Q61L-expressing tumors decreased by 28% in mice treated with GI-4014 yeast alone or in the GI-4001 plus GI-4014 treatment group, compared with a 4% decrease in the size of tumors expressing wild-type K-Ras. These results confirm other studies that tumor-protective immune responses were antigen-specific and could not be provided by mock treatment of administering yeast lacking the target antigen (Ref. 4; data not shown). The partial diminution in Q61L-expressing tumors suggests that the Q61L-containing epitope in GI-4014 yeast may be eliciting incomplete antigen-specific responses in immunized animals. The weaker Q61L-directed response may be attributable to three factors: the restricted size of the Q61L epitope

Table 1 Impact of four doses of GI-4001 yeast on control of urethane-induced tumors

<table>
<thead>
<tr>
<th>Immunotherapy</th>
<th>% reduction in average tumor volume/mouse versus mock-treated</th>
<th>% reduction in average no. tumors/mouse versus mock-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>GI-4001 (4 doses) initiated with urethane</td>
<td>22% ((P &lt; 0.05))</td>
<td>None</td>
</tr>
<tr>
<td>GI-4001 (4 doses) initiated post-urethane</td>
<td>29% ((P &lt; 0.01))</td>
<td>10% ((P &lt; 0.05))</td>
</tr>
</tbody>
</table>
provided by the Ras protein expressed in GI-4014 yeast (Fig. 2B), that fewer functional T cells are activated in A/J mice in response to immunization with the Q61L epitope, or that the tumors do not efficiently present the Q61L mutant epitope for T-cell recognition. The Q61L epitope expressed in GI-4014 yeast is appended as a 14 amino acid NH2-terminal fusion domain to the Ras protein. The Q61L Ras segment may not have provided a sufficiently long sequence for the presentation of a 9 amino acid peptide of the appropriate register for the mutant epitope to fit into the MHC-I receptor groove to stimulate a high affinity T-cell response. Ras protein constructs that harbor the Q61L mutant epitope in a manner analogous to that used for Q61R in GI-4001 (Fig. 2A) would distinguish between these possibilities.

The results of this study thus revealed that the dosing regimen of yeast-based immunotherapy was key to eliciting improved tumor control, in that the administration of yeast with elevated Ras antigen content led to complete remission of the Ras mutant Q61R-bearing tumors. This study represents the first report of immunotherapeutic control of established urethane-induced tumors in mice. However, several previous reports provided indications that successful immunotherapy against carcinogen-induced murine lung cancer might be possible. For example, one showed that the transplantation of immunocompetent lymphocytes reduced lung tumorigenesis (20) and another showed that lymphocyte depletion before carcinogen administration enhanced tumorigenesis (21). Therefore, a role for lymphocytes in tumor control has been well documented, although eliciting...
protective therapeutic immune responses had not been described previously.

An important question is the relevance of this animal model to human disease. The commonalities between mouse and human lung cancers have been reviewed in detail, including the classification of mouse tumors along the same lines as the current WHO classification for human lung cancers to coordinate these two classification schemes (14, 22). The principal difference between mouse and human lung cancers is that there is a continuum from hyperplasia to adenoma to carcinoma in the mouse that allows study of early stages, whereas in human adenocarcinomas, the only early lesions commonly observed are the atypical adenomatous hyperplasias. However, the distinction between focal hyperplasia and adenoma is not clear, and recent evidence suggests that a subclass of both the mouse and human early lesions will have a greater tendency for progression to malignancy. Therefore not surprisingly, this mouse model has generated pharmacological information that is now being applied to the human disease, such as clinical trials involving budesonide and Aptosyn (exisulind, sulindac sulfone). Thus, information contained in this paper reporting the success of therapeutic immunization for the rejection of mouse lung adenomas is likely to have significant benefit for the study and treatment of human cancer.

In summary, this report demonstrates that whole recombinant yeast provoke immune responses that therapeutically control established, mutated oncoprotein-expressing tumors. These immune responses are antigen-specific and result in complete remission of target antigen-bearing cells. The fact that the improved immune response was seen with repeated dosing of recombinant yeast over a 3-month period confirmed that the backbone yeast vector is not neutralized by the host immune system. These observations suggest that dosing with yeast immunotherapeutics is effective for both priming and boosting of the immune response. Because the Ras protein expressed in GI-4014 yeast is nearly identical to the sequences found in human cells, including the frequently observed G12V mutant epitope, this yeast strain is directly applicable for use in treating Ras mutation-bearing human cancers. In combination with genomic sequencing of tumors before initiation of immunotherapy, this approach has potential for immediate application to human cancers characterized by mutations in Ras.

Acknowledgments

We thank Bin Zou, Charles Kemmler, and Drs. S. Munson, L. Kisley, Z. Guo, D. Chan, A. Pardi, T. King, and A. Haller for contributions to this work, and the DNA sequencing core facility of the CU Cancer Center.

References

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Cancer Res 2004;64:5084-5088.

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