A Naturally Occurring Mutant Human Epidermal Growth Factor Receptor as a Target for Peptide Vaccine Immunotherapy of Tumors

David K. Moscatello, Gloria Ramirez, and Albert J. Wong

Department of Microbiology and Immunology [D. K. M., G. R., A. J. W.], Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Abstract

The type III EGF receptor (EGFRvIII) is the result of an in-frame deletion from nucleotides 275 to 1075 in the EGF receptor cDNA sequence creating a novel epitope at the fusion junction. This spontaneously occurring alteration is found in a high percentage of primary human brain, breast, lung and ovarian tumors. We have explored whether a peptide derived from the fusion junction could serve as the basis for an antitumor vaccine. Preimmunization of mice with this peptide substantially inhibited tumor formation by cells expressing EGFRvIII. Tumor cell inoculation followed by immunization could also enhance the regression of existing tumors. Antibody production was elicited in animals that was highly specific for the novel epitope and also a CTL response that was mediated by CD8+ T lymphocytes. The alteration present in EGFRvIII could serve as the basis for an antitumor vaccine with potentially wide application in humans.

Introduction

Vaccines directed against neoplastic cells offer the possibility of a simple but potentially efficacious means for the prevention or therapy of tumors. Vaccines using whole tumor cells modified with haptenes (1) or mixed with Bacillus Calmette-Guérin (2) have been successful in inducing tumor regression in human melanoma patients, indicating the potential of this approach. To enhance antitumor effects and avoid potential systemic problems, it would be highly desirable to use well-defined antigens. Vaccines based on peptides have been successful in the prevention (3) or regression of solitary masses in animals (4), and there has been at least one report of the treatment of micrometastatic disease (5). Ideally, such peptides should also be derived from antigens that are expressed strictly on tumor cells. The genetic alterations known to be present in human tumors provides a potentially rich source for targets (6). Peptide vaccines based on point mutations such as those found in p53 (7) or ras (8) have been shown to elicit both a MHC class I and II response that will lyse target cells. However, any antitumor effects of these peptides has not been established in animal model systems, although it has been shown that immunization with whole mutant ras protein can elicit a therapeutic response (9).

The EGFR receptor has been implicated in the pathogenesis of several human tumors, including those of the brain, breast, ovary, prostate, and skin (10). Several different types of alterations within the EGF receptor gene that result in aberrant protein products have been detected in human glial tumors (11-13). The EGFRvIII is the result of the deletion of exons 2-7 due to either alternative splicing or a rearrangement within the EGF receptor gene (11, 12). This results in the in-frame joining of nucleotides 275-1075 in the EGF receptor cDNA and the creation of a novel epitope (14). Subsequent studies have shown that this is the most prevalent EGF receptor alteration found in human cancers. The EGFRvIII protein has been detected in 57% of primary human glial tumors, 78% of breast carcinomas, 16% of non-small carcinoma of the lung tumors, 86% of medulloblastoma tumors, and 75% of ovarian tumors. It has been estimated that the incidence in the United States alone is >150,000 cases/year (15). Moreover, the EGFRvIII is tumor specific, given that it has not been detected in any normal tissue examined (14, 16). Using a peptide based on the sequence at the mutant junction, we have succeeded in raising both polyclonal and monoclonal antibodies that specifically recognize this mutant (14, 17). The fact that this mutant EGF receptor is tumor specific and at least capable of eliciting a humoral response in animals raises the possibility that a vaccine based on this alteration could be used as a peptide vaccine against tumors. In this report, we demonstrate the feasibility of using pepEGFRvIII for the prevention and regression of tumors expressing EGFRvIII.

Materials and Methods

Cell Lines. The HC2 20d2/c cell line, which expresses the EGFRvIII, and the CO12 20c2/b line, which expresses the normal human EGF receptor, have been described previously (18). Briefly, they were derived by transfection of NIH-3T3 cells with either the pLTR-HC2 or pLTR-CO12 (19) and pKomeo at a 20:1 ratio. G418-resistant colonies (500 µg/ml) were obtained and screened for protein expression to obtain clones that expressed similar numbers of normal or mutant receptors and then subcloned further to derive a pure population. Rat-1 fibroblasts expressing the EGFRvIII (rat-1 H2C C11) at levels similar to that of the HC2 20d2/c cell line were obtained in the same fashion.

Tumor Formation in Animals. Eight to 10-week-old female NIH Swiss mice and female Fischer 344 rats of similar age were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Tumor cells were injected s.c. into the right hind flank at the doses indicated in "Results." Tumor size were measured every day in three different axes; tumor size was calculated according to the formula: [(4/3π)(length x width x height)]/2. When the tumors exceeded 10% of body weight or the animal was moribund, it was sacrificed. Daily mean tumor volumes were calculated based on surviving animals.

Peptide Encompassing the EGFRvIII Alteration and Vaccination. A 14-amino acid peptide corresponding to the junction present in EGFRvIII was chemically synthesized and purified by high-performance liquid chromatography. In single-letter code, this peptide was LEEKGNYVYTDHC, where the underlined glycine represents the novel amino acid created by the fusion of these two normally distant sequences and the terminal cysteine was added for the purposes of conjugation (14). This peptide was conjugated to keyhole limpet hemocyanin at a 1:1 ratio (w/w) and used for immunization. Control animals were vaccinated with keyhole limpet hemocyanin at a 1:1 ratio (w/w) and used for immunization. Control animals were vaccinated with keyhole limpet hemocyanin only. Mice were injected s.c. with 100 µg of the immunogen in Freund's complete or incomplete adjuvant according to the schedule as described in "Results."

Western Blots. Cells were lysed or tumors were homogenized in PBS/TDS (Triton X-100/deoxycholate/SDS) buffer (15). Fifty µg of protein were electrophoresed in SDS-PAGE followed by Western blot analysis (15). Affinity-purified rabbit polyclonal antibody against pepEGFRvIII has been described...
VACCINE IMMUNOTHERAPY AGAINST EGFRvIII

Table 1  Summary of pre- and postvaccination experiments with pepEGFRvIII

<table>
<thead>
<tr>
<th></th>
<th>Prevaccination</th>
<th>Postvaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>pepEGFRvIII</td>
</tr>
<tr>
<td>Number dead or sacrificed</td>
<td>13 of 16</td>
<td>4 of 16</td>
</tr>
<tr>
<td>Tumor size distribution (mm³)</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>0—500</td>
<td>5,100</td>
<td>8</td>
</tr>
<tr>
<td>501—1000</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>1001—1500</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>&gt;1501</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

A Five tumors were recurrent; four did not express EGFRvIII.
B Maximum tumor size.

Immunization with a Peptide Vaccine from EGFRvIII Prevents Tumor Formation. We first generated cell lines expressing the EGFRvIII and tried to identify appropriate animal hosts in which the efficiency of tumor formation was reproducible and rapid. NIH-3T3 and Rat-1 fibroblasts were transfected with an expression vector containing the EGFRvIII cDNA, and two clones were derived, HC2 20d2/c (NIH-3T3) and HC2-C11 (Rat-1), which expressed nearly identical levels of the mutant receptor. Injection of 10⁶ HC2-C11 cells into syngeneic Fischer rats produced tumors in approximately one-half of animals but these regressed rapidly. In contrast, injection of 10⁷ HC2 20d2/c cells into NIH-Swiss mice produced tumors in >90% of animals within 6—8 days, which progressed as noted below. In this host, tumor formation was noted at doses down to 10⁵ cells, although 10⁶ cells provided the most rapid and uniform tumor production.

Immunohistochemistry. Tumors that were progressing or undergoing regression were excised from animals immunized with pepEGFRvIII or control vaccine and frozen rapidly. Because these animals were sacrificed early, these animals were not included in other results reported. Six-μm cryostat sections were cut onto slides, blocked with goat serum; incubated with biotinylated antimouse CD4⁺, CD8⁺ (both from Life Technologies, Inc.), or control antibody; washed in PBS; incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories); washed again; and then incubated with diaminobenzidine-H₂O₂. Slides were washed with water and counterstained with H&E.

Results

Immunization with a Peptide Vaccine from EGFRvIII Enhances Tumor Regression and Eliminates Cells Expressing EGFRvIII. A, mean daily tumor volumes following injection with HC2 20d2/c cells. Mice were injected with 10⁷ HC2 20d2/c cells and immunized with pepEGFRvIII 4 days later. ——, control vaccinated; --- ---, pepEGFRvIII vaccinated. B, EGFRvIII expression in recurrent tumors from five pepEGFRvIII-vaccinated animals. Tumors had regressed by day 20—25 but later recurred in the same location, necessitating sacrifice by day 40—50. Western blots containing 50 μg of lysate from either the HC2 20d2/c (HC2) cells or the tumors (Lanes 1—5) were incubated with the polyclonal antibody against EGFRvIII. Only one animal (Lane 1) was highly positive for EGFRvIII expression, whereas another animal had low levels of expression (Lane 5).
We then investigated whether vaccination with a peptide corresponding to the fusion junction present in EGFRvIII (pepEGFRvIII) could prevent the formation of tumors from HC2 20d2/c cells. A total of 32 mice were used. Equal numbers of mice were immunized with pepEGFRvIII or control vaccine in Freund’s complete adjuvant, which was followed by immunization 4 weeks later in incomplete adjuvant. After another 2 weeks, the animals were injected with $10^7$ HC2 20d2/c cells. Only 4 of 16 mice that received pepEGFRvIII developed tumors, and in 2 animals the tumor progressed and necessitated sacrifice (Fig. 1; Table 1). In contrast, 13 of 16 mice that received control vaccine developed tumors, and in 9 animals, the tumors progressed and required sacrifice (Fig. 1). Comparison of the maximum tumor volumes showed a statistically significant difference between the two groups ($P < 0.028$, unpaired Student’s $t$ test). These results demonstrate that prior vaccination with pepEGFRvIII can result in a significant decrease in the overall incidence of tumor formation and affect the ultimate tumor size. Seven of the animals that had received pepEGFRvIII were rechallenged with $10^7$ HC2 20d2/c cells at periods of 6 months to 1 year later. No tumor formation was ever noted.

**The Peptide Vaccine Enhances Tumor Regression and Eliminates Cells Expressing EGFRvIII.** Because pepEGFRvIII vaccination was effective at preventing tumors, we wished to determine whether this vaccine could induce the regression of existing tumors. A total of 60 mice were injected with HC2 20d2/c cells, and 4 days later the animals received a single injection of either pepEGFRvIII or control vaccine in a blinded fashion. This time period was chosen because, due to the aggressive nature of these cells, the tumors were visible by day 7, and would be at least $300 - 500$ mm$^3$ in size before any immune response would be seen. The average daily tumor volume for these two groups of mice is shown in Fig. 2A. Although both groups of mice had similar kinetics of tumor growth until day 12–13 (8–9 days postimmunization), substantial regression was evident thereafter in the group that received the pepEGFRvIII vaccine. There was a statistically significant difference in tumor volumes between the two groups 21 days postvaccination ($P < 0.05$). Vaccination also affected the ultimate tumor size, as animals receiving the pepEGFRvIII vaccine were skewed greatly toward smaller tumor volumes (Table 1).

Twenty-six of 29 mice in the pepEGFRvIII-vaccinated group showed regression of the original tumor, but only 15 of 29 in the control vaccinated group did. Thirteen animals required sacrifice in the control vaccinated group, whereas eight animals were sacrificed in the pepEGFRvIII group (Table 1). However, this includes five animals in the pepEGFRvIII group that showed complete regression of the original tumor but ~40–50 days later developed a second tumor that ultimately required sacrifice. This phenomenon was not noted in any animal that had received control vaccine. These recurrent tumors were examined for expression of EGFRvIII by Western blot analysis (Fig. 2B). Interestingly, only one of these tumors showed expression of EGFRvIII comparable to the HC2 20d2/c cell line (Lane 1), another tumor had barely detectable expression of EGFRvIII (Lane 5), and the rest were negative. This suggested that the immune system in four of these animals was largely successful at eradicating cells expressing this mutant receptor and that the second tumor arose from variant cells within the original tumor mass.

Evidence for a CTL Response Mediated by CD8+ Lymphocytes. Because it is thought that a CTL response is necessary to reduce large tumor burdens (3), we performed CTL assays to determine whether such a response was present in animals that had rejected their tumors. Lymphocytes were isolated from pepEGFRvIII or control vaccinated mice and used in CTL assays with $^{35}$Cr-labeled HC2 20d2/c and CO12 20c2/b, an NIH-3T3 cell-transfectant clone that overexpresses the normal EGF receptor, as the targets. Lymphocytes...
from all pepEGFRvIII-vaccinated mice tested showed a dose-dependent lysis of HC2 20d2/c cells but not of C012 20c2/b, whereas lymphocytes from control vaccinated mice did not show specific lysis of any of these target cells (Fig. 3A and B). We tested whether pepEGFRvIII was the specific target of these CTLs. C012 20c2/b cells were pulsed with pepEGFRvIII or a related peptide from the normal EGF receptor. Cells loaded with pepEGFRvIII showed enhanced lysis over cells containing the normal peptide (Fig. 3C). To determine whether this response was mediated by class I-restricted T cells, CTL assays were performed after the CD8+ population had been depleted by treatment with anti-CD8+ antibody and complement. Although the presence of complement alone did not affect cell killing, the addition of the anti-CD8+ antibody substantially reduced the percentage specific lysis (Fig. 3D).

We also examined the nature of the lymphocytic infiltrate in these tumors. Immunohistochemistry was performed on tumors from animals that had been immunized and showed active regression of their tumors. Staining with anti-CD8+ or anti-CD4+ antibodies revealed intense positive staining of numerous lymphocytes throughout the tumor (Fig. 4, A and B), with the CD8+ cells far more abundant than the CD4+ cells. In contrast, immunohistochemistry on progressing tumors from control immunized animals showed almost a complete lack of lymphocytic infiltrate (Fig. 4, C and D). No staining was seen in the absence of primary antibody (Fig. 4E). Taken together, these data suggest that mainly CD8+ restricted CTLs were generated and present in the tumor mass.

The Molecular Alteration Present in EGFRvIII Is the Target of the Antitumor Response. Because the amino acid sequence and the exon/intron boundaries of the EGF receptor gene that give rise to EGFRvIII are conserved from chickens to humans, the peptide-elicited immune response seen was not due to a reaction against nonnative sequences. Other experiments demonstrated further that it was the novel junction present in EGFRvIII that was the main determinant of the immune response.

Previous work has shown that immunization with pepEGFRvIII elicits antibodies specific for the mutant and normal EGF receptor. As expected, 19 of 20 mice tested that had received pepEGFRvIII generated an antibody response specific for the mutant EGF receptor. Because of the nearly uniform response against EGFRvIII, the presence of antibody alone did not appear to correlate with subsequent tumor rejection. Next, we explored the mechanisms for tumor regression in animals that had not been vaccinated with pepEGFRvIII. In Western blots performed using sera from these animals, 12 of 15 animals tested showed the presence of antibodies directed against EGFRvIII but very little recognition of the normal human EGF receptor or of other antigens in NIH-3T3 cells (Fig. 5A). CTL assays using cells isolated from animals showing spontaneous regression demonstrated that, in all three animals examined, there were lymphocytes that specifically lysed cells expressing EGFRvIII but not cells expressing normal human EGF receptor (Fig. 5B). Thus, despite lack of prior immunization against this molecule, the novel epitope in EGFRvIII appeared to be a major factor in eliciting an antitumor response in mice undergoing spontaneous regression.

We also determined whether the NIH-3T3 cells themselves or the presence of the human EGF receptor could affect tumor formation. Mice were injected i.p. with 107 parental NIH-3T3 fibroblasts or C012 20c2/b cells and 1 month later challenged with 107 HC2 20d2/c cells injected s.c. in the hind flank. Six of the eight mice that received NIH-3T3 cells were sacrificed eventually due to the progression of the HC2 20d2/c-derived tumors; the other two mice never developed a tumor. Thus, there was no significant immunological response raised against the NIH-3T3 cell background. None of the eight mice that first received C012 20c2/b cells developed HC2 20d2/c-related tumors, indicating that other epitopes of the human EGF receptor can confer immunity to the subsequent challenge with HC2 20d2/c cells. However, all eight animals were sacrificed due to progression of the C012 20c2/b tumor, suggesting that the presence of the human EGF receptor alone was not sufficient to result in tumor rejection. These observations, taken together with the fact that antisera from animals exhibiting spontaneous regression fail to recognize the normal human EGF receptor and that CTLs from such animals do not show specific lysis of C012 20c2/b cells, suggest that the EGFRvIII fusion junction represents the dominant epitope and significantly facilitates tumor rejection.

Discussion

We have shown that the alteration present in EGFRvIII can serve as the basis for a peptide vaccine that can both prevent and enhance the regression of tumors expressing this receptor. Because this is a well-defined, highly prevalent antigen, it presents many advantages for the vaccine therapy of cancer in humans. To be effective as a prophylactic agent in humans, ideally, a memory effect should be present. We found that injection of tumor cells into animals that had received prior vaccination never resulted in tumor formation even up to 1 year following initial immunization. A similar effect was seen in animals
that had successfully eradicated tumors following vaccination. For the treatment of existing neoplasms, it is thought that a CTL response is necessary to eradicate large tumors. In CTL assays, we demonstrated the presence of cells that specifically targeted cells overexpressing EGFRvIII but not normal human EGF receptor. Depletion of the CD8+ population suggested that class I-restricted T cells were the major effectors of cell killing. This was supported by immunohistochemistry analysis, which showed a preponderance of CD8+ cells in tumors undergoing active regression. These factors, combined with its lack of expression in normal tissues, make a vaccine directed against EGFRvIII a promising candidate for therapy in humans.

Because pepEGFRvIII was also capable of activating a humoral response, this may have also contributed to the antitumor effect. It has been shown that cross-linking of the EGF receptor by monoclonal antibodies can induce the down-regulation of the receptor (21) and that the removal of this constant growth stimulus by this means can result in tumor reduction in animal models (22). In contrast, the EGFRvIII is unusual in that the receptor is dimerized constitutively in HC2 20d2/c cells (18), but the internalization of receptor has a t1/2 of 5-7 h as opposed to 15-30 min for intact EGF receptor (23). Following incubation with anti-EGFRvIII antibody, there is no reduction in receptor number or phosphotyrosine levels in these cells (18). Furthermore, although we have found antibodies against EGFRvIII in both pepEGFRvIII-vaccinated and control mice, there was no strict correlation between antibody levels and tumor regression. For these reasons, we do not believe that down-regulation of the receptor makes a large contribution to the tumor regression effects seen, although antibody-dependent cell-mediated cytotoxicity may play a role.

Although these studies were done in mice, there are other indications that peptide vaccine therapy directed against EGFRvIII could be successful in humans as well. In preliminary experiments, we have found that patients with glioblastoma multiforme tumors have serum antibodies against EGFRvIII. This suggests that the immune system of patients can recognize this antigen. Patients with breast tumors show immunity against the neu/HER-2 protein, and on this basis it has been speculated that these patients may benefit from the boosting of innate immunity through peptide vaccination (24). It is also worth noting that the amino acids surrounding the mutant junction in EGFRvIII conform to the motif for binding to HLA-A3, a frequently occurring MHC class I molecule in humans (25), and that it could also match that for HLA-A2 in either a kinked or extended conformation (26). Recently, Coulié et al. reported the first case of a point mutation within a peptide derived from a human cancer that could stimulate autologous CTLs (27). The sequence of this peptide, EKLIVVLF, bears an interesting similarity to that of pepEGFRvIII. Potential peptide vaccines are not limited to pepEGFRvIII, because at least five other alterations within the EGF receptor have been described to occur in tumors (11), and it has been found that more than one alteration can be present within one tumor (12). Our work suggests that alterations within the EGF receptor certainly merit consideration as the basis for antitumor vaccines.

References


A Naturally Occurring Mutant Human Epidermal Growth Factor Receptor as a Target for Peptide Vaccine Immunotherapy of Tumors

David K. Moscatello, Gloria Ramirez and Albert J. Wong


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/8/1419

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.