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EGFRvIII-targeted immunotoxin induces antitumor immunity that is inhibited in the absence of CD4+ and CD8+ T cells

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Abstract

Purpose—Immunotoxins as anti-cancer therapeutics have several potential advantages over conventional agents including a high specificity, extraordinary potency, and a lack of an identified mechanism for resistance. It has been clearly demonstrated that *Pseudomonas*-based immunotoxins have a direct cytotoxic effect. However, delayed and often dramatic antitumor responses seen in human studies with targeted toxins led us to hypothesize that immunologic responses may be a secondary mechanism that enhances the therapeutic efficacy of these novel drugs.

Experimental design—This hypothesis was tested in a murine system using an immunotoxin, MR1-1 [MR1-1 (dsFv)-PE38KDEL], that targets a syngeneic murine homologue of the tumor-specific human epidermal growth factor mutation, EGFRvIII, expressed on a murine cell line.

Results—Intratumoral treatment with MR1-1 eliminated EGFRvIII-expressing tumors ($P < 0.0001$). The antitumor activity of MR1-1 was dependent on the expression of EGFRvIII on some, but not all tumors cells, and was significantly inhibited in the absence of CD4+ ($P = 0.0193$) and CD8+ ($P = 0.0193$) T cells. MR1-1 induced EGFRvIII-specific immunity ($P < 0.0005$) and produced long lasting immunity against tumors expressing EGFRvIII as well as EGFRvIII-negative tumors.

Conclusions—These data suggest that immunotoxins may not be strictly dependent on direct cytotoxicity for their efficacy, but may also be potent inducers of antitumor immunity active even against cells that do not express the targeted antigen.

Keywords

Astrocytoma; Epidermal growth factor receptor; Immunotherapy; Immunotoxins; T lymphocytes

Introduction

Immunotoxins are a novel class of therapeutic agents that consist of a modified form of a bacterial or plant toxin that is targeted to tumor cells by a monoclonal antibody or antibody fragment. Toxin-based therapy has several potential advantages over conventional therapies

because recombinant immunotoxins are highly specific, extraordinarily potent, and lack an identified mechanism for resistance [3,5,19–21]. As a result, these agents have shown promise in the treatment of a variety of human neoplasms [2,7,15,17,22,23,27,28].

It has been clearly demonstrated that *Pseudomonas*-based immunotoxins have a direct cytotoxic effect wherein the immunotoxin binds to the target cell through a high affinity internalizing antibody fragment, enters into the cytosol through interactions with domain II of the exotoxin, and then uses domain III to inactivate the ADP-ribosylation activity of elongation factor 2 thus blocking protein synthesis and leading to cell death [11,12,19–21]. However, in clinical trials using these agents, curious delayed regressions [23,27] of tumors and delayed toxic effects [18] are seen which appear inconsistent with a direct and immediate cytotoxic effect. We also suspect that tumor regressions occur even after suboptimal drug delivery or when heterogeneous expression of the targeted antigen exists within the tumor [27]. We, therefore, hypothesized that immunologic responses may be a secondary mechanism that enhances the therapeutic efficacy of these novel anti-cancer drugs. To test this hypothesis in a syngeneic murine system that would eliminate confounding xenogeneic immunologic responses, a cell line that expresses a murine homologue of the tumor-specific human epidermal growth factor receptor mutation, EGFRvIII, was created and tumors derived from this cell line were targeted with an EGFRvIII-specific immunotoxin.

These studies demonstrate that the antitumor responses seen after intratumoral treatment with the EGFRvIII-specific immunotoxin, MR1-1 [MR1-1(dsFv)-PE38KDEL], are significantly inhibited in the absence of CD4⁺ and CD8⁺ T cells. Furthermore, while the antitumor activity requires that the immunotoxin be targeted to an antigen expressed on a subset of tumor cells, other tumor cells not expressing the target antigen, that are otherwise resistant to the direct cytotoxic effects of the toxin, can be similarly eliminated.

Materials and methods

MR1-1(dsFv)-PE38KDEL and anti-Tac(dsFv)-PE38 immunotoxins

MR1-1(dsFv)-PE38KDEL (MR1-1) is a recombinant protein that consists of the single chain variable fragment (scFv) of the EGFRvIII-specific MR1 monoclonal antibody that was enhanced by a phage display library screening to form the disulfide-stabilized variable fragment (dsFv). MR1 was then fused at the genetic level to *Pseudomonas* exotoxin domains II and III to form the MR1-1 immunotoxin. This toxin was shown by competitive binding to specifically target cells expressing the EGFRvIII mutation [1,8,9,11,12]. Anti-CD25(dsFv)-PE38KDEL [anti-Tac(dsFv)-PE38] [16] is an immunotoxin specific for the human interleukin-2 receptor (CD25) that serves as a control in these experiments. The human interleukin-2 receptor is not expressed in the mice used in these experiments.

Tumor cell lines

SMA560 is a malignant astrocytoma cell line derived from a spontaneously arising intracerebral tumor in inbred VM/Dk mice [25]. SMA560msEGFRvIII is the SMA560 cell line stably transfected with a fully syngeneic mouse homologue of the EGFRvIII mutation (msEGFRvIII) [26]. The murine homologue of the human EGFRvIII mutation was created by using cDNA sequences spanning the murine EGFR. The precise cDNA sequences used to construct msEGFRvIII have been previously reported [26].

Animal model

Inbred female VM/Dk mice, 6–12 weeks old of age, were maintained in the Duke University Cancer Center Isolation Facility. For tumor therapy, SMA 560 or SMA560ms EGFRvIII cells were harvested in log phase growth and rinsed twice in PBS. Viable cells were counted by

using try-pan blue staining. To induce subcutaneous tumors, 1.5×10^5 cells in a volume of 0.5 ml were injected into the right flank. Intratumoral treatment was performed immediately after tumor cell inoculation with PBS, 1 or 3 μg of MR1-1 or anti-Tac(dsFv)-PE38 in a volume of 20 μl , using a 250 μl Hamilton syringe. Mice were re-challenged with tumor in an identical fashion in the contralateral flank.

In vitro proliferation assay

Splenocytes were harvested from untreated or tumor-bearing mice treated with MR1-1. Harvested splenocytes were washed, resuspended in RPMI 1640 medium containing 10% (v/v) FCS, and plated into 96-well plates (5×10^5 cells/100 μl per well). Various concentrations (20, 10, and 1 $\mu\text{g}/\text{ml}$) of PEPvIII (LEEKKGNVVT DHC, Anaspec, San Jose, CA), a peptide that spans the deletion mutation of EGFRvIII, were added to the wells and the plates were incubated at 37°C and 5% CO₂. After 72 h of incubation, 1 $\mu\text{Ci}/25 \mu\text{l}$ of methyl-[³H]-thymidine (Amersham Biosciences, Piscataway, NJ) was added into the each well and incubated again. After 18 h, splenocytes were harvested and radioactivity counted.

Lymphocyte depletion

Lymphocyte subsets were depleted in vivo using specific antibodies as previously described [24,26]. Anti-CD4 (GK1.5) and anti-CD8 antibody (2.43) hybridomas were obtained from the American Type Culture Collection and grown in intraperitoneal cavity of athymic mice for antibody production. Immunoglobulins were purified from ascites by protein G affinity chromatography. The depletion antibodies were injected intravenously 4 days before tumor cell inoculation and intraperitoneally at the time of tumor cell inoculation and once a week for 2 weeks thereafter. Cell depletion was confirmed in a matched cohort of mice as previously described [24,26].

Statistical analysis

For in vitro proliferation assays, groups were compared using Student's *t* test. For in vivo mouse studies, serial measures of the longest tumor diameter and the diameter perpendicular to that measurement were collected throughout each experiment. Tumor volume was calculated as the product of these two perpendicular measures using the formula ($\text{short}^2/2 \times \text{long}$). The date at which each tumor exceeded 1,000 mm³ was recorded according to the guidelines of the Duke University Animal Welfare Committee. For mice that remained tumor-free at end of an experiment, the time to first detection was arbitrarily set to one day greater than the length of the experiment. Growth patterns were compared across treatment groups using the non-parametric Kruskal–Wallis test. If the overall test was statistically significant, pairwise comparisons were then made using the Mann–Whitney test.

Results

MR1-1 specifically inhibits growth of EGFRvIII expressing tumor in vitro

To demonstrate the specificity of MR1-1 against SMA560msEGFRvIII cells, in vitro cytotoxicity assays were performed. These assays demonstrated that MR1-1 induced a 50% inhibition of protein synthesis at 112 ng/ml against SMA560msEGFRvIII but required >10,000 ng/ml to inhibit synthesis in the parental untransfected SMA560 cell line that does not express EGFRvIII.

Intratumoral treatment with MR1-1 protects mice against challenge with EGFRvIII-expressing tumors and induces long-lasting antitumor immunity

To determine if direct intratumoral injection of MR1-1 was capable of mediating antitumor responses against subcutaneous tumors expressing EGFRvIII in vivo, we challenged VM/Dk

mice with a lethal dose (1.5×10^5 cells) of SMA560msEGFRvIII cells, and then treated them once intratumorally with 1 or 3 μ g of MR1-1 or PBS. All VM/ Dk mice treated with PBS developed lethal tumors. In the MR1-1 treated groups, however, all mice treated at either dose of MR1-1 never developed palpable tumors, and all mice survived >55 days without evidence of clinical or histological toxicity ($P < 0.0001$, Fig. 1a). All mice initially treated with MR1-1 were then re-challenged in the opposite flank with SMA560msEGFRvIII cells (1.5×10^5 cells), and all survived re-challenge suggesting long-lasting antitumor immunity ($P < 0.0001$, Fig. 1b).

Intratumoral treatment of EGFRvIII-expressing tumors with MR1-1 enhances EGFRvIII-specific immunity

To determine if intratumoral treatment of EGFRvIII-expressing tumors with MR1-1 induced antigen-specific immunity specific for EGFRvIII, we isolated splenocytes from tumor-bearing mice 7 days after being treated with 3 μ g MR1-1 and determined their ability to proliferate in vitro in response to PEPvIII (LEKKKGNYVVT DHC), a 14 amino acid peptide that spans the tumor-specific region of the mutation. These studies demonstrated that splenocytes derived from mice treated with MR1-1 possess a significantly enhanced proliferation response to PEPvIII compared to the splenocytes from untreated mice ($P < 0.0005$, Fig. 2).

Intratumoral treatment of EGFRvIII-expressing tumors with MR1-1 primes mice for protection against EGFRvIII negative tumor cells

To determine if treatment with the EGFRvIII specific immunotoxin MR1-1 would protect mice from a heterogeneous tumor population where some tumor cells do not express the targeted antigen, we challenged VM/Dk mice with a lethal dose of SMA560msEGFRvIII cells (1.5×10^5 cells) mixed with a lethal dose of untransfected SMA560 cells (1.5×10^5 cells). These mice were then treated with intratumoral injection of MR1-1 or PBS. The time to detection of tumor growth differed significantly among the three treatment groups ($P < 0.0001$). All mice treated with PBS developed lethal tumors (Fig. 3). However, in the MR1-1 treated group, mice never developed palpable tumors and survived more than 65 days without evidence of toxicity. On day 65 after the initial tumor challenge, all mice initially treated with MR1-1 were then rechallenged subcutaneously with another dose of untransfected SMA560 cells (1.5×10^5 cells). All these mice rejected these EGFRvIII-negative tumors, suggesting that the intratumoral MR1-1 treatment provided long lasting antitumor immunity that extended to EGFRvIII-negative tumor cells as well ($P < 0.0001$).

Antitumor effect of MR1-1 is dependent on EGFRvIII-specificity

To determine if the antitumor effect of MR1-1 against the mixed EGFRvIII-positive and EGFRvIII-negative tumors was a non-specific function of the toxin moiety or if the antigen specificity of the toxin conjugate was important, we again challenged VM/Dk mice with a lethal dose of SMA560msEGFRvIII cells (1.5×10^5 cells) combined with a lethal dose of untransfected SMA560 cells (1.5×10^5 cells). Mice were then treated with intratumoral injection of PBS, MR1-1, or an otherwise identical toxin targeted to the human CD25 molecule, anti-Tac(dsFv)-PE38 (Fig. 4). The time to detection of tumor growth differed significantly among the three treatment groups ($P < 0.0001$). All mice treated with PBS or anti-Tac(dsFv)-PE38 developed lethal tumors. In the MR1-1 treated group, however, there was a significant tumor growth inhibition, and all mice survived more than 55 days without evidence of tumor growth or toxicity ($P = 0.0079$) indicating that a toxin specific for at least some molecule present on at least some tumor cells was needed for the specific antitumor effects.

Specific antitumor effect of MR1-1 is abrogated by CD4 and CD8+ T cell depletion

To define the effector cells responsible for the immune response in this model, we again challenged VM/Dk mice with a lethal dose of SMA560msEGFRvIII cells (1.5×10^5 cells) while simultaneously depleting T lymphocyte subsets. In tumor-bearing mice treated with PBS, depletion of CD4+ or CD8+ T cells produced no significant effects in this tumor model ($P > 0.5$). Similarly, in mice treated with anti-Tac(dsFv)-PE38, depletion of CD4+ ($P = 0.1292$) and CD8+ ($P = 0.0753$) also had no effect. However, depletion of CD4+ ($P = 0.0193$) or CD8+ ($P = 0.0193$) T cells, in mice treated with MR1-1, uniquely and significantly abrogated the therapeutic efficacy of this immunotoxin (Fig. 5).

Discussion

The data presented here raise the possibility that immunotoxins may not be strictly dependent on a direct cytotoxic effect of the conjugated *Pseudomonas* exotoxin for their antitumor activity, but may also function to enhance antitumor immunity. Our studies demonstrate that the specific antitumor responses induced by MR1-1 are significantly inhibited in the absence of CD4+ or CD8+ T cells. We also demonstrate that this response requires that only a subset of tumor cells express the targeted antigen.

Induction of an immune response by targeted toxins may have several advantages and may explain some of the dramatic clinical efficacy that is being observed with these novel therapeutic agents. All tumors display a significant heterogeneity in the protein targets they express. This is also true of oncogenes like EGFRvIII. This heterogeneity at multiple levels restricts the ability of a single targeted agent to completely eradicate a tumor. The inherent specificity of an immune response would have similar limitations if it were not for the well-established principal of cross presentation [4,6,10,14]. Cross presentation is the mechanism whereby the antigenic constituents present in dying cells are presented for immune response consideration. Whether or not an immune response is generated in this context depends on a set of currently ill-defined, but critically important, inflammatory “danger” signals [13]. Our data then, showing killing of tumor cells that do not express the targeted antigen are likely explained by a cross-presentation of associated antigens subsequent to the direct killing of antigen-expressing cells by the toxin. Something central to the mechanism of cell death induced by the immunotoxin or the inflammatory context the toxin induces is likely responsible for allowing this process to be successful. Although cells not expressing EGFRvIII will not internalize the toxin, it is possible that cross-presentation of internalized toxin molecules within EGFRvIII-expressing cells may enhance the antitumor immune response generated. Similarly, this process may also be responsible for potentially deleterious inflammatory reactions seen at the sites of delivery of these agents [18]. For example, it is likely that some of the toxic effects of transferrin-targeted toxins occur because of the non-specific binding of these toxins to the normal vasculature. Cloroquine can be used to alleviate this toxicity, however.

If intratumoral delivery of specifically targeted immunotoxins provides an environment and antigen source conducive to cross-presentation, as our data suggests, then our findings have broad and significant implications for the use of these agents. First of all, immune responses during toxin therapy should probably not be potently suppressed, as is currently done. Suppression of immune responses generated against the antigenic components of the toxin, which otherwise may limit multiple dosing with these agents, might need to be reconsidered. Furthermore, delivery of these agents throughout the tumor, which can be very challenging, but which is also necessary for efficacious direct cytotoxic activity, may not be as important as once thought. Finally, decisions regarding optimal dosing of these molecules may need to be viewed within the context of the immune response that they generate rather than escalating doses until toxicity is met. Our studies, however, do not address whether different doses would enhance or inhibit the immune responses generated.

One of the obvious shortcomings of the findings from the current study is that they may not apply to all toxins universally. However, there is nothing distinctive about the *Pseudomonas*-based MR1-1 toxin other than its targeting moiety. The MR1-1 toxin was chosen for these studies though because of the availability of a murine homologue of the EGFRvIII mutation so these studies could be conducted within the context of a syngeneic murine model. All other commercially available toxins that are being used in human trials target a molecule that is specific to the human species. Murine models that might be designed to test these toxins would be hampered by the potential generation of xenogeneic responses to these human molecules within the transfected murine tumor and might be misleading.

In summary, we have demonstrated that the intratumoral delivery of an EGFRvIII targeted toxin, MR1-1dsFvPE38KDEL, enhances antitumor immunity which destroys not only EGFRvIII expressing tumor cells but also tumor cells not expressing EGFRvIII. These data suggest that immunotoxin therapy has a potential to induce antitumor responses through a variety of mechanisms and may be able to overcome treatment failures caused by antigen heterogeneity within tumors.

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Abbreviations

EGFRvIII	Epidermal growth factor receptor variant III
Anti-Tac(dsFv)-PE38	Anti-CD25(dsFv)-PE38KDEL immunotoxin
MR1-1	MR1-1(dsFv)-PE38KDEL immunotoxin

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Fig. 1.

a Tumor volume (mm³) of subcutaneous SMA560msEGFRvIII tumors in VM/Dk mice treated with MR1-1. Mice are challenged with 1.5×10^5 SMA560msEGFRvIII cells subcutaneously and treated with intratumoral injection of MR1-1 (1 or 3 μg) or PBS on the day of tumor challenge. The time to detection of tumor growth differed significantly among the three treatment groups ($P < 0.0001$). Treatment with PBS failed to protect against tumor growth. Treatment with 1 or 3 μg of MR1-1 protected 100% of the mice from tumor growth and all mice treated with MR1-1 survived for >55 days without evidence of tumor ($P < 0.0001$). **b** At day 65 after the initial tumor challenge, mice were re-challenged with EGFRvIII-positive

(SMA560msEGFRvIII) tumors. Initial treatment with MR1-1 protected mice from subsequent challenge with EGFRvIII-positive (SMA560msEGFRvIII) tumors as well ($P < 0.0001$)

Fig. 2.

Proliferation of splenocytes to PEPvIII stimulation in vitro. Splenocytes were harvested from tumor-bearing mice 7 days after being treated with 3 µg MR1-1 and plated into 96-well plates (5×10^5 cells/100 µl per well), and incubated with various concentrations (20, 10, and 1 µg/ml) of PEPvIII (LEKKKGNYVVT DHC), a peptide that spans the deletion mutation of EGFRvIII, for 72 h. About 1 µCi/25 µl of methyl-[3 H]-thymidine was then added to each well. After 18 h, the splenocytes were harvested and radioactivity counted. Splenocytes from mice receiving intratumoral treatment with MR1-1 proliferate significantly in response to PEPvIII compared to splenocytes from untreated mice (* $P < 0.0005$)

Fig. 3.

Tumor volume (mm³) of subcutaneous mixed EGFRvIII-positive (SMA560msEGFRvIII) and EGFRvIII-negative (SMA560) tumors in VM/Dk mice treated with MR1-1. Mice were challenged subcutaneously with a equal dose of cells (1.5×10^5) of each type universally lethal in this mouse model for either tumor alone, and then treated with intratumoral injection of MR1-1 (1 or 3 µg) or PBS on the day of tumor challenge. Treatment with PBS failed to protect against tumor growth. Treatment with 1 or 3 µg of MR1-1 protected the mice from growth of either tumor type, and all mice treated with MR1-1 survived for >55 days ($P < 0.0001$)

Fig. 4.

Subcutaneous mixed EGFRvIII-positive (SMA560msEGFRvIII) and EGFRvIII-negative (SMA560) tumors in VM/Dk mice treated with MR1-1 or anti-Tac(dsFv)-PE38. Mice were challenged with a mixture of a lethal dose of SMA560msEGFRvIII cells (1.5×10^5) in combination with a lethal dose of SMA560 cells (1.5×10^5) subcutaneously and treated with intratumoral injection of MR1-1 (3 μ g) or an otherwise identical toxin targeted to the human CD25 molecule, anti-Tac(dsFv)-PE38 (3 μ g) on the day of tumor challenge. Time for mice to develop tumors $>1,000$ mm³ is plotted. Treatment anti-Tac(dsFv)-PE38 failed to protect against tumor growth. Treatment with MR1-1 inhibited tumor growth, and all mice treated with MR1-1 survived for 55 days without evidence of tumor formation ($P = 0.0079$)

Fig. 5.

VM/dK mice were challenged 1.5×10^5 of SMA560msEGFR^{III} cells subcutaneously and treated with intratumoral injection of MR1-1 on the day of tumor challenge. The antitumor effects of intratumoral MR1-1 were evaluated after depletion of CD4⁺ or CD8⁺ T cells. Efficacy of MR1-1 was significantly reduced in the absence of CD4⁺ ($P = 0.0193$) or CD8⁺ ($P = 0.0193$) T cells. (Statistical analysis represents results of individual experiments, but for clarity, MR1-1 treated mice are pooled from repeated experiments.)