EGFR-Homing dsRNA Activates Cancer-Targeted Immune Response and Eliminates Disseminated EGFR-Overexpressing Tumors in Mice

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Abstract

Purpose: The cause of most cancer deaths is incurable dissemination of cancer cells into vital organs. Current systemic therapies for disseminated cancers provide limited efficacy and are often accompanied by toxic side effects. We have recently shown that local application of epidermal growth factor receptor (EGFR)-targeted polynucleotide-antigen (polyIC) eradicates preestablished EGFR-overexpressing tumors. Here we show for the first time the high efficiency of systemic application of polyIC/melittin-polylethyleneimine-polylethylene glycol-EGF (polyIC/MPE) in combination with human immune cells.

Experimental design: Cancer-targeted activation of immune cells was examined in vitro and in vivo following transfection with polyIC/MPE. The therapeutic efficiency of the strategy was then examined on disseminated EGFR-overexpressing tumors grown in severe combined immunodeficient (SCID) mice.

Results: Intravenous delivery of polyIC/MPE followed by intraperitoneal injection of peripheral blood mononuclear cells induced the complete cure of SCID mice with preestablished disseminated EGFR-overexpressing tumors, with no adverse toxic effects. The immune cells and the cytokines they produce are localized to the tumor site of the treated animal and contribute decisively to the demise of the tumor cells. The immune system home to the tumors, due to the chemokines produced by the internalized polyIC.

Conclusion: The EGFR-homing vector loaded with polyIC can be used to treat and possibly cure patients with disseminated EGFR-overexpressing tumors. The possibility of adopting this strategy to treat other tumors that express a protein capable of ligand induced internalization is discussed. Clin Cancer Res; 17(5); 1033–43. ©2010 AACR.
Translational Relevance

Metastasis of cancer into internal organs is virtually impossible to treat by conventional methods. A significant fraction of all cancer-related deaths are associated with overexpression of epidermal growth factor receptor (EGFR). The current EGFR-targeted treatments induce partial therapeutic effects but do not actually cure patients. This is most likely because EGFR is not essential for the survival of the targeted cancer cells. Here we show a strategy that utilizes the high level of expression of EGFR, rather than its activity, as the Achilles' heel of the tumor. This is achieved by utilizing an EGFR-homing chemical vector loaded with polynosine-cytosine (polyIC). Targeted polyIC activates a cascade of antiproliferative mechanisms and induces expression of immunoactive cytokines, selectively in EGFR-overexpressing cells. This study shows high efficiency of the strategy in the treatment of disseminated tumor models in mice. The data presented here suggest that systemic treatment of EGFR-overexpressing metastatic tumors with EGFR-targeted polyIC may lead to a complete cure, in patients with a functional immune system.

Methods

PBMC extraction

PBMCs from healthy human donors were separated on Ficoll-Plaque (Pharmacia), washed twice with 50 mL RPMI 1640 medium, resuspended at a density of 4 × 10^6/mL and cultured in this medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, 100 µg/mL streptomycin.

Chemo taxis

MDA-MB-468, A431, and U138MG cells grown in 96-well plates in triplicates were transfected with 2.5 µg/mL polyIC or polyglutamic acid (pGlu) formulated with MPPE. Forty-eight hours after the transfection, 100 µL medium of the cells were transferred to the lower chamber of Millipore chemo taxis plates with 5-µm pores (Millipore). A total of 5 × 10^4 PBMCs in 100-µL medium were added to the upper chamber, and plates were incubated for 4 hours at 37°C. Both lower and upper plates were then subjected to CellTiter-Glo Luminescent Cell Viability Assay (Promega). The assay is a homogeneous method to determine the number of viable cells in culture on the basis of quantitation of the ATP present, which signals the presence of metabolically active cells. The procedure involves adding of single reagent (CellTiter-Glo reagent) directly to cells cultured in serum-supplemented medium. This results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. CellTiter-Glo reagent (100 µL) was added to the wells of both lower and upper plates. The contents were mixed for 2 minutes on an orbital shaker to induce cell lysis. Ten minutes later, 100 µL of the mixture from each well were transferred to the new single 96-well plate (Nunc) and the luminescence was counted (arbitrary units) in a luminometer.

PBMC-mediated bystander effect

A total of 100,000 MDA-MB-468 cells or A431 cells were seeded into 6-well plates and grown overnight with 2 mL medium per well (2). Cells were then transfected with polyIC/MPPE, to a final concentration of 0.1 or 0.5 µg/mL. Forty-eight hours after transfection, 0.5 mL medium from the transfected cells (“conditioned medium”) was added to 500,000 PBMCs, which had been seeded 24 hours earlier into 24-well plates and grown in 0.5 mL medium. From the challenged PBMCs, 0.1 mL of medium was then exchanged for 0.1 mL medium from additional nontransfected MDA-MB-468 cells and U138MG cells (“indicator cells”) on 96-well plates 24 hours earlier. Survival of these cells was determined by methylene blue assay (2), 48 hours after challenge with the medium from the PBMCs.

In parallel, to show the direct bystander effect, 0.1 mL of conditioned medium was used to replace 0.1 mL medium from nontransfected indicator cells, seeded 24 hours earlier onto 96-well plates and grown in 0.2 mL medium. Survival of these cells was determined 48 hours after addition of the conditioned medium using methylene blue.

wild-type EGFR (wtEGFR) and cells harboring the mutant EGFRvIII, which does not internalize the vector, were also completely eradicated (2). This “bystander effect” was due to the antiproliferative cytokines such as IFN-α, generated at the tumor site by the polyIC/MPPE affected tumor cells (2).

IFN-α and other cytokines induced by polyIC/MPPE are also potent immune activators. Activation of the immune system selectively against cancer should strongly increase efficiency of the polyIC/MPPE therapy. Higher therapeutic efficiency should allow an effective treatment of not only local but also distant disseminated tumors, which are far more difficult to treat. Recognizing that severe combined immunodeficient (SCID) mice possess a deficient immune system, we decided to examine the therapeutic efficacy of EGFR-targeted polyIC on disseminated EGFR-overexpressing tumors in SCID mice and in SCID mice with a reconstituted immune system. In this study, we show that polyIC/MPPE, together with a reconstituted immune system, can eradicate disseminated tumors in SCID mice, by systemic intravenous (i.v.) application. Intravenous delivery of polyIC/MPPE, followed by intraperitoneal (i.p.) injection of human peripheral blood mononuclear cells (PBMC), induced the complete cure of SCID mice with preestablished disseminated EGFR-overexpressing tumors, with no obvious adverse toxic effects. We show that the internalized polyIC attracts the immune cells to the tumor. The tumor-targeted activated immune cells increase the efficiency of the polyIC/MPPE therapeutic effects by several fold, yet preserve its selectivity. The data presented here strongly suggest that EGFR-targeted polyIC can be utilized to treat and possibly cure patients with metastatic tumors that overexpress EGFR.
In vitro cancer cell killing by activated PBMCs

A total of 20,000 A431, 30,000 MDA-MB-468, or 20,000 U138MG cells were seeded onto 24-well plates and grown overnight in 1 mL RPMI medium supplemented with 10% FCS and antibiotics. Cells were then transfected with polyIC/MPPE at 0.1 μg/mL. Twenty-four hours later, 500,000 PBMCs/well were added to the cancer cells and coincubated for another 24 hours. Apoptotic cells (red fluorescence) were visualized using an Annexin-V-Biotin kit (Biosource Inc.). To distinguish tumor cells from PBMCs, tumor cells were labeled with fluorescein isothiocyanate (FITC)–conjugated EGFR antibody (Biosource Inc., green fluorescence). Cells were visualized with a fluorescent microscope and photographed using a digital camera.

Effect of polyIC/MPPE/PBMC treatment on survival of mice with disseminated tumors

Female SCID-nonobese diabetic (NOD) mice (Harlan) were injected i.v. with 1 million A431 or MDA-MB-468 or 1 × 10^5 U138MG cells suspended in 200 μL PBS. Ten or 15 days later, the animals were randomly divided into groups (5 mice per group) and treatment was initiated with a series of i.v. injections of 20 μg polyIC/MPPE. Twenty-four hours after the last polyIC injection, the animals were injected once with 4 million PBMCs. Survival of the mice was analyzed afterwards. Animals were checked daily for the development of symptoms associated with the progression of implanted tumors: weight decrease, moribund state, slow movement, or inability to feed or drink. Animals were sacrificed with overdose of anesthetic immediately after appearance of the above signs. Internal organs (lungs, liver, and others) were examined for the presence of tumor.

Results

PolyIC/MPPE induces expression of immunoactive cytokines in A431 and MDA-MB-468 cells

In our previous study (2), we showed that low concentration of EGFR-targeted polyIC induced expression of IFN-α, Interferon γ-induced protein 10 kDa (IP-10), and growth-regulated protein α (Gro-α) in EGFR-overexpressing glioblastoma cells (U87MGwtEGFR), but not cells with low levels of EGFR (U87MG). These data support the notion that cells produce these cytokines only when a certain threshold level of dsRNA has been internalized, and that this threshold is achieved only in cells overexpressing EGFR. In this study, we extended the analysis to two additional EGFR-overexpressing cancer cell lines: A431 (vulval epidermoid carcinoma) and MDA-MB-468 (breast carcinoma). When these cells were transfected with polyIC/MPPE, we detected up to 5.1 pg/mL of IFN-β; 148 pg/mL of Gro-α and 188 pg/mL of IP-10 in the growth medium (Supplementary Table S1). Gro-α and IP-10 are chemokines responsible for the recruitment of T cells to the area in which they are expressed (4, 5). Thus, A431 and MDA-MB-468 cells, like U87MGwtEGFR cells, secrete cytokines into the medium, following challenge with polyIC/MPPE.

In vitro activation of human immune cells

Given the above results, we hypothesized that the cytokine-enriched medium from A431 and MDA-MB-468 cells treated with polyIC/MPPE should attract and stimulate the immune system. We examined whether this was so, by testing the effect of medium from polyIC-transfected cancer cells on healthy human PBMCs (6). PBMCs consist of several types of immune cells [NK (natural killer), T cells, NK-T cells, and macrophages]. The antitumor effect of these cells has been extensively studied and many cell killing mechanisms are well established. When activated, PBMCs produce toxic cytokines, such as IFN-γ and TNF-α (6), known to be effective against various cancer cells (7, 8). Other cell killing mechanisms include perforin/granzyme (9, 10) and Fas ligand/Fas (10), which efficiently destroy tumor cells (10). PBMCs also interact with each other, leading to a synergistic, highly antiproliferative effect. For example, activated T cells and NK cells produce IFN-γ, which activates macrophages (11) and stimulates the production of TNF-α (12). Release of interleukin 2 (IL-2) into the medium correlates directly with PBMC activation (6) and can be conveniently quantified by ELISA. Thus PBMCs are a convenient system for studying the selective immune reaction against polyIC-transfected tumor cells.

First, we examined whether the medium from polyIC/MPPE-transfected cancer cells attracts PBMCs by chemotaxis (Fig. 1A). Using chemotactic chambers (Millipore), we show that the medium of A431 and the medium of MDA-MB-468 cells transfected with polyIC/MPPE strongly stimulate chemotaxis of PBMCs. In contrast, medium of transfected U138MG cells virtually did not affect chemotaxis (Fig. 1A). Thus PBMCs are attracted only to the medium of EGFR-overexpressing cells, which have been treated with polyIC/MPPE.

Next, we examined whether the medium from polyIC/MPPE-transfected cancer cells activates PBMCs. Activation of PBMCs was measured using IL-2, IFN-γ, and TNF-α ELISAs. PBMCs were challenged with medium from polyIC/MPPE-transfected cancer cells. Figure 1B shows the induction of IL-2 expression by PBMCs 48 hours after the challenge. Medium from A431 and MDA-MB-468 cells transfected with polyIC/MPPE (0.1 μg/mL) led the PBMCs to produce up to 165 pg/mL of IL-2. In contrast, medium from polyIC/MPPE-treated U87MG cells (with ~12 times lower expression of EGFR than A431 and MDA-MB-468 cells) or U138MG cells (no EGFR expression; ref. 13) did not affect PBMCs. Similar results were obtained when the expression of other cytokines was examined: Both IFN-γ (Fig. 1C) and TNF-α (Fig. 1D) were induced in PBMCs challenged with the medium from polyIC/MPPE-transfected A431 and MDA-MB-468 cells, but not from polyIC/MPPE-transfected U87MG and U138MG cells transfected with 0.1 μg/mL of polyIC/MPPE. Treatment of the cells with naked polyIC or pGlu formulated with MPPE did not induce any production of the cytokines and no production of cytokines was noted in U138MG cells (that are devoid of EGFR; ref. 13; Fig. 1E) and low levels of IL-2 and IFN-γ in U87MG, which express about 80,000...
receptors, were observed only at higher concentrations of polyIC/MPPE (Fig. 1).

**Activation of PBMCs in vivo**

The selective expression of cytokines in EGFR-overexpressing tumors was also confirmed in vivo (Table 1). SCID-NOD mice bearing EGFR-overexpressing subcutaneous (s.c.) tumors on the right flank and U138MG tumors on the left flank were i.v. treated with 3 consecutive daily injections of polyIC/MPPE followed by a single i.p. injection of 10 million PBMCs. Expression of cytokines was examined within the tumors and in blood from the animals, indicating the homing of the immune cells to the tumors. IFN-β was expressed in the EGFR-overexpressing tumors only. IP-10 and Gro-α, potent T-cell chemokines, were detected in the blood and, at much higher concentrations,
bystander effects are clearly shown in Figure 3. The PBMC-468 cells (Fig. 3A and C). Both direct and PBMC-mediated medium from polyIC/MPPE-treated A431 and MDA-MB-compared with the "direct" bystander effect, mediated by was examined 24 and 48 hours after medium exchange and challenged PBMCs was added to newly seeded, nontrans-(Methods). After another 48 hours, medium from the transfected cells were challenged with the medium from the transfected cells (polyIC/MPPE and 48 hours later, PBMCs transfected with polyIC/MPPE and 48 hours later, PBMCs challenged PBMCs strongly enhance polyIC/MPPE cancer cell killing

Table 1. In vivo cytokine expression pattern

<table>
<thead>
<tr>
<th>PolyIC/MPPE/PBMC</th>
<th>IP-10 (pg/g)</th>
<th>Gro-α (pg/g)</th>
<th>IFN-β (pg/g)</th>
<th>IL-2 (pg/g)</th>
<th>IFN-γ (pg/g)</th>
<th>TNF-α (pg/g)</th>
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<tr>
<td>MDA-MB-468</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>8.6</td>
<td>±2.2</td>
<td>10.6</td>
<td>237.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>89.3</td>
<td>±11</td>
<td>24.7</td>
<td>±2.6</td>
<td>0</td>
<td>71</td>
<td>0</td>
</tr>
<tr>
<td>14.5</td>
<td>±3.8</td>
<td>8.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.5</td>
<td>±3.8</td>
<td>8.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blood</td>
<td>10.3</td>
<td>4.8</td>
<td>8.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2.4</td>
<td>±3.6</td>
<td>±13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| U138MG           |             |             |             |            |             |             |
| 13.4             | ±1.6        | 14.5        | ±6.8        | 9          | ±14.5       | ±13.02      |
| 5.5              | ±2.8        | 77          | ±7.2        | 0          | 0           | 0           |
| 9.5              | ±3.8        | 8.7         | 0           | 0          | 0           | 0           |
| Blood            | 3.1         | 7.7         | 6            | 0          | 0           | 0           |
| 1.4              | 12.3        | 7.7         | 10.6        | 0          | 0           | 0           |
| 1.3              | 6.9         | 4.2         | ±7.3        | 0          | 0           | 0           |

| A431             |             |             |             |            |             |             |
| 1.5              | ±1.6        | 9.4         | ±6.8        | 77         | 0           | 0           |
| 108.1            | ±2.8        | 77          | ±7.2        | 0          | 0           | 0           |
| Blood            | 2.9         | 7.7         | 6            | 0          | 0           | 0           |
| 1.4              | 12.3        | 7.7         | 10.6        | 0          | 0           | 0           |
| 1.3              | 6.9         | 4.2         | ±7.3        | 0          | 0           | 0           |

NOTE: Cells were s.c. injected into right (A431 or MDA-MB-468) and left (U138MG) flanks of SCID-NOD mice. Twenty days later, when the tumors reached approximately 100 to 300 mm³, treatment was initiated as follows: 3 consecutive polyIC/MPPE i.v. injections of 20 μg(mouse/day. Twenty-four hours after the last polyIC/MPPE injection, 10 million PBMCs were injected i.p. Seven days later animals were sacrificed, tumors were extracted, homogenized with 1.5 mL extraction buffer [containing 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100] per gram of tissue, using a homogenizer. Homogenates were centrifuged at 13,000 g for 10 minutes at 4 °C, stored at −70 °C, then subjected to ELISAs. ELISAs were also carried out on blood samples. “PolyIC/MPPE/PBMC –” shows negative control with tumors extracted from untreated animals (3 animals per group); "PolyIC/MPPE/PBMC +" shows cytokine expression from the animals treated as described above (4 animals per group). Data represent mean ± SD of 3 to 4 animals. The numbers show pg of cytokine per 1 g of tissue (1 mL of blood = 1 g).

within the EGFR-overexpressing tumors (Table 1). These cytokines were expected to attract PBMCs selectively to the EGFR-overexpressing tumors, where the PBMCs would be activated. In separate experiments, infiltration of the PBMCs (Supplementary Fig. S1) into the EGFR-overexpressing tumors of the polyIC/MPPE+PBMC-treated animals was detected. No immune cell infiltration was detected in U138MG tumors, which do not overexpress EGFR (Fig. 2; Supplementary Fig. S1).

PBMC-mediated bystander effect

Expression of IFN-γ and TNF-α, potent antitumor cytokines, should strongly enhance bystander killing of untransfected cancer cells. To examine PBMC-mediated bystander effects, A431 or MDA-MB-468 cells were first transfected with polyIC/MPPE and 48 hours later, PBMCs were challenged with the medium from the transfected cells (Methods). After another 48 hours, medium from the challenged PBMCs was added to newly seeded, nontransfected cells (Fig. 3). The PBMC-mediated bystander effect was examined 24 and 48 hours after medium exchange and compared with the "direct" bystander effect, mediated by medium from polyIC/MPPE-treated A431 and MDA-MB-468 cells (Fig. 3A and C). Both direct and PBMC-mediated bystander effects are clearly shown in Figure 3. The PBMC-mediated effect was particularly strong, killing up to 90% of the nontransfected cells. U138MG cells, which do not express EGFR at all, were also efficiently inhibited by both types of medium (Fig. 3B and D). No effect was observed when polyIC was replaced by pGlu, which similarly (to polyIC) forms particles with MPPE, but does not induce an immune response.

As expected, preincubation of PBMC medium with neutralizing IFN-γ and TNF-α antibodies strongly inhibited PBMC-mediated bystander effects (Supplementary Fig. S2). When added together, the protecting effect of the antibodies was very strong. In contrast, neutralization by single antibody, either to IFN-γ or to TNF-α, had only a slight effect. It was reported previously that IFN-γ and TNF-α synergize in their killing effects (14, 15). It seems that at least in the present setting each cytokine has its own strong antiproliferative effect.

These results show that the combination of polyIC/MPPE and PBMC synergize effectively killing EGFR-overexpressing cancer cells.

PBMCs strongly enhance polyIC/MPPE cancer cell killing in vitro

To examine the synergistic cancer killing effect, tumor cells were transfected with polyIC/MPPE at low dose,
followed by addition of PBMCs (Fig. 4). To distinguish tumor cells from PBMCs, tumor cells were labeled with FITC-conjugated EGFR antibody (green fluorescence). Tumor cells undergoing apoptosis were detected with an Annexin-V-Biotin kit (Biosource Inc.; red fluorescence). Cells treated with either polyIC/MPPE alone at low dose of 0.1 μg/mL or PBMCs alone showed a very weak apoptotic signal. In contrast, a strong apoptotic signal was detected when the EGFR-overexpressing cells were treated with both polyIC/MPPE (0.1 μg/mL) and PBMCs (Fig. 4A and B). U138MG cells did not undergo detectable apoptosis (Fig. 4C). Thus, the addition of PBMCs to polyIC/MPPE-treated tumor cells strongly enhanced tumor cell apoptosis. Apoptotic death of EGFR-overexpressing tumors was also confirmed in vivo (Supplementary Fig. S3).

Systemic application of polyIC/MPPE combined with PBMCs cures mice with disseminated tumors

In view of our earlier finding that the polyIC-loaded EGFR-homing vector had no toxic effects on normal brain cells in tissue culture or in vivo (2), we examined whether EGFR-targeted polyIC/MPPE could be applied systemically, for the treatment of disseminated EGFR-overexpressing tumors in vivo. In the absence of a mouse model of EGFR-overexpressing tumors, we injected i.v. 1 million human A431 or MDA-MB-468 cells into SCID-NOD mice. Ten days after cell injection, treatment was initiated, with two 3-day cycles and one 4-day cycle of daily injections of 20 μg polyIC/MPPE, with a 24-hour interval between each cycle (i.e., a total of 10 injections, spread over 12 days). Mice bearing A431 tumors that received polyIC/MPPE survived at least 3 times longer than untreated mice, and 3 mice were completely cured (Fig. 5B). Mice bearing MDA-MB-468 tumors treated with polyIC/MPPE survived up to twice as long as untreated mice (Fig. 5C).

These results, combined with the finding that PBMCs strongly enhance the effect of polyIC/MPPE in vitro, encouraged us to test whether PBMCs would similarly enhance the effect of polyIC/MPPE in vivo. For these experiments, we waited 15 days after injection of A431 or MDA-MB-468 cells in SCID-NOD mice, at which point large tumors of up to 500 mm3 could be detected in the lungs (Fig. 5A). Mice were then treated with 4 consecutive, daily i.v. injections of 20 μg of polyIC/MPPE. Twenty-four hours after the final polyIC injection, the mice were injected once with 4 million human PBMCs (16). Reconstitution of the SCID-NOD mouse immune system using human PBMCs is a common practice (17–19). As in the earlier experiment, polyIC/MPPE-treated mice bearing A431 tumors survived longer than untreated mice. Mice that were treated with both polyIC/MPPE and human PBMCs survived for more than a year, and did not show any signs of tumors (Fig. 5D). Similarly, mice bearing MDA-MB-468 tumors treated with polyIC/MPPE alone survived up to twice as long as untreated mice, whereas mice treated with both polyIC/MPPE and PBMCs survived more than a year and did not show any signs of tumors (Fig. 5E). In contrast, there was virtually no change in survival rate of the mice bearing U138MG tumors, which do not express EGFR (Fig. 5F) and treated at the exactly same manner with polyIC/MPPE and PBMCs. No visible signs of toxicity such as reduction in weight or abnormal behavior were detected,
either during the treatment or afterwards. Thus, by introducing human PBMCs, we were able to significantly reduce the dosage and duration of treatment with polyIC/MPPE and to eliminate established disseminated tumors. Hence, PBMCs play a crucial role in tumor eradication.

Discussion

Most cancer deaths are caused by metastases, and there is a great need for effective therapies. A large fraction of the most frequent and the deadliest cancers overexpress the EGFR, including lung cancer (215,020 estimated new cases and 161,840 estimated deaths in 2008 in the United States of America), breast cancer (184,450 estimated new cases, 40,930 estimated deaths), colon and rectal cancer (148,610 estimated new cases, 55,170 estimated deaths). EGFR overexpression is often correlated with metastasis and poor prognosis. Here we show that targeted polyIC, delivered systemically, mobilizes immune cells to achieve complete regression of disseminated tumors.

We earlier showed that EGFR-targeted polyIC leads to complete regression of localized xenografts in mice (2). EGFR-targeted polyIC exerts a strong bystander effect, that is, it kills EGFR-overexpressing cells and neighboring tumor cells, whether or not they express EGFR or its mutated version EGFRvIII (2). This is crucial, because even tumors showing strong overexpression of EGFR are commonly heterogeneous with respect to EGFR expression. At the same time, EGFR-targeted polyIC is highly selective for tumor cells, with minimal toxic effects on the surrounding...
normal tissues (2) and on distant normal tissues. In the future, more extensive toxicity studies will examine this issue, in particular attention to skin and other EGFR-rich organs, this is especially important prior to clinical development. The targeted polyIC quickly activates multiple antiproliferative/proapoptotic pathways, minimizing the likelihood of mutations leading to drug resistance. In this study, we have been able to show that the polyIC-loaded vector targeted to EGFR, can be applied systemically and therefore be utilized to treat disseminated EGFR-overexpressing tumors (Fig. 5). Furthermore, we show an additional advantage of the EGFR-targeted polyIC strategy, namely, the activation of an immune reaction selectively against the tumor (Fig. 5). Treatment of mice harboring tumors lacking EGFR with polyIC/MPPE did not result in any cure, with or without PBMC (Fig. 5F). In our previous study, we used nude or SCID mice with significantly impaired immune system. We did not detect infiltration of the (remaining) mouse immune cells into the polyIC/MPPE-treated glioblastoma model, despite induction of human IFN-α and other cytokines (2). Here, reconstituting the murine immune system with human PBMCs resulted in a strong, yet selective immune reaction against cancer (Fig. 5D and E). This is also shown by the expression of IL-2, TNF-α, and IFN-γ within the tumor, in EGFR-overexpressing tumor bearing mice, treated with polyIC/MPPE and PBMC (Table 1) but not in tumors lacking EGFR. Infiltration of immune cells, including T cells is also shown histologically (Fig. 2; Supplementary Fig. S1).

It should be noted that systemic application of nontargeted polyIC has been attempted to treat cancer (20, 21). The survival benefit was minimal although pronounced systemic toxicity was observed (20, 21). The weak effect was most likely caused by the failure to introduce a sufficient dose of polyIC into the tumor cells. Most of the nontargeted polyIC probably scattered through normal tissues, entering into noncancer cells and inducing toxic reactions. On the other hand, EGFR-targeted polyIC affects cancer cells only, leaving normal cells unharmed.

For a tumor to become established, it must avoid elimination by the immune system. Many cancers develop mechanisms of inhibiting immune surveillance and can even grow in the presence of immune lymphocytes that recognize cancer antigens (22, 23). Furthermore, elements of the immune system can be co-opted for tumor growth (24), supporting the Virchow hypothesis (1863) that the numerous immune cells found in the vicinity of practically all malignant tumors, attest to the role of inflammation in the generation of cancer. It seems however that some of these cells possess antitumor activities (25), and can be "woken up" by tumor-localized polyIC, as shown here. PolyIC, a potent adjuvant, and IFN, a strong immune activator, may activate preexisting cancer-specific immune lymphocytes, in addition to attracting and activating other antitumor immune cells. Here, we show that the cytokines induced by targeted polyIC attract immune cells to the tumor and strongly enhance the efficiency with which cancer cells are killed. This induces a significant additive or even synergistic effect with the direct effects induced by the internalized polyIC, leading to the complete elimination of disseminated tumors, even when treated from a distance with a low total dose of EGFR-targeted polyIC (Fig. 5D and E). Activated immune cells strongly enhance the bystander effect (Fig. 3), which should facilitate the killing of heterogeneous cancers. The relatively fast immune response (Figs. 3 and 4) indicates that primarily the innate immunity is activated. It is likely though that at later stage adoptive immunity against cancer could be developed as well. Infiltration of antigen presenting cells, such as macrophages, into the tumor and induction of
MHC of both classes by IFNs eventually should lead to an adoptive immune response against cancer. Although our models preclude study of adoptive immune response, induction of IFNs (Table 1; Supplementary Table S1) and infiltration of PBMCs (Fig. 2; Supplementary Fig. S1) into the tumor implies possible activation of adoptive immunity that should further enhance efficiency and selectivity of the EGFR-targeted polyIC therapy.

Because polyIC/MPPE is targeted selectively to cancer cells, we do not expect significant systemic immunotoxic reaction, while inducing a strong antitumor reaction, supports this theory.

Intratumoral or peritumoral administration of nontargeted polyIC has been shown to be effective in antitumor immunotherapy (26). Such treatment is limited to localized tumors only. In contrast, EGFR-targeted polyIC is effective both by local treatment (2) and in the treatment of disseminated tumors as shown in this study.

EGFR-targeted polyIC could potentially be combined with several cancer immunotherapies available today. These include cancer vaccines and cancer-targeted (engineered or extracted) T cells. To mediate antitumor effects

Figure 5. Effect of polyIC/MPPE/PBMC treatment on survival of mice with disseminated tumors. Disseminated tumors were established as described in Methods. A, histopathologic analysis of mouse lungs at the time of treatment initiation (15 days after cell injection). Yellow arrows point to a tumor in a lung capillary. B, C, and F, 15 days after cell injection, the animals were randomly divided into groups (5 mice per group) and the treatment was initiated with 4 consecutive i.v. injections of 20 µg polyIC/MPPE at 24-hour intervals. Twenty-four hours after the last polyIC injection, the animals were injected once with 4 million PBMCs. Control groups included mice treated with pGlu/MPPE to determine the effect of the conjugate without polyIC. B, survival of animals with A431 tumors. C, survival of animals with MDA-MB-468 tumors. F, survival of animals with U138MG tumors. D and E, 10 days after cell injection, the animals were randomly divided into groups (5 mice per group) and the treatment was initiated with 3 cycles of 3 or 4 consecutive i.v. injections of 20 µg polyIC/MPPE at 24-hour intervals (total 10 injections). The interval between cycles was 48 hours. Control groups included mice treated with pGlu/MPPE to determine the effect of the conjugate without polyIC and HBG buffer (HEPES buffered glucose; ref. 2). D, survival of animals with A431 tumors. E, survival of animals with MDA-MB-468 tumors.
in vivo, cancer-targeted T cells must travel to the tumor site, extravasate from the circulation, and then mediate effector functions to cause destruction of cancer cells (25). IP-10 and Gro-α strongly induced by targeted polyIC selectively in tumor cells (Table 1; Supplementary Table S1), should facilitate both traffic to tumor and extravasation (4, 5), whereas IFN should enhance T-cell–mediated cancer killing.

In addition, allergenic immune cell transplantation to activate graft versus tumor reactions (27) could be combined with the strategy described here. Injection of foreign PBMCs into polyIC/MPPE-treated mice in this study (Fig. 5D and E) actually resembles such a combination. Grafted immune cells should confer a stronger antitumor effect than the patient’s own immune cells.

The absence of an immunocompetent mouse model of EGFR-overexpressing cancer precludes study of the activation of the mouse’s own immune system by polyIC/MPPE. However, the data presented here suggest that systemic treatment of EGFR-overexpressing metastatic tumors with EGFR-targeted polyIC may lead to a complete cure, in patients with a functional immune system. In view of the success of the EGFR-targeted polyIC therapy, we propose to explore the validity of the approach for the treatment of other cancers. The chemical vector we utilize is actually built like Lego, such that the homing ligand can be modified, whereas the other elements remain constant. Any tumor that overexpresses a surface protein that can be internalized on ligand binding is a candidate for such therapy, once the coupling conditions have been optimized for the relevant ligand.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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