A Single Vaccination with Polyomavirus VP1/VP2Her2 Virus-Like Particles Prevents Outgrowth of HER-2/neu–Expressing Tumors

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Abstract

Murine polyomavirus (MPyV) VP1 virus-like particles (VLPs), containing a fusion protein between MPyV VP2 and the extracellular and transmembrane domain of HER-2/neu (Her2), Her21-683PyVLPs, were tested for their ability to vaccinate against Her2-expressing tumors in two different in vivo models. Protection was assessed both against a lethal challenge with a BALB/c mammary carcinoma transfected with human Her2 (D2F2/E2) and against the outgrowth of autochthonous mammary carcinomas in BALB-neuT mice, transgenic for the activated rat Her2 oncogene. A single injection of Her21-683PyVLPs before tumor inoculation induced a complete rejection of D2F2/E2 tumour cells in BALB/c mice. Similarly, a single injection of Her21-683PyVLPs at 6 weeks of age protected BALB-neuT mice with atypical hyperplasia from a later outgrowth of mammary carcinomas, whereas all controls developed palpable tumors in all mammary glands. VLPs containing only VP1 and VP2 did not induce protection. The protection elicited by Her21-683PyVLPs vaccination was most likely due to a cellular immune response because a Her2-specific response was shown in an ELISPOT assay, whereas antibodies against Her2 were not detected in any of the two models. The results show the feasibility of using MPyV-VLPs carrying Her2 fusion proteins as safe and efficient vaccines against Her2-expressing tumors. (Cancer Res 2005; 65(13): 5953-7)

Introduction

HER-2/neu (Her2), a proto-oncogene overexpressed in many epithelial carcinomas, including breast cancer, and associated with increased metastatic potential and poor prognosis (1), is considered a candidate for immunotherapy. Vaccination strategies have been tested in animal models and these show that tolerance to Her2 can be broken, resulting in tumor resistance and induction of CTL and T helper cell responses (2).

Several different vaccination strategies have been evaluated in transgenic BALB-neuT mice, where a mutated rat Her2 transgene is expressed in the mammary glands by week 3 (3) and foci of atypical hyperplasia appear by week 6, resulting in carcinoma in situ around week 10 and invasive lobular carcinomas by week 20 (4). Vaccination has shown it possible but difficult to completely inhibit tumor development in this model (5).

Murine polyomavirus-like particles (MPyV-VLPs) are devoid of viral DNA and enter cells similar to native virus (6). They can also potentially introduce molecules bound to VLPs into cells because the MPyV receptor is abundant on most cells in different species, including mouse and man (7).

Here, the ability of the minor capsid proteins VP2 and VP3 to bind to the internal side of VP1 (8) was exploited in the production of MPyV-VLPs carrying a fusion protein between MPyV-VP2 and the extracellular and transmembrane domains of human Her2. These Her21-683PyVLPs were used to immunize against Her2-expressing tumors. One immunization with Her21-683PyVLPs inhibited both the outgrowth of a transplantable Her2-expressing tumor in BALB/c mice and the development of multiple autochthonous carcinomas in BALB-neuT mice.

Materials and Methods

Mice

Female BALB/c mice, bred and maintained, pathogen-free, at the Microbiology and Tumor Biology Center, Karolinska Institutet, were used for rejection tests.

Inbred BALB-neuT mice overexpressing the transforming rat Her2 oncogene (neuT/neuT) were produced, screened and maintained, pathogen free, at the Department of Clinical and Biological Science, University of Turin (3).

Cells and Cell Lines

D2F2, a murine mammary carcinoma cell line, and D2F2/E2, obtained by transfection of D2F2 with a Her2 expressing plasmid (9), N202.1A and N202.1E cells (10) were maintained in medium supplemented with 10% FCS and for D2F2/E2 with 800 µg/ml of G418. Her2 expression was confirmed by anti-human Her2 antibody Ab5 staining (Oncogene, Boston, MA).

Baculovirus Constructs

For construction of baculoviruses coexpressing two separate proteins, the baculovirus transfer vector pAcDB3 (BD Biosciences PharmMingen, San Diego, CA) was used. The MyIPyVP1 gene was cloned into pAcDB3 generating the plasmid pAcDB3PyVP1. At a second promoter, a fusion gene was inserted, coding for VP2 fused to the NH2-terminal of Her2 amino acids 1 to 683 (derived from pVAX/E2A, ref. 11), with three alanines as linker in between. The VP2 gene was also cloned into a separate pAcDB3PyVP1 plasmid. Recombinant baculoviruses expressing VP1 and VP2 or VP1 and VP2HER21-683 were acquired by cotransferring the pAcDB3 constructs with BD BaculoGold-linearized AcNPV baculovirus DNA in Spodoptera frugiperda cells (SF9) according to the manufacturer (BD Biosciences PharmMingen).

Expression and Purification of Virus-like Particles

VP2PyVLPs and Her21-683PyVLPs were produced in SF9 cells infected with recombinant baculoviruses and purified by a CsCl gradient (12). MPyV-VP1 VLPs were produced as described (12).

SDS-PAGE and Her2-specific Immunoblot

SF9 cell extracts or CsCl-purified VLP preparations were analyzed for protein content using SDS-PAGE. For Her2 detection, proteins were transferred to a nitrocellulose filter and incubated with αErbB2 (BD Biosciences PharmMingen) followed by incubation with alkaline phosphatase.
Vaccination and Tumor Challenge

BALB/c mice challenged with D2F2/E2 or D2F2 cells. Each immunization group contained 10 mice, Her21-683PyVLPs or VP2PyVLPs (50 μg) were given s.c. 21 days before s.c. challenge with 2 × 10^5 of D2F2/E2 or D2F2 cells. Mice were followed for tumor outgrowth and sacrificed when their tumor diameters reached 15 mm. In a second and third test, each group contained 10 female or 10 male mice, respectively. In both of these tests, 1 × 10^6 D2F2/E2 cells were given 13 days after VLP vaccinations.

**BALB-neuT mice.** At 6, 10, or 14 weeks of age, female BALB-neuT mice were immunized i.p. with 50 μg of VP2PyVLPs or Her21-683PyVLPs. Mammary glands were palpated weekly for tumor appearance. Progressively growing masses of >1 mm mean diameter were regarded as tumors. Tumor multiplicity was calculated as the cumulative number of tumors / total number of mice and was reported as mean ± SE. Growth was monitored until all mammary glands displayed a palpable tumor or until a tumor exceeded 10 mm at which time the mice were sacrificed. Mice were bled 2 weeks after immunization.

**Measurement of Her2- and VP1-specific Antibodies**

Antibodies to Her2 were screened by fluorescence-activated cell sorting analysis as described in ref. (11) for human Her2 and in ref. (13) for rat Her2. VP1 antibody titers were measured by ELISA (14).

**ELISPOT Assay**

ELISPOT assay was done according to Mabtech’s guidelines for Mouse IFN-γ ELISPOT (Mabtech, Nacka, Sweden). Briefly, splenocytes (4 × 10^6) from nonimmunized and immunized mice in 1 mL complete medium were incubated in 24-well plates alone or together with different stimulatory agents: D2F2 cells (3 × 10^5), D2F2/E2 cells (3 × 10^5), VP2PyVLPs (10 μg/mL), or Her21-683PyVLPs (10 μg/mL). The plates were incubated 20 to 24 hours at 37°C, and cells (1.2 × 10^5) in triplicate from each well were transferred to an anti-mouse IFN-γ antibody-coated ELISPOT plate and incubated 20 to 24 hours at 37°C before spots were detected and counted in an ELISPOT reader (Zeiss, München-Hallbergmoos, Germany).

**Statistical Analysis**

Differences in takes of transplantable tumors in Table 1 were calculated by Fischer’s exact test. Differences in tumor incidence in BALB-neuT mice were calculated by Mantel-Haenszel log-rank test (14). Differences in tumor multiplicity and in ELISPOT data were evaluated by Student’s t test.

**Results**

**Generation and characterization of Her21-683PyVLPs and VP2PyVLPs.** To obtain MPyV-VLPs as carriers, the ability of VP2 to bind to VP1 was used. A VP2/Her2 fusion gene (VP2Her21-683) between the NH2-terminal half of the Her2 gene, coding for amino acids 1 to 683, including the extracellular and transmembrane domains, and the full-length VP2 was made. For Her21-683PyVLP production, a baculovirus expressing both VP2Her21-683 and VP1 was constructed. For comparison, a baculovirus expressing full-length VP1 and VP2 for the production of VP2PyVLPs was constructed.

Her21-683PyVLPs and VP2PyVLPs were purified and analyzed for their content of VP2 or VP2Her21-683 on SDS-PAGE and by Her2-specific immunoblot (data not shown). The number of VP2Her21-683 molecules, migrating as a 120-kDa band, per Her21-683PyVLP was estimated to around three. The appearance of Her21-683PyVLPs was confirmed by electron microscopy to be identical to VLPs made by MPyV-VP1 alone (data not shown).

**Immunization with Her21-683PyVLPs protects BALB/c mice from the outgrowth of a Her2-expressing tumor.** To test if Her21-683PyVLPs could induce protective immunity, three tests were done. In each test, 10 BALB/c mice were immunized with one s.c. injection of 50 μg of Her21-683PyVLPs (corresponding to around 800 ng Her21-683). As controls, 10 mice were immunized with 50 μg VP2PyVLPs and 10 mice were left untreated. Thirteen or 21 days later, mice were challenged with a lethal dose of Her2-positive D2F2/E2 cells (15) and monitored for tumor outgrowth.

**Table 1. Immunization with Her21-683PyVLPs protects mice against a challenge with D2F2/E2 tumor cells**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Takes*</th>
<th>Total takes, (Experiments 1-3)</th>
<th>Takes (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 3</td>
</tr>
<tr>
<td>Naive</td>
<td>8/10</td>
<td>9/10</td>
<td>10/10</td>
<td>27/30</td>
</tr>
<tr>
<td>Her21-683PyVLPs</td>
<td>0/10</td>
<td>1/10</td>
<td>3/10</td>
<td>4/30</td>
</tr>
<tr>
<td>VP2PyVLPs</td>
<td>9/10</td>
<td>9/10</td>
<td>10/10</td>
<td>28/30</td>
</tr>
</tbody>
</table>

*Results are reported as the number of tumor-positive animals per total number of animals challenged.

†Ps were calculated based on the comparison between the specified group and the naive mice.
The first test is illustrated in Fig. 1A, and all three tests are presented in Table 1. In summary, 10% of the naive mice and 7% of the VP2PyVLP-immunized mice survived a challenge, indicating that there was no unspecific protection following VLP immunization. However, after Her21-683PyVLP immunization, 87% of the mice rejected the tumor inoculum. This protection was significant both when the Her21-683PyVLP-immunized group was compared with the naive group and to the VP2PyVLP-immunized group \((P < 0.0001)\). Her21-683PyVLP or VP2PyVLP immunization did not confer immunity to a challenge with D2F2 tumor cells, the Her2-negative parental cell line of D2F2/E2 (Fig. 1B), indicating that the protection was Her2-specific and not due to unspecific stimulation of the immune system by Her21-683PyVLPs.

Immunization with Her21-683PyVLPs protects BALB-neuT mice from outgrowth of autochthonous mammary carcinomas. Groups of six BALB-neuT transgenic mice were immunized i.p. at 6, 10, or 14 weeks of age with 50 \(\mu\)g Her21-683PyVLPs or VP2PyVLPs.

Her21-683PyVLPs, given on week 6, conferred complete protection against tumor development in five of six mice, whereas all VP2PyVLP-immunized and nonimmunized mice developed tumors in each mammary gland (Fig. 2A and B). Notably, VP2PyVLP immunization caused a 4-week delay in tumor appearance (Fig. 2B).

Her21-683PyVLP given on week 10 gave a 3-month delay in tumor outgrowth but did not confer complete protection (Fig. 2C and D); Her21-683PyVLP given on week 14 did not provide any protection (Fig. 2E and F).

Antibodies to VP1, but not to Her2, are present in Her21-683PyVLP-immunized mice. To assess if rejection of D2F2/E2 was antibody mediated, sera obtained before tumor challenge were screened for Her2 and VP1 antibodies. Her2 antibodies were not detected in sera from Her21-683PyVLP-immunized BALB/c or BALB-neuT mice, whereas all VLP-immunized mice had high VP1-specific antibody titers (data not shown).

Her21-683PyVLPs induce Her2-specific T cells. To evaluate and quantify Her2-specific T cells, an ELISPOT assay was done. Spleens from BALB/c mice, immunized one to two times with VP2PyVLPs or Her21-683PyVLPs, were taken 10 to 14 days after the last immunization. Splenocytes were incubated with or without different stimulating agents such as D2F2 cells, D2F2/E2 cells, VP2PyVLPs or Her21-683PyVLPs, and number IFN-\(\gamma\)-producing cells (spots) were counted.
or VP2PyVLPs and the frequency of specific T cells was calculated. Only Her2\(_{1-683}\)PyVLP-immunized T cells elicited a significant (P < 0.01) response to D2F2/E2 cells, whereas no response was observed to D2F2 cells, indicating a specific Her2 response (Fig. 3). This response did not increase with two immunizations. T cells from both Her2\(_{1-683}\)PyVLP- and VP2PyVLP-immunized mice responded to VP2PyVLPs, and this response increased after two immunizations. Both immunogens responded also to Her2\(_{1-683}\)PyVLPs. However, the response from the Her2\(_{1-683}\)PyVLP-immunized mice was significantly (P < 0.01) higher indicating T-cell recognition of the Her2 component of the Her2\(_{1-683}\)PyVLPs.

Discussion

In this study, a single vaccination using Her2\(_{1-683}\)PyVLPs conferred complete protection in vivo both against a subsequent challenge with a Her2-expressing tumor in BALB/c mice and against the aggressive development of mammary carcinomas in BALB-neuT mice. Protection in BALB-neuT mice was achieved, although the target cells expressed the rat Her2 and not the human Her2 present in the VLPs. However, for complete protection in these mice, it was necessary to vaccinate at an early age. Vaccination with Her2\(_{1-683}\)PyVLPs against Her2-negative tumor cells did not affect tumor outgrowth showing the Her2 specificity of the rejection response.

An injection of Her2 DNA has been shown not to induce complete rejection of a D2F2/E2 challenge in BALB/c mice. However, complete protection can be achieved by coimmunization of DNA expressing granulocyte macrophage colony-stimulating factor (11, 15). We were unable to observe a synergistic effect by combining Her2 DNA with Her2\(_{1-683}\)PyVLP immunization (data not shown). This may be due to the very high efficiency that Her2\(_{1-683}\)PyVLP immunization alone had in this model. In BALB-neuT mice, many vaccination strategies have been tested allowing a comparison with the strategy used here. Immunization with allogenic cells expressing rat Her2 followed by the administration of IL-12 (10) and electroporation of DNA plasmids coding the extracellular + transmembrane domain of Her2 (5) is among the most efficient strategies. However, immunization had to be repeated to completely inhibit tumor outgrowth in contrast to the complete protection achieved after only a single injection at 6 weeks of age in the present study. Furthermore, BALB-neuT mice vaccinated on week 10 had a 3-month delay in tumor outgrowth. This can be compared with BALB-neuT mice electroporated with neu DNA starting from week 10 (5). However, in that study, eight DNA vaccinations were necessary to achieve protection extending beyond 3 months and four DNA vaccinations gave lower protection than the one injection of Her2\(_{1-683}\)PyVLPs given on week 10 in the present study.

The high efficiency of the immune response induced by the VLPs despite the relatively low amount of Her2 protein in the vaccine can be explained by at least two mechanisms. First, due to the ubiquitous expression of the MPyV receptor on different cell types, including cells of the immune system (16), the VP2Her2 fusion proteins are likely to be delivered efficiently by Her2\(_{1-683}\)PyVLPs into cells where they can be processed and presented to the immune system. Second, VLPs most likely also act as an adjuvant inducing a nonspecific stimulation of the immune response (17), in this study indicated by the delay in the outgrowth of mammary carcinomas in BALB-neuT mice immunized with VP2PyVLPs. In the BALB-neuT system, the importance of the combined administration of the Her2 antigen and a nonspecific cytokine has been noted in several studies (10).

Because a Her2-specific T-cell response upon Her2\(_{1-683}\)PyVLP vaccination was shown using an ELISPOT assay, whereas no antibodies against Her2 were detected in either of our two tumor models, tumor rejection was likely to be T cell mediated. In other studies, different parts of the immune system have been shown involved depending on the nature of the tumor rejection model. In BALB/c mice immunized with Her2 DNA and challenged with D2F2/E2 cells, the rejection was shown to require the presence of both CD4+ and CD8+ T cells and antibody independent (11). However, in another tumor transplantation model, rejection of established Her2-expressing carcinomas after gene-gun vaccination with plasmids coding for the extracellular + transmembrane domains of the rat Her2 required antibodies, cytokines, and perforin (13). Furthermore, in the BALB-neuT system, neu-specific antibodies have been suggested to be responsible for inhibition of tumor outgrowth after vaccination using Her2 DNA (5, 18). It is therefore particularly interesting that our data in the BALB-neuT transgenic model show for the first time that a strong tumor protection can be induced in this model in the apparent absence of antibodies. However, the final analysis of this question awaits further studies in B-cell knockout mice.

MPyV-VLPs are attractive vaccine vehicles for use in humans, because MPyV is nonhuman and preexisting immunity to MPyV-VLPs is not expected to inhibit the efficiency of such a vaccine. Furthermore, although MPyV-VLPs have not been used in humans, VLPs from several other viruses (e.g., human papillomavirus; ref. 19) have been used in clinical trials and this mode of vaccination is now established as safe and often also efficient.

In summary, a single injection of MPyV-VLPs carrying a VP2/Her2 fusion protein constitutes a potent way of inducing both a rejection response against a tumor inoculum, as well as inhibition of the aggressive spontaneous tumor outgrowth in a mutant Her2 transgenic mouse model. Similar VLPs should also be possible to use as prophylactic vaccine in healthy persons. Finally, this vaccination strategy, avoiding “viral vectors” and DNA and eliminating all risks connected with this type of therapy, should also be possible to use to elicit an immune response towards other human tumor antigens.

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