Specific immune response induced in mice by immunization with the human Epidermal Growth Factor Receptor extracellular domain

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ABSTRACT

Epidermal Growth Factor Receptor (EGFR or HER1) is considered a tumor associated antigen. HER1 is overexpressed in many human epithelial tumors and involved in crucial cellular functions for tumor development. For this reason HER1 is considered a good target for cancer immunotherapy and some passive agents are being evaluated in clinical trials. However, HER1-based active immunotherapy has not been clinically explored. To develop an active immunotherapy approach based on HER1 for the treatment of HER1+ cancer patients, we cloned and expressed in mammalian cells the HER1 extracellular domain protein (HER1-ECD), which was specifically recognized by an anti-EGFR monoclonal antibody. HER1-ECD was purified by affinity chromatography and adjuvated in Very Small Size Proteoliposomes (VSSP) or Complete Freund adjuvant to immunize C57BL/6 mice. Immunization elicited specific humoral and cellular immune response. The polyclonal antibodies induced by immunization recognized the full length HER1 on the surface of a lung tumor cell line. These results suggest that HER1-ECD could be an appropriated antigen to induce specific immune response in patients with HER1+ tumors.

Keywords: HER1, immunization

Introduction

Cancer cells have many distinctive properties in which the Epidermal Growth Factor Receptor (EGFR or HER1) is involved. These include the capacity of autocrine growth stimulation, apoptosis evasion, unlimited replication, angiogenesis promotion, and capacity for tissue invasion and metastasis [1]. HER1 is overexpressed in many human epithelial tumors, such as lung [2], breast [3], ovary [4], colon [5], head and neck [6], prostate [7, 8], bladder [9], and pancreatic [10] tumors. This overexpression has been associated in clinical studies with bad prognosis of the disease in bladder, cervical, ovarian and esophageal cancers, and with reduced survival rates in breast, gastric and colorectal cancers [11]. The apparent association of HER1 with poor patient outcome may also reflect its role in the promotion of tumor invasion and metastasis [12]. Besides, much evidence suggests that activation of HER1 may promote resistance to the conventional cytotoxic therapies [13]. For these reasons, HER1 is considered a tumor associated antigen, and consequently, a good target for cancer immunotherapy.

HER1 belongs to a type I tyrosine kinase family, named HER1 family, which includes four transmembrane growth factor receptors: HER1 [14], HER2/neu, HER3 and HER4 [15]. These receptors are structurally related, and each of them is composed of an extracellular domain (ECD) and a conserved cytoplasmic signal transduction domain, separated by a hydrophobic membrane-spanning region. The ECD is the ligand binding domain, and is less conserved among the four receptors of the family, suggesting that they have different specificities in ligand binding. The more relevant HER1 ligands for tumor growth are the EGF [16] and the transforming growth factor α (TGFα) [17]. This ECD

region includes four subdomains. The subdomains I and III, contain the binding site, and subdomain II is responsible for receptor dimerization. Binding of the ligands to the HER1-ECD results in receptor homo- or hetero-dimerization, tyrosine kinase activation and initiation of signaling cascades [18, 19].

HER1 targeting immunotherapies have been limited to passive therapy with monoclonal antibodies (MAb) and small tyrosine kinase inhibitors (TKI). Some of these anti-HER1 agents have produced good clinical results. Cetuximab is a chimeric IgG1 MAb that has been approved by the US Food and Drug Administration (FDA), either as monotherapy or in combination with irinotecan, for the treatment of advanced colorectal carcinoma with detectable HER1 expression [20]. Another anti-HER1 MAb with relevant clinical results is the hR3/Nimotuzumab MAb (hr3), which has been approved combined with chemotherapy for the treatment of advanced head and neck tumors by the Cuban Center for State Control of Drug Quality (CECMED) [21]. These MAbs recognize the ligand binding domain, and inhibit HER1 phosphorylation. In contrast to MAbs, HER1-TKI exerts its activity intracellularly at the level of tyrosine kinase phosphorylation. The best characterized anti-HER1 TKI is gefitinib. This agent has entered phase II/III clinical testing in prostate, breast, bladder and renal cell carcinoma [22]. In contrast to passive therapy, anti-HER1 active immunotherapy has not been clinically tested, and only limited to preclinical studies [23, 24]. We have recently reported that vaccination of mice with the ECD of autologous EGFR overcomes the tolerance to the self EGFR and has an anti-metastatic effect on an EGFR+ tumor [25]. These results suggest that immunization of cancer patients with the ECD of human HER1 could result in a good clinical outcome. This paper reports the cloning of the gene encoding HER1-ECD in a mammalian expression vector. The recombinant protein was expressed in HEK293 cells and purified by affinity chromatography. Mice were then immunized with HER1-ECD adjuvanted in Complete Freund Adjuvant (CFA) or in Very Small Size Alum and purified by affinity chromatography. Mice were then immunized with HER1-ECD/HEK293 stable cell lines. The mock transfection with the pcDNA3 vector was used as a negative control.

HER293 cells were grown in 6-well plates (1.75 x 10^6 cell/mL) and 8 hours later they were transfected with 4 mg of the HER1-ECD/pDNA3 plasmid using the calcium phosphate transfection procedure. Plates were incubated overnight at 3% CO2, and transferred to 5% CO2, 16 hours later. Transfected cells were grown in a selective medium (containing 1 mg/mL of G418; Geneticin, Sigma, USA) starting 48 hours after transfection for the generation of HER1/ECD/HEK 293 and HER1-ECD/HEK293 stable cell lines. The mock transfection with the pcDNA3 vector was used as a negative control.

HER293 transfectants were grown in a SFM4-CHO serum-free medium (HyClone, USA) supplemented with 4 mM glutamine, 1 g/L Pluronic F68, and 26 mM of NaICO3.

Immunoprecipitation

The supernatant from the HER1-ECD/HEK293 transfectant cultures (2 mL) was mixed with 1 mg of hR3 MAb (specific for HER1-extracellular domain, CIM, Cuba) and 20 mL of Protein A-Sepharose (Amersham-Pharmacia Biotech, Upsalla, Sweden). Samples were gently shaken overnight at 4°C and then centrifuged 1 min at 11 000 g. The precipitated recombinant protein was separated on 7.5% SDS-PAGE and visualized by silver staining.

Purification

Recombinant HER1-ECD was purified from confluent cultures of the transfectant agent by affinity chromatography using EAH-Sepharose 4B (Amersham Pharmacia Biotech, USA) covalently coupled to hR3 MAb. Equilibration and washing steps were performed with PBS/NaCl 1 M pH 7.0 and protein elution with Glycine 0.2 M pH 2.8. Purity was assessed by densiometry, using a personal densiometer SI (Amersham Pharmacia Biotech, USA) and Imag Quant Software. Protein concentration was assayed by Lowry’s method.

Western blot

Purified HER1-ECD protein (15 μg) was applied into 7.5% SDS-PAGE gels and transferred to Hybond-ECL Nitrocellulose membrane (Amersham Pharmacia Biotech, USA, UK). Membranes were blocked with NEGT buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl (pH 7.5), 0.02% Tween 20, 0.04% Gelatin) and incubated with 10 μg/mL of hR3 MAb. The pro-
Immune response induced by EGFR immunization

Enzyme immunoassay for testing hR3 MAb recognition

Microtiter plates (High binding, Costar, USA) were coated with 5 μg or 10 μg of HER1-ECD in carbonate buffer, 0.1 M, pH 9.6, and incubated overnight at 4°C. Plates were blocked with 5% calf serum in PBS/Tween-20, and hR3 MAb at different concentrations added into the plate and incubated 1 hour at 37°C. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added and incubated 1 hour at 37°C. After the addition of P nitrophenylphosphate (1 mg/mL) (Sigma, USA) the Optical Density (OD) was measured at 405 nm using a micro well system reader (Organon Teknika Inc., Salzburg, Austria). All washes were made with PBS/Tween-20. ELISA test background was two times the OD at 405 nm for PBS.

Mice and immunization protocols

Female C57BL/6 mice, aged 8-12 weeks old, were purchased from the National Center for Laboratory Animals Production (CENPALAB, Havana, Cuba). All mice were kept under pathogen-free conditions. Animal experiments were approved by the Center of Molecular Immunology’s Institutional Animal Care and Use Committee (CIM, Havana, Cuba).

Mice (n = 10) were immunized four times biweekly with 50 μg of HER1-ECD in Freund Adjuvant (FA; complete for the first immunization and incomplete for the rest) (HER1-ECD/FA) or in VSSP adjuvant [31, 32], in water/oil (Montanide ISA 51, Seppic, France) emulsion (HER1-ECD/VSSP). The routes of vaccine administration were subcutaneous (sc) or intramuscular (im) respectively. Sera samples were obtained on days 0, 21, 35, and 56.

T-cell proliferation assay

Inguinal lymph nodes (LN) were harvested from mice immunized with 50 μg of HER1 ECD adjuvanted in VSSP or FA. Single cell suspensions were plated at 5 x 10^4 cells/well in flat-bottomed 96-well microculture plates (TPP, Switzerland), using DMEM plus 4% FCS. Cells were cultured in triplicate with 50, 75 and 100 μg of HER1-ECD at 37°C in a 5% CO₂ incubator. On the fourth day of the culture, wells were pulsed with 1μCi [³H]thymidine for 18 hr. Plates were harvested and the incorporated [³H]thymidine was measured using a scintillation counter. Results were expressed as stimulation index (SI).

DTH test

Mice were immunized three times with 50 μg of HER1-ECD adjuvanted in VSSP or FA as described above. Seven days after the last immunization mice were sensitized by intradermal injection with 50 μg of HER1-ECD in 50 μL of PBS in the right hind foot pad and the same volume of PBS in the left foot pad. After 48 hours foot swelling was measured using a plethysmometer (Ugo Basile, Italy). Mice inoculated with PBS in VSSP and sensitized with HER1-ECD in PBS were used as negative control. Differences in DTH between treated and control groups were translated into differences in displaced liquid volume and were statistically validated by Kruskal Wallis and Dunn’s Multiple Comparison test.

Enzyme immunoassay for testing biological activity

Microtiter plates (High binding, Costar, USA) were coated with 10 mg/mL of HER1 ECD in carbonate buffer, 0.1 M, pH 9.6, and incubated overnight at 4°C. Plates were blocked with 5% calf serum in PBS/Tween-20, and sera dilutions in duplicate, from immunized mice (n = 10), or pre-immune sera (as negative control) were added and incubated 1 hour at 37°C. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added and incubated 1 hour at 37°C. After the addition of P nitrophenylphosphate (1 mg/mL) (Sigma, US) the Optical Density (OD) was measured at 405 nm using a micro well system reader (Organon Teknika Inc., Salzburg, Austria). All washes were made with PBS/Tween-20. The Mann Whitney U test was used to assess statistical differences between individual time points in the humoral response kinetics. ELISA test background was two times the OD at 405 nm of pre-immune sera.

RT-PCR

Total RNA from H125, U1906 and H661 cell lines was extracted using the RNAeasy minikit (QIAGEN, Germany) followed by reverse transcription kit (Qiagen Germany) in accordance with the manufacturer’s protocols. To amplify cDNAs, 1 μg of the reverse-transcribed cDNA was subjected to PCR in 50 μL of PCR buffer [10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, and 2.5 mM MgCl₂] containing 0.8 mM deoxynucleotide triphosphates, 0.5 mM of each of set of primers, and 2.5 units of Taq DNA polymerase (QIAGEN, Germany). The specific primers for HER1 gene amplification were: sense 5´-CTGAGGAC-3´ and antisense 5´-C GACCCTTA-3´. The specific primers for HER2 and β-actin gene amplification (used as control of the experiment) were designed from published sequences [33]. After PCR amplification, 10 μL of the RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized with Ethidium bromide.

FACS Analysis

Cells were stained with sera from immunized mice (1/200 dilution) or 5 μg/mL of hR3 Mab (Center of Molecular Immunology, Cuba), or 5 μg/mL of anti HER2 Mab (Oncogene, US), followed by FITC-goat anti-mouse IgG (Jackson, Immunoresearch laboratories Inc, USA). Up to 10 000 cells were acquired using a FACScan flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA, USA).

Results

HER1-ECD cloning and expression

PCR amplified DNA encoding HER1-ECD was cloned into pcDNA3 expression vector, and HEK293 cells
were transfected with the resulting plasmid. Protein expression by stable HEK293 transfectants was evaluated by immunoprecipitation with the hR3 MAb, which is specific for HER1. Mock transfection was used as a negative control. A protein band of approximately 105 kDa corresponding with the expected size of HER1-ECD was visualized in SDS PAGE (Figure 1).

**HER1-ECD purification**

HEK293 transfectant was grown in an SFM4-CHO serum free medium, and the supernatant was used to purify the soluble HER1-ECD recombinant protein. The purification was made by affinity chromatography using EAH sepharose covalently coupled to hR3 MAb. The purity after one step of purification was 98% (Figure 2a), determined by densitometry. HER1-ECD identity was determined by western blot using hR3 MAb, from transfectant culture supernatants. The arrow indicates the band corresponding to HER1-ECD. A mock transfectant culture supernatant was used as the negative control.

**Immune response induced by immunization with HER1-ECD**

C57BL/6 mice were immunized four times biweekly with the HER1-ECD in FA or the VSSP adjuvant, which has been tested in humans. To evaluate the immunogenicity of HER1-ECD adjuvanted in VSSP, the stimulation of T cells was measured in vitro. For this purpose, LN cells from immunized mice were isolated and stimulated in vitro with different concentrations of HER1-ECD purified protein. T cells from immunized mice with HER1-ECD/VSSP had a significant stimulation index (S.I) compared with control mice, reaching up to 9.53 S.I (Mann Whitney U test, p < 0.05) (Figure 3a). The specificity of the lymphocyte stimulation was verified using EGF as an irrelevant stimulation antigen (Figure 3b).

The activation in vivo of a cellular immune response was measured by the DTH test. Mice immunized with HER1 ECD in VSSP or FA were sensitized at the foot pad with the HER1-ECD protein, and swelling was measured after 48 hours. Animals immunized with HER1-ECD in VSSP and FA showed inflammation 30 times higher than the negative control group (p < 0.05, Dunn’s multiple comparison test) (Figure 4).
To evaluate the humoral induced immune response, sera from mice immunized four times biweekly were tested on day 56. All immunized mice developed high serum IgG polyclonal antibody (PAb) levels against HER1-ECD using both VSSP and the FA adjuvant, as determined by ELISA (Table 1).

Specific polyclonal antibodies recognize full length HER1 in cancer cells, but not HER2

To determine whether polyclonal antibodies (PAb) induced by immunization with a truncated HER1 could bind a full length receptor on the cell surface, three lung carcinoma cell lines were characterized in terms of HER1 and HER2 (homologous members of the EGFR family) expression. All tested cell lines (H125, U1906 and H661) expressed HER1 and HER2 at the mRNA level (Figure 5a). However, while all cells showed HER2 expression by FACS, only H125 cells expressed HER1 on the membrane (Figure 5b).

PAb induced in mice by the immunization with HER1-ECD adjuvanted in VSSP were evaluated by FACS for their capacity to bind the above mentioned cell lines. As shown in figure 6, induced PAb recognized the HER1+ H125 cell lines but did not bind to U1906 which was selected as negative control of HER1 expression.

Discussion

HER1 is a well-characterized tumor associated antigen. It has been widely explored as a target for cancer immunotherapy, but these studies have been limited to a passive immunotherapy with MAbs [21] and TIK [22, 34]. Active immunotherapy based on HER1 would need to circumvent the tolerance to the self protein, but previous studies where mice were immunized with murine EGFR-ECD and generated an immune response with antiinflammatory effect on an EGFR+ tumor [25], pointed out the potential effectiveness of a vaccine based on autologous HER1.

The present study was conducted to further explore the efficacy of active immunotherapy based on human HER1-ECD in the adequate adjuvant to generate a specific immune response in mice. DNA encoding HER1 ECD was cloned into a pcDNA3 mammalian expression vector and HEK293 human cells were successfully transfected. The HER1-ECD recombinant protein expression was verified by immunoprecipitation using hR3 MAb and was then purified from the culture supernatant by affinity chromatography. The high purity protein identity was checked by using hR3 MAb.

The HER1-ECD protein was mixed with the VSSP adjuvant and then emulsified in Montanide ISA 51. VSSP was the selected adjuvant due to its capacity to promote a proinflammatory context and avoid the tolerance to poorly immunogenic proteins. VSSP/Montanide ISA 51 has been clinically tested [35] without showing high toxicity. The HER1-ECD/VSSP vaccine preparation elicited specific cellular and humoral immune response in immunized mice. Lymph node cells from immunized mice were stimulated in vitro in a concentration depending manner with HER1-ECD. CD4+ T cell proliferation could be induced by the inflammatory context provided by VSSP in the formulation. The induction of specific cellular immune response was confirmed by the DTH test. Mice immunized with HER1-ECD/VSSP and then sensitized with the same antigen developed inflammation, indicating that T CD4+ cells were activated.

The induction of specific humoral immune response was also evaluated in C57BL/6 mice. FA, even when it can not be used in humans due its high toxicity, was included in our studies as a reference TH1 adjuvant. Both HER1-ECD/VSSP and HER1 ECD/FA vaccine formulations elicited specific IgG antibodies in immunized mice, with titers reaching 1/320 000 after four inoculations. The induction of high antibody titers were expected from our previous studies immunizing mice with the murine EGFR-ECD in VSSP which generated very high specific IgG response [25]. Varying results have been published by other authors when HER2 has been used as the immunization antigen. Vaccination of monkeys with HER2-ECD in Detox [36], a potent adjuvant [37], induced no more than 1/100 000 antibody titer. Vaccination of monkeys with HER2-ECD in Detox/FA [38] also evoked very low IgG response [25]. Varying results have been published by other authors when HER2 has been used as the immunization antigen. Vaccination of monkeys with HER2-ECD in Detox [36], a potent adjuvant [37], induced no more than 1/100 000 antibody titer. Vaccination of monkeys with HER2-ECD in Detox/FA [38] also evoked very low IgG response [25].

*Measured by ELISA.

Table 1. Frequency of responder animals and IgG titer* of C57BL/6 animals on day 56 after 4 immunizations with HER1-ECD/VSSP or HER1-ECD/FA

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Frequency</th>
<th>5000</th>
<th>10 000</th>
<th>20 000</th>
<th>40 000</th>
<th>60 000</th>
<th>80 000</th>
<th>160 000</th>
<th>320 000</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER1-ECD in FA</td>
<td>10/10</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER1-ECD in VSSP</td>
<td>10/10</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
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10,000 specific IgG titers, indicating that HER2 could be less immunogenic than HER1.

The immunization with the truncated HER1-ECD protein did not affect the recognition of the PAb of the full-length protein on the tumor cell surface. Specificity for HER1 was also demonstrated as shown in the FACS experiments with human tumor cell lines. Using FACS the anti-HER1 PAb only recognized the H125 cell line, which is HER1+/HER2+, but did not recognize the U1906 cell line, which is HER1-/HER2+.

In summary, the present study demonstrates that a vaccine composition containing HER1-ECD adjuvanted in VSSP/Montanide ISA 51 is able to induce specific cellular and humoral response in mice. These results, together with the previously demonstrated anti-metastatic effect of autologous EGFR-ECD vaccination in mice, suggest that this novel therapeutic approach could be used to target EGFR+ tumors in patients.

Received in September, 2007. Accepted for publication in June, 2008.