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Abstract

Purpose: Epidermal growth factor (EGF) might be a suitable immunotherapeutic target in non–small-cell lung cancer (NSCLC). Our approach consists of active immunotherapy with EGF. The aim of the study is to characterize the humoral response and its effects on signal transduction in relation with the clinical outcome.

Experimental Design: Eighty NSCLC patients treated with first-line chemotherapy were randomized to receive the EGF vaccine or supportive care. EGF concentration in sera, anti-EGF antibodies and their capacity to inhibit the binding between EGF/EGF receptor (EGFR), and the EGFR phosphorylation were measured.

Results: Seventy-three percent of vaccinated patients developed a good antibody response, whereas none of the controls did. In good antibody-responder patients, self EGF in sera was significantly reduced. In 58% of vaccinated patients, the post-immune sera inhibited EGF/EGFR binding; in the control group, no inhibition occurred. Post-immune sera inhibited the EGFR phosphorylation whereas sera from control patients did not have this capacity. Good antibody-responder patients younger than 60 years had a significantly better survival. A high correlation between anti-EGF antibody titers, EGFR phosphorylation inhibition, and EGF/EGFR binding inhibition was found. There was a significantly better survival for vaccinated patients that showed the higher capacity to inhibit EGF/EGFR binding and for those who showed an immunodominance by the central region of EGF molecule.

Conclusions: Immunization with the EGF vaccine induced neutralizing anti-EGF antibodies capable of inhibiting EGFR phosphorylation. There was a significant positive correlation between antibody titers, EGF/EGFR binding inhibition, immunodominance of anti-EGF antibodies, and survival in advanced NSCLC patients.

Non–small-cell lung cancer (NSCLC) is one of the most common malignant diseases with a high mortality rate worldwide. Only 30% of patients can be treated surgically. For the majority of patients, traditional treatment options have modest efficacy (1). Over the last 20 years, elevated levels of the epidermal growth factor (EGF) receptor (EGFR) and its cognate ligands have been identified as a common component of numerous cancer types (2). The extracellular domain of EGFR is a ligand-binding site for various polypeptide growth factors: EGF, transforming growth factor-α (TGFα), amphiregulin, betacellulin, heparin-binding protein, epiregulin, and vaccinia virus growth factor. EGFR plays a role in cell motility, adhesion, invasion, and angiogenesis (3). The binding of a ligand to the extracellular region of EGFR induces a dimerization of EGFR, resulting in autophosphorylation and activation of cytoplasmic signal proteins that are involved in transmitting a mitogenic signal (4). In fact, EGFR is overexpressed in 40% to 80% of NSCLC (5), and this overexpression is associated with a poor prognosis and resistance to cytotoxic agents (6). Several studies in gastric cancer that examined concurrent expression of EGFR and its ligands (2, 7, 8) showed that coexpression of EGFR and either EGF or TGFα was associated with a marked overall survival or relapse-free survival disadvantage. Many clinical trials are under way using agents that target this receptor, such as monoclonal antibodies (i.e., C225) or gefitinib (Iressa) and erlotinib (Tarceva), two low molecular weight inhibitors of EGFR (9). We have been exploring strategies based on vaccination with EGFR ligands. A cancer vaccine based on an immunogenic conjugate of human EGF is

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currently in clinical trials. Additionally, recent preclinical studies to assess the potential of an active specific immunotherapy approach to block the TGFα/EGFR autocrine loop have been reported (10). Our group also obtained a TGFα fusion protein that could be a potential immunogen for the development of a new cancer vaccine. The ultimate goal has been to exploit autoreactivities as a way to remove or inactivate EGF or TGFα, aiming to reduce the growth rate of human EGF-dependent tumors (11, 12). Our clinical trials based on vaccination with autologous EGF in patients with epithelial tumors have shown that the vaccine is immunogenic and well tolerated. A trend to increased antibody titers was observed and there was a significant increase in survival of patients who maintained antibody responses (13). Furthermore, those who developed a “good antibody response” seemed to have significantly better survival compared with patients who had lower anti-EGF antibody responses. A recent report showed that high anti-EGF antibody titers and low EGF levels in serum correlated with patient survival (14). All these results are consistent with our hypothesis that anti-EGF antibodies block the binding between EGF and its receptor, slowing down tumor cell proliferation.

The aim of the present report was to study the humoral immune response of patients enrolled in a randomized phase II clinical trial using EGF vaccination and its relation with the survival of treated patients.

Patients and Methods

Patients. Eighty patients with histologically or cytologically confirmed stage IIIb or IV NSCLC, who completed first line of oncosepnic therapy at least 4 weeks before participation, were included. Patients with known brain metastases were ineligible. However, after an external review by an expert committee, 74 patients (37 in each arm) were considered fully evaluable as per protocol; 4 patients did not comply with protocol entry criteria whereas 2 subjects refused to continue participating in the trial after randomization before initiating treatment.

The study was approved by the Institutional Review Boards of the participating centers and by the National Regulatory Authority. All patients provided informed consent before inclusion in the study.

Vaccination protocol. The EGF vaccine was composed by human recombinant EGF (expressed in yeast) chemically conjugated to a recombinant protein (P64k) from Neisseria meningitides (expressed in E. coli) as carrier protein and emulsified with the adjuvant Montanide ISA51 as previously described (12, 15). The recombinant human EGF, produced in the Center of Genetic Engineering and Biotechnology (CIGB, Havana, Cuba), expressed on S. cerevisiae, is composed by a mixture of EGF 51 and EGF 52. This nonglycosylated molecule has shown a biological activity equivalent to the one displayed by the full-length human EGF (53 amino acids; ref. 16). Eligible patients were randomly assigned to groups designated to receive supportive care (control group) or four immunizations (induction phase) and reimmunizations with EGF vaccine (vaccine group) monthly until disease progression. One dose of the vaccine was equivalent to 50 μg of EGF and the vaccine was administered i.m. in upper limbs.

Immunologic assays. Patients for whom three or more serum samples were collected until 6 months post-immunization (42 patients, 26 vaccinated and 16 controls) were included in the evaluation of the kinetic of the immune response (antibody response and EGF concentration). Four control patients refused frequent sampling even after signing the informed consent, whereas for the remaining patients (11 vaccinated and 17 controls) two or less samples were collected on account of tumor progression and the deterioration of the general patients’ condition. Indeed, 23 patients (vaccinated and controls) died within 3 months of randomization due to the natural course of the disease.

From the 26 vaccinated with three or more serum samples selected for the kinetic study, we chose 13 good responders (50% of the total sample) to characterize the influence of vaccination on TGFα concentration, EGFR phosphorylation, and binding inhibition capacity, as well as peptide immunodominance. Sera from five control patients were used as negative controls.

Measurement of antibody titers by ELISA. Blood samples were collected every 15 days for 60 days and then monthly. Antibody titers against human EGF were measured by ELISA as previously described (12, 15). Anti-EGF antibody titer was defined as the inverse of the highest serum dilution, yielding a final absorbance value higher than the blank absorbance plus thrice the SD. Patients were classified as good antibody responders if anti-EGF antibody response reached titers ≥1:4,000 and were at least four times the pre-immunization value at any time during the study. Patients were considered poor antibody responders if titers did not meet these values. Anti-TGFα was measured by ELISA. The microtiter plates were coated with 50 ng/well human TGFα (R&D Systems). The geometric mean of antibody titer was used to compare the magnitude of antibody response in each patient group.

To identify the epitopes recognized by the sera of immunized patients, six peptides that represent different zones of the EGF molecule, as previously described (17), were synthesized. Both pre- and post-immune sera (after 3-6 months; 1:100 dilution) from vaccinated patients were tested for antibody responses specific to different peptides from EGF molecule. The absorbance of the natural antibody response from healthy donors was subtracted from the response versus each peptide to establish the cutoff value.

Measurement of EGF and TGFα concentrations in serum. EGF and TGFα concentrations in serum were measured with commercial kits (Quantikine Human EGF Kit and Quantikine Human TGFα, R&D Systems). The assay uses a quantitative sandwich enzyme immunoassay, with plates precoated with anti-TGFα or anti-EGF monoclonal antibody. After adding the standard calibration curve and the patients’ samples, an enzyme-linked polyclonal antibody specific for the specific ligand was added to the wells. After a washing step, the enzymatic reaction was visualized with a substrate (tetramethylbenzidine) solution and the absorbance was measured at 450 nm.

Radioimmunoassay. To assess the capacity of the generated anti-EGF antibodies to inhibit EGF/EGFR binding, we used a BIA as follows: the A431 human epidermoid carcinoma cell line (American Type Culture Collection) was maintained in DMEM (Hyclone) supplemented with 10% FCS (Hyclone). A431 cells were seeded in 75-cm² culture flasks and kept overnight with 5% CO₂ at 37°C. The next day, the cells were washed twice with PBS and preincubated (2 × 10⁵ cells) with 1:100 dilution of sera (immune or pre-immune) for 30 min at 37°C, and then the cells were incubated with 100,000 cpm of [125I]-EGF (61.5 μCi/μg) for 1 h at room temperature. Binding inhibition by an excess of nonradioactive EGF was used as the positive control. After three washes, total [125I]-EGF bound to cell membranes was measured in an automatic gamma counter (Wallac). The EGF was radioiodinated by the Chloramine T method (18). The inhibition occurred when values were higher than the mean of inhibition percentages at baseline from all patients plus twice the SD.

Cell lysates and Western blotting. An immunoblotting assay, which detects phosphorylated EGFR, was used to evaluate the capacity of the anti-EGF antibodies to inhibit the EGFR activation in the presence of EGF. A431 cells were serum starved for 24 h and then incubated with sera from control or vaccinated patients for 1 h at 37°C. Incubation...
with 1 mol/L tyrphostin AG1478 (tyrosin kinase inhibitor) for 1 h was used as the positive control. Cell lysates were prepared using 50 mmol/L HEPES (pH 7.4), 0.15 mol/L NaCl, 1% Triton X-100 buffer containing 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L Na3VO4, and then clarified by centrifugation. The protein concentration of the lysates was determined with a bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein were resolved on SDS-PAGE, transferred onto polyvinylidene difluoride nitrocellulose membrane (Gelmar), followed by blocking with NEGT buffer [0.15 mol/L NaCl, 5 mmol/L Tris-HCl (pH 7.5), 0.02% Tween 20, and 0.04% gelatin] overnight at 4°C.

Then the membranes were incubated with specific anti-phosphotyrosine antibody (Santa Cruz Biotechnology) at room temperature for 1 h. After washing with NEGT buffer, the membranes were incubated with secondary antibody (antimouse or antirabbit antibodies conjugated with horseradish peroxidase) for 1 h at room temperature. The signal was visualized by enhanced chemiluminescence according to the manufacturer's instruction (Amersham Biosciences UK) and band intensity was quantitated using a personal densitometer SI (Pharmacia Biotech) and ImageMaster 1D prime Software. To normalize the protein loading on the gel, the membranes were stripped and reprobed with anti-EGFR antibodies for 1 h at room temperature. Antimouse antibodies conjugated with horseradish peroxidase were used as secondary antibodies. We used ECL Plus Western blotting detection reagents (Amersham Biosciences) as detection system. The inhibition of phosphorylation occurred when values were higher than the mean of the percentages of inhibition reached in the control cohort plus 2 SD.

**Results**

**Patients.** Patients were randomized to receive the EGF cancer vaccine or best supportive care. The vaccine was well tolerated; the most common adverse events were chills, fever, and nausea and were classified as grade 1 or 2 according to the National Cancer Institute Common Toxicity Criteria. The clinical data have been described in a previous report.4

**Antibody response.** Antibody response against EGF was repeatedly measured in 42 patients (26 from the vaccinated group and 16 from the control group). The results are presented in Fig. 1. Preexisting reactivity to EGF that was previously described by Crombet et al. was detected in 13 patients (31%; 9 in the vaccine group and 4 in the control group).

The geometric mean of the baseline anti-EGF antibody titer was 1,244 for the vaccine group and 1,158 for the control group. Gradual increases in the geometric mean of anti-EGF titers were observed in the vaccine group after repeated immunizations. More than 70% of vaccinated patients (n = 19; 73%) were classified as good antibody responders. None of the control group met this criterion. As expected from results obtained previously4 by our group, significant differences in antibody titers were obtained between vaccinated and control patients (P < 0.0001, Mann-Whitney U test).

We also evaluated the presence of antibodies against TGFα, looking for the possibility of cross-reactivity induced by EGF vaccination. Sera were taken before vaccination (day 0) and post-immunization (between 3 and 6 month). Before vaccination, natural TGFα-specific antibodies were found (geometric mean, 1:202) and antibody titers >1:100 were detected in 35% of the patients. After EGF immunization, anti-EGF antibody titers (geometric mean, 1:8724) were significantly higher than anti-TGFα titer (geometric mean, 1:208) in the vaccinated subjects (P < 0.0001). No induction of anti-TGFα antibodies occurred as a consequence of EGF vaccination and no significant differences in the specific anti-TGFα response

**Statistical methods.** Medians of continuous variables were compared by the Mann-Whitney U test and Dunn test. Survival time was calculated from patients’ randomization. Survival data were analyzed using the Kaplan-Meier method and the log-rank test. Pearson’s correlation coefficient was used to estimate the correlation between the immunologic and clinical variables with survival time. All analyses were done using SPSS for windows version 10.0 and GraphPad Prism version 4.0.

**Fig. 1.** Anti-EGF humoral responses. Antibody titers were detected by ELISA in serially diluted sera from NSCLC patients not treated (n = 16) or treated (n = 26) with 50 μg equivalents of human EGF, with four doses weekly initially and then one dose monthly. Y-axis, geometric mean of antibody titers obtained from each group of patients. X-axis, different time points after the first immunizations. The vaccinated patients (■) developed a high anti-EGF antibody response compared with the natural antibody responses found in control patients (○) over time.

**Fig. 2.** Relationship between the anti-EGF antibody titers and sEGF concentration in sera from NSCLC patients. Right ordinate shows the log of geometric mean of antibody titers (■) and left ordinate shows the sEGF concentration (▲). Abscissa is time after the first immunization. There was a statistically significant inverse correlation between sEGF concentration and anti-EGF antibody titers in the vaccinated group (A, P < 0.05; Pearson correlation) but not in the control group (B).
EGF Vaccination Effectiveness in Advanced NSCLC

Inhibition of phosphorylation. The capacity of anti-EGF antibodies to inhibit the activation of EGFR in the presence of EGFR was examined by Western blotting assay to detect phosphorylated EGFR. Sera from vaccinated patients significantly abrogated EGFR phosphorylation in the presence of EGF as compared with control patients (P < 0.05, Mann-Whitney U test; Fig. 4A). In the vaccine group, post-immunization samples of 8 of 13 evaluated subjects inhibited the EGFR phosphorylation (range, 13.1-62.5% inhibition). Neither sera before immunization nor sera from patients in the control group were able to hamper the EGF-induced EGFR phosphorylation. In addition, a high correlation between anti-EGF antibody titers and the capacity to inhibit phosphorylation was found in post-vaccination sera (P = 0.01, Pearson). However, in some individual patients, serum samples taken at different time points and showing similar anti-EGF antibody titers showed different EGFR phosphorylation inhibition capacity. One of these cases is exemplified in Fig. 4B. For this particular patient, no inhibition of the receptor phosphorylation was evident at month 3 with an anti-EGF titer of 1:16,000, whereas a 30% of inhibition of EGFR activation was detected at month 6 after vaccination although the anti-EGF antibody titer was the same. Moreover, 11 months later, the percent of inhibition was increased to 100% with a higher antibody titer (Fig. 4B).

Immunodominance of EGF peptides. We tested sera from 13 patients classified as good antibody responders against six peptides previously designed to cover different regions of the TGFα concentration was also measured in sera collected from 18 patients (13 vaccinated and 5 controls). Mean pre-vaccination TGFα concentration was 8.48 pg/mL for all patients. EGF vaccination did not provoke any reduction in TGFα serum concentration (data not shown).

EGF/EGFR binding inhibition capacity. The capacity to inhibit the binding of EGF to its receptor was assessed in 18 patients (13 vaccinated and 5 controls). Sera from 11 (76.9%) vaccinated patients, collected at least 3 months after vaccination, were able to inhibit EGF/EGFR binding (range, 22.4-58%). A direct correlation between percentage of binding inhibition and the anti-EGF antibody titer was shown with the Pearson correlation coefficient (P < 0.05), as shown in Fig. 2. Vaccination resulted in a reduction of sEGF in the serum; in 94% of good antibody-responder patients, the sEGF concentrations decreased to values <168 pg/mL. No correlation between sEGF concentration and anti-EGF antibody titers was found at any time in patients from the control group.

Serum EGF and TGFα concentrations. All tested patients (26 in the vaccine group and 16 in the control group) showed high levels of self EGF (sEGF) concentration before treatment. The mean sEGF concentration was 1,893.65 pg/mL (range, 124.3-5,000 pg/mL). There was a statistically significant inverse correlation between the sEGF concentration and the anti-EGF antibody response over time in the vaccine group according to the Pearson correlation coefficient (P < 0.05), as shown in Fig. 2. Vaccination resulted in a reduction of sEGF in the serum; in 94% of good antibody responders against six peptides previously designed to cover different regions of the EGF membrane for immunoblotting.
EGF molecule (17) to evaluate the immunodominance of the antibody response induced by vaccination. Antibodies to all peptides existed in the natural response before vaccinations. However, after vaccinations, a clear dominance of immune response was observed in some patients. For 46% (n = 6) of the analyzed patients, the anti-EGF antibody response was focused on the central peptide corresponding to loop B (region 15-33), which is also the main region involved in the binding of EGF to the EGFR. In these patients, the absorbance of the antibody ELISA against the B-loop was at least twice the mean absorbance of the response against the other peptides. In the remaining patients, the antibody response was evenly distributed against all regions of the EGF molecule without a clear dominance. All samples that preferentially recognized the central loop also showed a strong inhibition capacity for the binding of EGF to the EGFR.

**Immune response and relation to clinical outcome.** In this study of 42 patients, there was a trend toward better survival in all vaccinated patients as compared with the control group, but it did not achieve the level of statistical significance. However, the survival advantage for vaccinated patients was statistically significant for the subgroup of patients ages <60 years.

Confirming a previous finding of our team in other clinical trials with this vaccine, within the vaccinated subgroup (n = 26), patients who elicited a good antibody response had a significantly better survival compared with patients who did not. Moreover, inside the good antibody responder group, patients younger than 60 years (n = 12) had a larger increase in median survival (median, 15.03 versus 7.43 months in the oldest patients; Fig. 5A).

The decrease in sEGF serum concentration elicited by vaccination was also associated with survival in this subgroup of vaccinated patients (n = 26). Patients in which the serum EGF concentration decreased below 168 pg/mL had significantly longer overall survival (median, 13 months) than patients in which sEGF concentration remained above that level (5.6 months; P = 0.0023; Fig. 5B).

![Fig. 5. Survival curves according to the humoral immune response generated in NSCLC patients after EGF vaccination.](image-url)
The inhibition of the binding of EGF to EGFR can be linked to longer survival; vaccinated patients (n = 10) whose sera showed inhibition of EGF/EGFR binding had a longer survival than patients (n = 3) whose post-immunization sera did not inhibit the binding (median 11.77 versus 5.63 months; \( P = 0.0001 \); Fig. 5C). This result should be confirmed in a larger patient sample.

Additionally, patients (n = 6) whose sera preferentially recognized central loop (B-loop) had a median survival equivalent to 33.5 months, whereas those patients (n = 7) who did not preferentially recognize this peptide had a median survival of 7.43 months (\( P = 0.012 \); Fig. 5D).

Finally, as expected, survival of control patients showed no correlation with any of these variables.

**Discussion**

NSCLC is the leading cause of cancer death worldwide (19). Approximately 80% to 85% of lung cancer cases are NSCLC, and roughly 65% of these patients have advanced-stage (IIIB/IV) disease at diagnosis. Prognosis of these patients is still very bad. Despite the introduction of more effective chemotherapeutic agents, it seems that a survival plateau has been reached.

The median survival for patients with advanced-stage NSCLC treated with platinum-based chemotherapy is a disappointing 8 to 10 months (20). New treatment strategies are undoubtedly needed. EGFR is overexpressed in 70% of NSCLC, and therefore an appealing therapeutic strategy would be the use of agents targeting specific pathways related to the EGFR. Several small-molecule tyrosine kinase inhibitors and monoclonal antibodies have already been introduced in clinical trials (21).

Our team is pursuing an innovative therapeutic approach by means of an EGF based cancer vaccine, which intends to reduce the availability of the main EGFR ligand.

This article summarizes the results of the immunologic evaluation of the NSCLC patients randomized to be treated either with the EGF cancer vaccine or with best supportive care. It also illustrates the effect of the anti-EGF antibodies on the EGFR signal transduction cascade.

After vaccination, 70% of the patients achieved a very high antibody response against self-EGF whereas none of the controls did. In a previous nonrandomized clinical trial, we had already reported that the magnitude of antibody response against EGF in vaccinated patients is positively associated with survival (14). Although no reproducible effects of age on survival are found in our control groups, the survival advantage elicited by vaccination was concentrated in the age group <60 years. Moreover, we found that inside the good antibody responders, patients younger than 60 years had a better survival compared with the older ones. A growing body of literature already exists (22–24) about the changes in the immune system related to senescence, which affects both T and B cells. Reduced responses to autoantigens and foreign antigens in aged individuals with consequently less protective immunoglobulins of lower titers and decreased affinities have been described (25). It is therefore not surprising that this cancer vaccine produced a therapeutic effect mainly in younger patients. However, the fact that there is an effect of age on survival within the good antibody responders suggests that the influence of age cannot be attributed exclusively to the capacity of the individual to generate anti-EGF antibodies.

In addition to measuring the antibody titers, EGF concentration can also be taken as a surrogate marker of the effect of the vaccine because serum EGF concentration dramatically decreased with vaccination, showing an inverse correlation with antibody response.

However, anti-EGF antibody response did not cross-react with TGFα. Despite the fact that EGF and TGFα are structurally and functionally related growth proteins (26), they are immunologically unrelated. Anti-EGF vaccination did not produce anti-TGFα antibodies and did not reduce TGFα concentration.

Although EGF and TGFα share similar secondary and tertiary structures imposed by three highly conserved intramolecular disulfide bonds, they only have 30% to 40% overall sequence identity. This suggests the necessity of a complementary vaccine with TGFα as immunogen. Our group is already evaluating a TGFα-based vaccine in the preclinical setting (10).

In the present article, we show for the first time data on the relationship between the anti-EGF antibodies and the functionality of the signal transduction cascade.

Sera from the vaccinated patients (but not sera from control patients) blocked the EGF/EGFR binding and inhibited the EGF-induced EGFR phosphorylation. There was a significant direct correlation between the antibody titer and both the inhibition of EGF binding and the abrogation of EGFR phosphorylation. This relationship validates the concept that the magnitude of the antibody response is critical for the end result of target inhibition. However, this association is not linear. We had serum samples with similar antibody titers but had very different percentage of phosphorylation decrease, suggesting diverse “qualities” of the antibodies and the need to pay closer attention to the measurement of the antibody affinities.

In addition to antibody specificities, unpublished data from our group suggested that the antibody affinities also seem to be important in the effectiveness of this vaccine.

Finally, we characterized the immunodominance of the antibody response. Although vaccination might induce a partially artifactual response against the truncated human recombinant EGF used in the vaccine or the neo-epitopes arising after the conjugation process, our evidence shows that vaccination induces a strong response against the EGF, which is relevant for its biological function. In half of the evaluated subjects, there was a clear dominant response against the central peptide corresponding to the B-loop β-sheet, which is the major structural element in EGF molecule (27) and also the main region involved in the binding of EGF to the EGFR. This finding might explain the neutralizing capacity of the induced antibodies and could also be considered as a surrogate marker for target inhibition.

Good antibody responses, EGF decrease, immunodominance by loop B, as well as the EGF/EGFR binding blockade capacity were all significantly associated with survival.

In summary, our results suggest that vaccination leads to safely generated anti-EGF neutralizing antibodies that inhibit EGFR activation. Patients with higher antibody response had a significantly better survival. We are now working on improving the vaccination dosage and schedule to further amplify the immune response. A larger biomarker panel and also “immunosenesence” markers are currently under evaluation as surrogates of clinical benefit in vaccinated patients.
References


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