Effects of an epidermal growth factor receptor-based cancer vaccine on wound healing and inflammation processes in murine experimental models

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Key words
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Abstract
Anti-epidermal growth factor receptor (EGFR) therapies have been proven clinically effective for a variety of epithelial tumours. Vaccination of mice with the extracellular domain (ECD) of autologous EGFR overcomes the tolerance to self-EGFR and has antimitastatic effect on EGFR+ tumour. Because EGF/EGFR-signalling plays an important role in the inflammation stage of wound healing, the main objective of this study was to explore the possible role of murine (m) EGFR-ECD vaccine in the croton-oil-induced ear oedema and wound healing process in mice as autologous experimental models, mimicking the possible post-surgical wound complication in patients treated with human EGFR-ECD/VSSP vaccine. Mice were intramuscularly immunised four times; biweekly with the mEGFR-ECD/VSSP/Mont. Seven days later, an 8 mm diameter, full-thickness skin wound was created on the back of each animal. Immunisation induced a strong specific humoral response against the mEGFR-ECD protein and a DTH dose–response curve but interestingly, animals treated with mEGFR-ECD/VSSP/Mont had similar inflammatory and healing speed responses compared to control ones. These data suggest that application of mEGFR-ECD/VSSP vaccine as a therapeutic approach in cancer patients could not elicit a poor healing process after surgery.

Introduction
The epidermal growth factor (EGF) family comprises four proteins: EGF, tumour growth factor (TGF), heparin-binding EGF and amphiregulin. All are similar in structure, act upon the same cell-surface receptor and have similar biological effect (1). The role of EGF has been extensively investigated in normal and pathological wound healing; EGF facilitates epidermal cell regeneration and plays an essential role in the process of dermal wound healing through stimulation of proliferation and migration of keratinocytes. It also promotes formation of granulation tissue and stimulates fibroblast motility (2). Activation of the EGF receptor (EGFR) launches a cascade of events that lead to angiogenesis, proliferation and cell migration (3).

Key Messages
- the aim of this article was to elucidate the effect of an autologous HER-1 cancer vaccine in experimental animal models of wound healing and inflammation
- immunisation induced in mice a strong specific humoral response against the murine EGFR-ECD protein and a DTH dose–response curve which did not affect skin wound repair or inflammatory oedema

In tumourigenesis, EGFR expression and its downstream growth promoting processes are altered, causing metastasis and unregulated cell growth (4). EGFR is involved in cancer cells’ distinctive properties. These include the capacity of
autocrine growth stimulation, apoptosis evasion, unlimited replication, angiogenesis promotion, and capacity for tissue invasion and metastasis (5). The aberrant activation of the key pathways of intracellular signalling is believed to be central to malignant transformation which is associated with the aberrant expression of oncoproteins. EGFR is overexpressed in many human epithelial tumours, such as those of lung (6), breast (7), ovary (8), colon (9), head and neck (10), pancreatic (11), bladder (12), vulva and ovary (8).

Because of their role in these processes, anti-EGFR therapies have been developed and have been proven effective in clinical trials for a variety of tumour types (13,14). EGFR-based active specific immunotherapy may be an alternative and complementary approach for the treatment of epithelial tumours, provided that induction of an immune response against self-EGFR is feasible. Recently it was reported that vaccination of mice with the extracellular domain (ECD) of autologous EGFR overcomes the tolerance to self-EGFR and has antimitastatic effect on EGFR+ tumour (15). These previous results indicate that immunisation of cancer patients with the human EGFR-ECD could result in a good clinical outcome.

Because EGFR-signalling is important in wound healing and EGF also plays an important role in the inflammation stage of wound healing (16), it is unknown whether this EGFR therapy poses an additional risk for the wound healing process. The main objective of this study was to explore the possible role of murine EGFR-ECD vaccine in croton-oil-induced ear oedema and wound healing in experimental animal models.

Materials and methods

Materials

The vaccine was prepared by mixing murine EGFR-ECD (50 µg) with very small-sized proteoliposomes (VSSP) adjuvant, which is obtained from the combination of the outer membrane proteins of Neisseria meningitidis with GM3 ganglioside (17). Then, the EGFR-ECD/VSSP preparation was emulsified (water in oil emulsion) in Montanide (Mont) (Sep-Igoside, France). This vaccine formulation will be referred to as mEGFR-ECD/VSSP/Mont.

Acetone, 12-O-tetradecanoylphorbol-13-acetate and croton oil were obtained from Sigma Chemical Co. (St. Louis, MO).

Mice and immunisation protocols

Female BALB/c mice, aged 13–14 weeks, were purchased from the National Center for Laboratory Animals Breeding (CENPALAB, Havana, Cuba). Food and water were administered ad libitum. All mice were kept under pathogen-free conditions. All animal studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Mice were immunised every 2 weeks with 50 µg of mEGFR-ECD/VSSP/Mont or with normal saline/VSSP/Mont intramuscularly four times.

Measurement of antibody titers

Sera were extracted on day 60 after the first immunisation. Microtiter plates (High binding, Costar, Corning, NY) were coated with 5 µg/ml of mEGFR-ECD in carbonate buffer, 0.1 M, pH 9.6, and incubated overnight at 4°C. Plates were blocked with 5% FCS in PBS/0.05% Tween 20. The sera dilutions (immune or control) were incubated for 1 hour at 37°C. Alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma) was added and incubated for 1 hour at 37°C. After addition of p-nitrophenylphosphate (1 mg/ml) (Sigma) in diethanolamine buffer pH 9.8, the absorbance at 405 nm was measured using a microwell system reader (Organon Teknica, Salzburg, Austria). ELISA test background was two times the absorbance at 405 nm of negative control sera, which coincides with the absorbance value for PBS (15).

Wound healing model

Seven days after the last immunisation, all animals were anaesthetised with intramuscular ketamine chloride (50 mg/kg) and their dorsal regions were depilated and washed with NaCl, 0.9% and ethanol 70%. Then, an 8-mm diameter, full-thickness skin wound was created on the back of each animal with a biotome (Acu Punch, Aucderm Inc., Fort Lauderdale, FL) in aseptic conditions. Animals were observed daily up to the 11th day post-surgery, when they were euthanised.

Wound closure dynamics were measured with a caliper at days 0, 4, 7 and 11. Furthermore, digital photographs of the wounds were taken on days 0 and 7 after skin wound and a planimetry study was carried out on skin images (two images/animal). Digitalised images were treated with a DIGIPAT IBM/PC computer system (18) and the following parameters were determined:

1. Percent of total re-epithelised area, the percentage of wound closure was calculated as: (area of original wound – area of actual wound)/area of original wound × 100.

2. Percent of reduction in wound perimeter was calculated as (perimeter of original wound – perimeter of actual wound)/perimeter of original wound × 100.

DTH test

Sixteen days after the last immunisation, mice were sensitised by intradermal injection with 50, 100 or 200 µg of mEGFR-ECD in 50 µl of PBS in the right hind foot pad and by the same volume of PBS in the left foot pad. After 48 hours, mice foot swellings were measured using a plethysmometer (Ugo Basile, VA, Italy). Mice injected with PBS/FA and sensitised with mEGFR-ECD were considered as negative controls. Differences in DTH between treatments groups were statistically validated by ANOVA and Tukey’s multiple comparison tests.
Measurement of Oedema

Because inflammation (measured as oedema) is a phase of the wound healing process, 18 days after the last immunisation, croton oil in acetone (3%, 0.6 mg/ear), in both vaccine-treated and control mice, was administered via an inflammation-inducing irritant to the inner surface of the right ears in a volume of 20 μl by a micropipette. Left ears were treated with saline. Four hours after the application, the animals were euthanised and 6 mm diameter disc from each ear was removed with a sharp metal punch. The weights of these disks were measured to mg accuracy. The oedema was calculated in terms of weight difference between the right ears and the left ones. Previous works have shown that acetone vehicle has no effect by itself (19).

Histological preparation

The ulcer area and a portion of surrounding tissue were excised using surgical scissors on day 11 after skin wounding. The samples were fixed in 10% buffered formalin and paraffin-embedded sections were stained with haematoxylin/eosin. Samples were blindly evaluated by a pathologist for determining the extent of the healing process. Also, histological score for wound healing was determined by two independent observers under a dissecting microscope and semi-qualitatively graded as follows (20):

Epidermis

Grade 1: Incomplete reepithelialisation, scanty projection of the epidermis with thin edges.
Grade 2: Complete reepithelialisation with less epidermal thickness and permanence of the desiccated clot.
Grade 3: Complete reepithelialisation with moderate thickness of the regenerated epidermis. Absence of the desiccated clot.

Dermis

Grade 1: Some collagen fibres in the neomatrix with no organisation and focally distributed. The infiltration of macrophages and angiogenesis is evident.
Grade 2: Presence of more collagen fibres, partially orientated in location parallel to the epidermis. Persistence of some dilated blood vessels.
Grade 3: Complete restitution of the new matrix with collagen fibres horizontally orientated. Absence of macrophages and scanty collapsed blood vessels.

The quantitative image morphometric analysis was carried out on skin sections (three sections/animal) after being stained with haematoxylin and eosin. The images data for morphometry were studied with the DIGIPAT IBM/PC computer system (18). Epidermis and dermis were evaluated based on gross appearance.

Statistical analysis

All statistical analyses were carried out using Minitab Data Analysis Program version 14 (Minitab Inc for Windows, 2003, USA). Statistical evaluation was performed by a randomised complete analysis of variance design with significance assessed at \( P < 0.05 \) level (ANOVA) or by the unpaired \( t \)-test. When data did not have a normal distribution, the Kruskall–Wallis test and the two-tailed Mann–Whitney test were used. The statistical evaluation of histological semi-qualitative analysis was performed by means of \( \chi^2 \) test.

Results

mEGFR-ECD immunisation induces a strong specific humoral response

Vaccine-related humoral responses were explored by immunising BALB/c mice four times every 2 weeks with 50 μg of mEGFR-ECD in VSSP/Mont. Inoculated mice developed high serum IgG antibody levels against the immunising protein 60 days after the first immunisation (Figure 1). No significant differences were observed in the body and spleen weights of the immunised mice in comparison with control animals (data not shown).

mEGFR-ECD/VSSP/Mont immunisation induces dose dependent DTH response

Generation of specific DTH responses was considered as a primary endpoint for the mEGFR-ECD vaccine ability to induce cellular immune response in immunised mice. Mice were intramuscularly injected with mEGFR-ECD/VSSP/Mont and then sensitised with 50, 100 or 200 μg of the nominal antigen on day 16 after the last immunisation. After 48 hours, mice foot swellings were measured. Whilst mice sensitised with 50 μg of mEGFR-ECD did not show statistical differences with respect to control group, those sensitised with 100 and 200 μg resulted in dose-dependent higher inflammations (\( P < 0.05 \), Tukey’s multiple comparison test) (Figure 2). DTH test was safe and well tolerated, with no adverse events such as blistering or ulceration.

Figure 1 Anti-mEGFR-ECD humoral response. BALB/c mice were immunised four times with 50 μg of the mEGFR-ECD in VSSP/Mont biweekly. Antibody titers were quantified by ELISA in sera collected on day 60. Data was log transformed (Log 1/titer + 1) for graphic representation (Mann–Whitney U-test, \( P \leq 0.05 \)).
mEGFR-ECD/VSSP/Mont immunisation does not affect the inflammatory oedema provoked by croton oil

For measurement of oedema, 11 days after the last immunisation, croton oil in acetone (3%, 0.6 mg/ear) was applied to right ears of all animals, while left ears were treated with saline. Four hours after the application, the animals were euthanised and 6 mm diameter disc from each ear was removed with a sharp metal punch. No statistical significant changes (Mann–Whitney test, \( P > 0.05 \)) were noted in terms of weight difference between the right ears and the left ones; mEGFR-ECD/VSSP/Mont-treated mice had a similar inflammatory response compared to control ones in the croton oil inflammation model (Table 1).

mEGFR-ECD/VSSP/Mont immunisation does not affect skin wound repair

A full-thickness skin wound was performed on the back of each animal in aseptic conditions 7 days after the last immunisation. Animals were observed daily; wound size was measured with a caliper at days 0, 4, 7 and 11 and the planimetry study was developed at 0 and 7 days.

No differences in healing speed, measured with a caliper, were found for the skin wounds inflicted in the mice vaccinated with mEGFR-ECD, with respect to the non-immunised control animals (data not shown). Furthermore, Table 2 shows that no statistical significant changes (\( P > 0.05 \)) were detected by planimetry study, in the healing indexes of wounds of immunised animals as compared with placebo-treated animals.

Table 2 Wound planimetry values 7 days after surgery in control and mEGFR-ECD/VSSP/Mont treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Re-epithelised area (%)</th>
<th>Wound perimeter reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSSP/Montanide</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>mEGFR-ECD/VSSP/Montanide</td>
<td>72.40</td>
<td>4.88</td>
</tr>
</tbody>
</table>

Wound healing is unaffected by mEGFR-ECD/VSSP/Mont treatment

Histopathological examination of the resected skin specimens revealed no wound healing complications in any animal. This study also evidenced that inflammation, fibroblasts and collagen were all in accord with the degree of fibrosis indistinctly in the control group or in mice immunised with mEGFR-ECD/VSSP/Mont or placebo. Besides, no significant differences in skin structure were evidenced in vaccinated mice, with respect to the non-immunised control animal.

Histological semi-qualitative analysis of wound repair (Table 3) did not reveal statistical differences (\( P > 0.05 \)) between wounds from mEGFR-ECD vaccine-treated and control animals. Furthermore, no significant difference in size of the skin layers (\( P > 0.05 \)) were observed between control and mEGFR-ECD/VSSP/Mont-treated mice by quantitative image morphometric analysis (Table 4).

Discussion

Dermal wound healing is a very orderly and efficient process characterised by four distinct but overlapping phases: haemostasis, inflammation, proliferation and remodelling (21). The wound-healing response begins the moment the tissue is injured. The initial inflammatory phase of wound healing...
is cell-mediated and depends on the interactions between chemokines, cytokines and growth factors to recruit phagocytes and fibroblasts at the wound site and to help promote remodelling of damaged tissue. This early response is pivotal because when it is impaired, the entire healing cascade is disrupted (22).

In the early stages of wound healing, fibroblasts interact with surrounding cells that initiate numerous cell-signalling cascades, resulting in the synthesis of extracellular matrix (ECM), glycoproteins, adhesive molecules and various growth factors. During the proliferative phase of wound healing, fibroblasts infiltrate the wound area and begin to deposit new ECM. The newly deposited collagen matrix then becomes cross-linked and organised during the final remodelling phase (23). Thus, extensive proliferation and migration of fibroblasts is closely linked with granulation-tissue formation, the first manifestation of the early matrix, and is also associated with deposition of collagen fibres at later stages of the wound-healing process (24).

Evidences suggest that this process may be regulated by growth factors; among those, the healing promoting effect of EGF has been reported (20,25). Besides, it has been reported that lack of EGF provokes retarded development of foetal tissue, but no damage on healthy adult tissues (26,27). Then, it could be theoretically argued that an EGFR-targeting drug may affect the wound healing process by inhibiting the binding of the ligand to its receptor, even in case it does not provoke damage effect on tissues.

Immunisation of mice with the ECD of murine EGFR (mEGFR-ECD) in adjuvants stimulates a potent antimetastatic effect in the EGFR+ Lewis lung carcinoma model, by inducing specific humoral and cellular immune responses (15,28). These results strongly suggest that EGFR could be a significant target not only for passive but also for active immunotherapy. It further encouraged the development of the EGFR-ECD/VSSP/Mont vaccine for patients with EGFR+ tumours. Hence it is very important to study the potential collateral effects of EGFR tolerance rupture by vaccination.

In a recent research (29) on the effect of agents targeting EGF on wound healing it was demonstrated that the evidence of targeted agents causing surgical complications is limited. There are limited data for cetuximab, sorafenib and sunitinib and very little for other solid tumour targeted agents. In other studies of monoclonal antibody and gefitinib, a specific inhibitor of EGF-R-tyrosine kinase treatments, wound healing complication rates have not been shown to differ from those of other EGF monoclonal antibodies (35).

Dean et al. (32) demonstrated that in head and neck cancer patients cetuximab did not significantly increase the risk of post-surgical wound complications, although a higher absolute number of wound complications was observed in the group treated with cetuximab and radiation therapy (13%, 2 of 15 patients), compared with the group treated with radiation alone (0%, 0 of 20 patients).

Panitumumab is a monoclonal antibody targeting the ECD of the EGFR (36). No guidelines have been published regarding the cessation of panitumumab therapy prior to surgery. However, according to Parikh et al., there does not, as yet, appear to be an increase in perioperative complications with this EGFR inhibitor (37).

Clinicians must keep these potential complications in mind in the clinical practice and in clinical trials with these kinds of drugs and provide greater support during any postoperative period to identify possible adverse drug-related events.

The majority of today’s non-clinical and clinical data suggest that anti-EGFR drugs, an anti-EGF vaccine (31,38,39) or an anti-EGFR vaccine (present data) do not elicit a serious deleterious wound healing process and therefore, the role of the EGF/EGFR system in wound healing needs to be re-dimensioned; this idea was previously considered by Govindan et al. (30,34).

In summary, our data showed that antibodies to EGFR did not have a deleterious effect on the healing process in adult mice. These data suggest that clinical application of EGFR-ECD/VSSP vaccine as a therapeutic approach in cancer patients could not elicit a poor healing process after surgery or other invasive procedures. An immune-active dose-escalation Phase I Clinical Trial in hormone refractory prostatic cancer patients treated with the ECD of the human EGFR is on-going in Cuba, and special emphasis is being made on detecting any skin or wound healing adverse effect.

References


