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TITLE: Targeting Mutated Epidermal Growth Factor Receptor

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designated by other documentation.
This project is aimed at developing specific and effective vaccines for immunotherapy of breast cancer targeting mutated epidermal growth factor receptor (mEGF-R) in a rat model. During the first 2 years of funding, vaccines of mEGF-R (peptides, recombinant protein and viral vector) were produced and shown to induce humoral immunity to rat mEGF-R in rats. Furthermore, rat mammary carcinoma cells were transfected with rat mEGF-R cDNA to provide target cells for in vivo vaccinations. During the past (third) year of funding, our efforts to isolate transfectants expressing the mutated epitope were unsuccessful, presumably due to mutations in the rat mEGF-R vector.

We have evaluated breast carcinoma patients' humoral and cellular immune responses to mEGF-R protein and peptides. Four of four patients tested had circulating antibodies that bound to mutated, but not normal, EGF-R protein. Neither of the 2 healthy donors' sera bound to mEGF-R. The peripheral blood lymphocytes of 2 of 5 breast cancer patients significantly proliferated to stimulation with mEGF-R protein and/or peptides. These data suggest that the mEGF-R epitope is immunogenic in breast cancer patients and, therefore, it should be possible to boost this immunity with mEGF-R vaccines.
FOREWORD

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TARGETING MUTATED EPIDERMAL GROWTH FACTOR RECEPTOR

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**Introduction** (adapted from previous report)

Clinical trials of active immunotherapy in breast carcinoma patients have suffered from the use of vaccines that induce only humoral, but not cellular, immunity (1,2) or lack specificity (3). Preclinical and clinical studies with cancer vaccines have demonstrated a correlation between the induction of humoral or cellular immunity and tumor growth inhibition (4-12). Thus, tumor vaccines ideally should induce both humoral and cellular immunity and induced immunity should be specific for the tumor cells. The major goal of this study is to test tumor-specific vaccines against breast carcinoma in a relevant rat model. Mutated epidermal growth factor receptor (mEGF-R) is expressed by a high proportion of breast carcinoma tissues derived from various patients, but not several normal tissues tested (13; and our unpublished data described in the original proposal). mEGF-R is expressed both on the surface and in the cytoplasm of tumor cells (13), rendering it a target for both B and T cells. Furthermore, targeting of mEGF-R may exert direct anti-proliferative effects (14).

We have chosen a rat model of mEGF-R vaccines for the proposed studies because of the availability of cloned normal rat EGF-R (15) and MHC class I and II positive rat mammary carcinoma cells with either high or low metastatic capability (16,17). The most specific vaccine of mEGF-R consists of the minimal sequence, including the mutation, that elicits B- and/or T-cell responses. We have chosen peptides of mEGF-R for induction of T cells, analogous to studies performed successfully with peptide vaccines by other groups (18-24) and our collaborators (25-27) in various antigen systems. In addition, recombinant, extracellular mEGF-R protein and full-length mEGF-R expressed in adenovirus vector have been chosen for induction of protective humoral and cellular immunity, respectively.

In conclusion, mEGF-R is a unique target for active specific immunotherapy of breast carcinoma, based on its specificity, frequency of expression, potential for activating both B and T cells, and availability of an ideal animal model of active immunotherapy. The studies will provide the rationale for specific active immunotherapy of breast carcinoma patients. The results we will obtain with mEGF-R in the rat mammary carcinoma model may be applicable to other tumor systems, such as lung carcinomas and gliomas which also express mEGF-R (13,28).

**Body of Work**

During the first year of funding (July 96-June 97), we made the following achievements:

a. Rat mammary carcinoma transfectants were generated which expressed the 145 kDa rat mEGF-R protein by Western blot analysis of whole cell extract, but did not express the protein in membrane extract. The transfectants did not react with murine monoclonal antibody (MAb) L8 directed to the human mEGF-R epitope. They were tumorigenic in syngeneic rats.

b. Rat mEGF-R and human mEGF-R proteins, both specifically reactive with MAb L8 were produced in recombinant baculovirus.

c. Peptides of rat mEGF-R were synthesized.

During the second year of funding (July 1997-June 1998) we focused our efforts on:

a. The generation of rat mammary carcinoma transfectants MTLN3 with stable expression of the mEGF-R epitope (defined by MAb L8 to the human mEGF-R epitope) following rat mEGF-R cDNA transfection. However, such transfectants could not be obtained.

b. Production of rat and human mEGF-R peptides and recombinant rat mEGF-R adenovirus vector for vaccination purposes.

c. Immunizations of rats with mEGF-R protein and peptides. Immunized rats produced antibodies binding to mutated and/or normal EGF-R. Thus, rats are not immunologically tolerant to
normal EGF-R and induction of immunity to this protein was not accompanied by toxicity to those organs which express the protein.

During the past funding period (July 1998-June 1999) we have performed the following studies:

a. Transfection of rat mammary carcinoma cells with rat mEGF-R cDNA in PcDNA3 vector. We had attributed our failure to obtain murine mammary carcinoma transfectants stably expressing rat mEGF-R protein to mutational events that possibly had occurred in the transfectants in vivo. Therefore, in order to obtain stable transfectants, a much larger number of mammary carcinoma cells needed to be transfected with the mEGF-R cDNA (see previous report for details). Thus, we have performed 4 independent transfections of 115 million cells total using various methods of transfection (liposomes, FuGene and calcium phosphate). A total of 516 colonies were isolated and tested for expression of the mEGF-R epitope defined by MAb L8A4. Forty-eight of these colonies had 40% or more of the cells within a colony expressing the mEGF-R epitope. However, all colonies lost mEGF-R expression after 1-3 months in culture. Furthermore, attempts to isolate mEGF-R+ cells by fluorescence-activated cell sorting were unsuccessful. In parallel, we sequenced the mEGF-R PcDNA3 vector used for cell transfection. There were two mutations in the extracellular domain of mEGF-R (approximately 1,000 amino acids distant from the mEGF-R epitope, between this epitope and the transmembrane domain of mEGF-R) and two mutations in the intracellular domain (approximately 100 amino acids distant from the transmembrane domain). This mutation must have occurred at some time point during mEGF-R vector propagation in vitro as it was not found in the vector when it was originally constructed and sequenced. This mutation may explain our difficulties in obtaining transfectants that stably express the mEGF-R epitope. We are currently sequencing the original normal EGF-R vector which was used to construct the mEGF-R vector. If the normal EGF-R vector shows the correct sequence, it will be used to correct the mEGF-R vector.

b. Evaluation of breast cancer patients' immune responses to mEGF-R expressed by their growing tumors (3rd and last proposed aim). We have evaluated humoral and/or cellular immune responses of 5 breast cancer patients to mEGF-R. Four of the patients (DI, MC, VG, KG) had mEGF-R+ lesions and one patient (TF) had mEGF-R- lesions. Presence or absence of mEGF-R on the tumors was determined by reverse transcriptase polymerase chain reaction and immunohistochemistry with mEGF-R-specific MAb L8A4. Patients' sera were evaluated for the presence of antibodies binding specifically to mEGF-R in enzyme-linked immunosorbent assay. However, this assay showed high non-specific binding of the sera. We have therefore developed a radioimmunoassay which determines the inhibition of binding of MAb L8A4 to mEGF-R by patients' sera (see Fig. 1A legend for details). As controls, inhibition of binding of MAb NCL to normal EGF-R (Fig. 1B), or anti-colorectal carcinoma MAb GA733 to the GA733-2E antigen (Fig. 1C), or polyclonal antibody to carcinoembryonic antigen (CEA; Fig. 1D) by the breast carcinoma patients' sera was determined. Additional control experiments determined whether sera from healthy donors bound to mEGF-R as determined by inhibition of binding of MAb L8A4 to mEGF-R by the sera (Fig. 1E). Sera from all 4 breast cancer patients with mEGF-R+ lesions specifically bound to mEGF-R (Fig. 1A), but not to normal EGF-R (Fig. 1B) or CEA (Fig. 1D). The CEA system is a valid negative control antigen system as <0.01% of human sera bind to this antigen (29). However, two of the four breast cancer patients' sera bound to the colorectal carcinoma-associated antigen GA733-2E (Fig. 1C), in agreement with our previous unpublished results indicating that a significant fraction of sera from untreated colorectal cancer patients and healthy donors bind to this antigen. None of the two sera from healthy donors bound to mEGF-R (Fig. 1E).

We then tested lymphoproliferative responses of 5 breast carcinoma patients (all with EGF-R+ lesions except for patient TF who had EGF-R- lesion) to stimulation with mEGF-R protein, normal EGF-R protein, mEGF-R peptides in PLG microspheres or combined with β2-microglobulin, or the corresponding control preparations (for details see Fig. 2 legend). Two patients (TF and KJ; Fig. 2) demonstrated statistically significant and specific lymphoproliferative responses to stimulation with mEGF-R protein (both patients) and, in addition, mEGF-R peptide in PLG microspheres (patient TF only), but not to stimulation with the various control preparations. Notably, patient TF had a mEGF-R-negative lesion. Thus, it is possible that the response of this patient's lymphocytes to mEGF-R protein and peptide stimulation represents a primary response, in the absence of in vivo exposure of the
lymphocytes to the antigen. This possibility will be addressed by stimulating the lymphocytes of additional breast cancer patients demonstrating mEGF-R-negative lesions and the lymphocytes from healthy donors with mEGF-R preparations.

Conclusions

During the past funding period, our efforts focused on the establishment of rat mammary carcinoma cells expressing rat mEGF-R after transfection with rat mEGF-R cDNA. Unfortunately, rat mEGF-R expression was unstable in each of the 48 transfected colonies tested. Our preliminary data suggest that instability of the rat mEGF-R epitope in the transfectants is due to a mutation of the rat mEGF-R cDNA in the extracellular, and possibly also intracellular, domains. Current efforts are aimed at correcting this mutation. Once we have established stable transfectants these will serve as targets in approaches to active immunotherapy of rat mammary carcinomas using available vaccines (mEGF-R protein, peptides and viral vector) generated during the initial funding period.

We have shown that breast cancer patients produce antibodies specific for mEGF-R. These antibodies do not bind to normal EGF-R or CEA. Furthermore, antibodies to mEGF-R were absent in healthy individuals' sera. The antibodies found in breast cancer patients' sera may be elicited by the mEGF-R epitope expressed by the patients' tumors. Interestingly, lymphoproliferative responses to in vitro stimulation with mEGF-R protein and peptide not only were found in a breast cancer patient with mEGF-R⁺ lesion, but also in a patient with a mEGF-R⁻ lesion. Thus, mEGF-R stimulation of lymphocytes may induce a primary response, although this hypothesis will have to be investigated further. Collectively, these results suggest that it should be possible to vaccinate breast cancer patients against mEGF-R in vivo.

Thus, we have fulfilled most of the goals originally proposed for the 4 years of study, with the exception of cellular and protective immune response evaluation in immunized rats. The latter studies could not be performed because we currently lack the appropriate tumor cells expressing rat mEGF-R epitope.

The goal for the last year of this funding period is to generate transfectants that stably express the mEGF-R epitope. These transfectants will be used to test the available mEGF-R vaccines for their protective effects in the rat model.

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A. Binding of breast cancer patient's sera to mEGFR

Inhibitors:
- MAb L8A4
- DI sera
- MC
- GV
- KJ

% inhibition of ¹²⁵I-MAb L8A4 binding

B. Absence of binding of breast cancer patients' sera to nEGF-R

Inhibitors:
- MAb NCL
- DI sera
- MC
- GV
- KJ

% inhibition of ¹²⁵I-MAb NCL binding

C. Binding of breast cancer patients' sera to GA733-2E

Inhibitors:
- MAb GA733
- DI sera
- MC
- GV
- KJ

% inhibition of ¹²⁵I-MAb GA733 binding

D. Absence of binding of breast cancer patients' sera to CEA

Inhibitors:
- anti-CEA PAb
- DI sera
- MC
- GV
- KJ

% inhibition of ¹²⁵I-PAb AntiCEA binding

E. Absence of binding of healthy donors' sera to mEGFR

Inhibitors:
- MAb L8A4
- DC sera
- DR

% inhibition of ¹²⁵I-MAb L8A4 binding

Figure 1

Reciprocal serum dilution Concentration (µg/ml)
Fig. 1. Immunoreactivity of breast cancer patients' sera with human mutated (m) or normal (nl) EGF-R.

A. Sera from breast cancer patients with mEGF-R⁺ lesions or healthy donors were tested for their capacity to inhibit binding of ¹²⁵I-labelled anti-mEGF-R monoclonal antibody (MAb) L8A4 to mEGF-R in radioimmunoassay. Briefly, wells coated with 10 µg/ml of anti-EGF-R MAb 425 were incubated first with 10 µg/ml of purified mEGF-R protein and then with inhibitors (serum dilutions or 1-20 µg/ml of MAb L8A4). 20K cpm per well of ¹²⁵I-labelled MAb L8A4 were added and % inhibition of binding of ¹²⁵I-MAb L8A4 to mEGF-R by sera or MAb L8A4 was calculated relative to buffer control.

B. As in A, except that wells coated with MAb 425 were incubated with 10 µg/ml of purified normal (nl) EGF-R protein followed by sera (or 1-20 µg/ml MAb NCL directed to the normal EGF-R) and 20K cpm per well of ¹²⁵I-labelled MAb NCL.

C. Wells were coated with 10 µg/ml chimeric MAb CO17-1A directed to the colorectal cancer-associated GA733 antigen, followed by the addition of 10 µg/ml of GA733-2E protein, sera (or 1-20 µg/ml of MAb GA733) and 20K cpm per well of ¹²⁵I-labelled MAb GA733.

D. Wells were coated with 10 µg/ml of anti-carcinoembryonic antigen (CEA) MAb followed by incubation with 10µg/ml of CEA, sera (or 1-20 µg/ml anti-CEA polyclonal antibody (PAb)) and 20K cpm per well of ¹²⁵I-labelled anti CEA PAb.

E. As in A, except that the sera were derived from healthy donors.

Conclusions - All four breast cancer patients' sera specifically bound to mutated, but not normal, EGF-R; two of these sera also bound to GA733-2E protein, consistent with our previous findings that sera from a fraction of colon cancer patients or healthy donors bind to GA733-2E antigen. However, none of the breast cancer patients' sera bound to CEA. Neither of the two sera derived from healthy donors bound to mEGF-R.
Fig. 2. Breast cancer patients' lymphoproliferative responses to stimulation with mEGF-R protein or peptide.

PBMC from patient TF (A) or KJ (B) were stimulated for 10 (TF) or 5 (KJ) days with 10 μg/ml of mutated (m) and normal (n) EGF-R protein, 25 μg/ml of mEGF-R peptide (ALEEKKGNY), 25 μg/ml of mEGF-R peptide in presence of 1 μg/ml of β2 microglobulin (mgl), 1 μg/ml of mEGF-R peptide in PLG microsphere or control (co) peptides at the same concentration as the specific peptides. PBMC were then pulsed with ³¹H-thymidine and ³¹H-thymidine incorporation was measured. Values with identical letters differ significantly from each other (A: a, p = 0.003; b, p = 0.018; c, p = 0.02; d, p = 0.05; B: a, p = 0.05) in one sided student's t-test.

Conclusions: Patient TF has specific lymphoproliferative responses to stimulation with mutated EGF-R protein and mEGF-R peptide in PLG microspheres, but not to normal EGF-R or control peptide. Patient KJ has a response to mEGF-R protein only, but not to normal EGF-R or mEGF-R peptides.