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Peptide Vaccines and Peptidomimetics of EGFR (HER-1) Ligand Binding Domain Inhibit Cancer Cell Growth In Vitro and In Vivo

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Epidermal growth factor receptor (EGFR) is central to human tumorigenesis and dysregulation of this receptor is mainly due to increased expression (1) and mutations in different domains of the receptor (2). EGFR is overexpressed in most epithelial cancers including breast/triple-negative breast cancer (TNBC; 14–90%), non–small cell lung (40–80%), colorectal (25–77%), and other cancers. EGFR overexpression in tumors is responsible for aggressiveness, poor prognosis, decreased survival, poor response to therapy, and development of resistance (6). EGFR forms heterodimers with other human epidermal growth factor receptor (HER) family members like HER-2, HER-3, and HER-4 resulting in aggressive forms of cancer with lower survival rates (7). The development of Abs targeting EGFR is mainly dependent on structural studies that help outline the details of the receptors and other conformational changes affecting its activation and downstream signaling. EGFR signaling is highly dependent on ligand binding, which is a key factor in releasing the dimerization arm of the receptors (8), and this explains why many of the anti-EGFR Abs are directed toward the ligand-binding region. Cetuximab, a humanized mAb, binds EGFR, prevents ligand binding, and is Food and Drug Administration–approved for the treatment of metastatic colorectal cancers with high EGFR expression (9). However, the emergence of resistance to cetuximab has led to the proposal that other mechanisms exist that are independent of ligand-binding and this has led to the development of other Abs with key epitopes that are different from cetuximab. For instance, activation of other HER family receptors may help in the stabilization of EGFR even in the absence of ligand binding (10). These studies simply illustrate that multiple EGFR targeting strategies can be employed in targeting EGFR signaling mechanisms in cancer, and this may avoid resistance and additively or synergistically inhibit tumor growth. mAbs have additional limitations, such as repeated frequency of i.v. treatments and infusion reactions that can be lethal in some patients (11). Inhibition of metastasis by these agents has been shown to be nonspecific in both preclinical and clinical settings, and the toxicity caused by these agents clearly illustrates the potential risks of their continuous use in the clinic. Given these caveats, there is an urgent unmet need for developing more efficacious, safer, and less toxic anti-EGFR agents. We decided to tackle these drawbacks by designing peptide epitopes from different regions of the EGFR ligand binding domains in an attempt to engineer efficacious inhibitors of EGFR. Our well-established strategies [reviewed by Kaumaya et al. (12)] have been validated in previous studies by engineering conformational epitopes specific for the extracellular domain of oncogenic Ags. Our novel peptide epitopes exhibit several advantages over mAbs such as cetuximab: 1) the engineered conformational peptides can elicit high-affinity and high-

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Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; EGFR, epidermal growth factor receptor; HER-1, human epidermal growth factor receptor 1; MVF, measles virus fusion protein; rh, recombinant human; TNBC, triple-negative breast cancer; VEGF, vascular endothelial growth factor.

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specificity polyclonal Abs that bind and destroy the tumor; 2) peptide epitopes spanning different biologically relevant regions of EGFR could provide additional and superior antitumor effects; 3) immunization with chimeric EGFR peptide vaccines can train the human immune system to produce high-affinity EGFR Ab, establish memory, and serve as a better agent than presently available agents; and 4) by combining different peptide inhibitors from the same oncogenic protein together may provide a means of circumventing emergence of resistance.

In this study, we selected three critical epitopes of EGFR (347–374, 382–410, and 418–435) as potential candidates for both the vaccine and therapeutic approach (Table I). The choice was based on: 1) the crystal structure of the complex between EGF and EGFR and analysis of the contact residues on both sides of the interface (13) that are all involved in ligand binding; and 2) computer-aided analysis of various algorithms of immunogenicity and antigenicity (14). The chimeric B cell epitopes were synthesized incorporating the measles virus fusion protein (MVF) promiscuous T cell epitope as previously described (15). Sequence-specific antipeptide Abs to the EGFR ligand binding domain were elicited by immunization of rabbits and mice. The peptide vaccines were highly immunogenic in both mice and rabbits, and the Abs elicited were able to specifically recognize the native EGFR receptor as shown by their reactivity in MDA-MB-468 (TNBC) cells and A549 lung cancer cells in flow cytometry and their binding to recombinant human (rh)EGFR in ELISA. We evaluated the in vitro antitumor effects of the Abs and the peptide mimics (B cell epitope alone) in different signaling assays: cellular proliferation, apoptosis induction, EGFR dephosphorylation, and Ab-dependent cellular cytotoxicity (ADCC). The peptide mimics and antipeptide Abs to all three epitopes inhibited cancer cell growth, prevented EGFR-specific phosphorylation, downregulated EGFR signaling pathways, and caused increased apoptosis and ADCC in EGFR-expressing cells. In mouse model studies, two of the vaccine epitopes were able to delay tumor growth in a met-1 transplantable breast cancer model and in SCID mice that were inoculated with A549 lung cancer cells. The combined in vitro and in vivo results obtained showed that the HER-1 (382–410) and HER-1 (418–435) sequences were the best can-

### Materials and Methods

#### Peptide synthesis and characterization

Peptide synthesis was performed using 9600 Milligen/Biosearch solid-phase peptide synthesizer (Millipore, Bedford, MA) using Fmoc/-Bu chemistry. Clear amide resin (0.32 mmol/gm) (Peptide International, Louisville, KY) was used for synthesis of all of the peptides. In the case of the peptide vaccines, the B cell epitopes were co-linearly synthesized with the promiscuous Th MFV epitope using regioselective side chain protections. After synthesis, the peptides were cleaved from the resin as previously described (16). The crude peptides were purified by reverse-phase HPLC in a gradient system using a C-4 vydac column in water/acetoniitrile (0.1% trifluoroacetic acid) on a Waters system. At the end of purification, the pure fractions were then analyzed in analytical HPLC, and fractions of interest were pooled together and lyophilized in 1% acetic acid solution. The final purified peptides were then identified using electrospray ionization mass spectrometry (Campus Chemical Instrumentation Center, The Ohio State University, Columbus, OH).

#### Animals

FVB/n wild-type inbred mice and BALB/c SCID mice were purchased from Charles River Laboratories, whereas female New Zealand White outbred rabbits were purchased from Harlan breeders. Animal care and use was in accordance with the International Animal Care and Use Committee institutional guidelines.

#### Cell lines and Abs

All cell-culture medium, FBS, and supplements were purchased from Invitrogen Life Technologies. The human TNBC cell line MDA-MB-468 and lung cancer cell line A549 were purchased from American Type Culture Collection and maintained according to the supplier’s guidelines. Met-1 cells are a mouse mammary cell line established in vitro from PyMT-transgenic mice with an FVB/n background, in which tumor growth and development are dependent on high expression of the HER family members (17, 18). Cetuximab was purchased from The James Cancer Hospital pharmacy of The Ohio State University Wexner Medical Center.

#### Immunoassays

To determine the Ab response to the peptide vaccine in both mice and rabbits, experimental procedures were performed as previously described (16). ELISA was performed to evaluate the binding of the vaccine Abs to EGFR (R&D Systems) (as described in Ref. 20). The 96-well plates were coated with 100 μL 2 μg/ml peptide Ag in PBS and refrigerated overnight. The plates were then washed with PBT/human serum, and nonspecific binding was blocked by adding 200 μL PBS/1% BSA with 0.02% sodium azide. After washing, 200 μL serum dilution starting at 1/500 for mice and 1/4000 for rabbits was added to the peptide-coated plates in duplicate wells, serially diluted 1:2 in phosphate buffer with Tween 20/human serum wash buffer, and incubated for 2 h at room temperature. The plates were washed, and 100 μL HRP-conjugated secondary Ab to a dilution of 1/500 was added and incubated for 1 h. After incubation, the plates were washed, and 50 μL substrate solution was added to each well for bound Ab detection and incubated for 10 min in the dark before stopping the reaction with 25 μL 1% SDS and reading the absorbance at 415 nm using a Bio-Rad microplate reader (Bio-Rad). Titers were considered as the highest dilution of sera with an absorbance of 0.2 after subtracting the blank.

#### Rabbit immunization and Ab purification

Female New Zealand White rabbits were used for immunization. Groups of rabbits (two per each vaccine epitope) were immunized as previously described (16). For each rabbit, 1 mg peptide vaccine was dissolved in water with 100 μg nor-MDP, and the total volume was mixed with an equal volume of ISA 720 by plunging back and forth at least 50 times. The mixture was injected into the bicep muscle of the rabbit. The rabbits are immunized three times at 3-wk intervals, and 4 wk after the third immu-

#### Flow cytometry

The experiment was performed as previously described (21) using 5 × 10^5 MDA-MB-468 breast cancer cells or A549 lung cancer cells. Cells were trypsinized, resuspended in ∼10 ml appropriate growth media, and then counted by trypan blue staining. The cells were then resuspended such that a single solution of 100 μL will contain 1 × 10^6 cells in 5-ml polystyrene culture tubes. The primary Ab was then added to each tube, gently vortexed, and then incubated for 2 h at 4°C. The tubes were then washed twice in 1 ml ice-cold PBS, spun at 1700 rpm for 5 min, and then decanted. The secondary Ab (anti-rabbit IgG-FITC conjugate) made up at 1:50 in a final volume of 10 μL was then added to each tube and incubated in the dark for 30 min after vortexing. The tubes were then washed twice in 1 ml ice-cold PBS and spun at 1700 rpm for 5 min after each wash before resuspending in 500 μL 1% formaldehyde made in PBS. Samples were then analyzed by a Coulter ELITE flow cytometer (Beckman Coulter), and 10,000 events were counted per treatment. Single-parameter histograms were drawn after gating selection of healthy cells through light scattered assessment.

#### MTT proliferation assay

The MTT proliferation assay was performed as previously described (22). Briefly, MDA-MB-468 (1 × 10^5 cells/well) and A549 cells (5 × 10^4 cells/well) were incubated overnight before changing media to low FBS and then incubated for another day. The following day, the inhibitors were added at different concentrations in low FBS media and incubated for 1 h before stimulating with 50 ng/ml EGF and then incubated for 3 d. The MTT reagent was then added and incubated for 2 h followed by addition of extraction buffer and then incubating overnight. The following day, the plate was read at 570 nm using a spec-

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EGFR-specific phosphorylation

A549 and MDA-MB-468 cells (1 × 10^6) were seeded on six-well plates in growth media supplemented with 10% FBS and incubated overnight at 37°C. Culture media was then removed, and the cells were washed with 1 ml PBS before adding low serum media (1% FBS) and incubating for another day. Culture media was then removed and the cell layer washed with PBS before adding different 100 µg inhibitors in binding buffer (0.2% w/v BSA and RPMI 1640 medium with 10 mM HEPES [pH 7.2]) to the wells and incubating at room temperature for 1 h. The cells were then stimulated by adding 50 ng/ml EGF per well and then incubated for another 10 min at room temperature. Binding buffer was removed and the cell layer washed with PBS before adding 1 ml RIPA lysis buffer. Plates were then rocked at 4°C for 1 h, lysates were removed from wells and spun at 13,000 × g for 10 min, and supernatants were transferred into clean tubes and stored at −80°C until usage. Protein lysates were used to measure phosphorylated EGFR using the Duoset IC human phospho-ErbB1 ELISA kit (R&D Systems), and inhibition of phosphorylation was calculated using the formula: (ODTREATED − ODUNTREATED)/ODUNTREATED × 100.

Caspase assay

The caspase assay was performed exactly like the proliferation assay, except that after treatment, the cells were incubated for just 24 h. After incubation, the Caspase Glo reagent substrate (Promega) was prepared according to the manufacturer’s instructions and then added to the wells, mixed, and incubated for 2 h before measuring luminescence using a luminometer.

ADCC

ADCC was performed as previously described (20), in which target cells (A549 and MDA-MB-468 cells) were placed on 96-well translucent plates, and the vaccine Abs were added to the cells and equilibrated for 25 min before adding effector cells (human PBMCs) at different E/T ratios. The plate was then incubated for at least 2 h before adding the ADCC reagent mixture and luminescence was measured in a luminometer.

Antitumor effects of immunization with HER-1 peptide vaccines and peptide mimics in Fvb/n breast cancer model

The Fvb/n transplantable model of HER-family dependent breast cancer represents a good model for human breast cancer. The peptide vaccines were dissolved in water and emulsified in Montanide ISA 720 (1:1) and 100 µg nor-MDP (N-acetylglucosamine-3-yl-acetyl-l-alanyl-D-isoglutamine). Female wild-type FVB/n mice (Charles River Laboratories) at the age of 5 to 6 wk were immunized three times at 3-wk intervals with 100 µg peptide vaccine, and 14 d after the third immunization, the mice were challenged s.c. with Met-1 cells (200,000 per mouse). Met-1 cells were derived from the PyMT-transgenic mouse model with FVB/n background (17). Tumor growth was monitored for up to 6 wk after challenge. During immunization, blood was drawn biweekly and used in ELISA to monitor Ab titers. In the case of treatment with the peptide mimics, the mice were simply challenged with Met-1 cells and treated weekly i.v. with 200 µg peptide mimics for up to 6 wk beginning at day 0. The mice were euthanized at the end of treatment and tumors extracted and weighed.

BALB/c transplantable lung tumor model

BALB/c SCID mice 5 to 6 wk old were s.c. injected with 3 × 10^6 A549 lung cancer cells, and starting from day of tumor injection, mice were treated i.v. with 200 µg each peptide mimic or cetuximab. In the case of the Abs, the mice were treated i.p. with 500 µg starting from day 0, and all treatments were repeated weekly right up to week 6. Tumor growth was measured twice weekly using vernier calipers, and at the end of the experiment, the tumors were extracted and weighed.

Immunohistochemical analysis

Tumor tissues were formalin fixed, embedded in paraffin, and stained immunohistochemically for CD3 and Ki-67. The tissue blocks were cut into 4-µm sections, deparaffinized in xylol, and then rehydrated with different grades of alcohol. The rehydrated sections were then washed with distilled water and placed in a microwave oven for 15 min. The slides were then blocked and incubated for 40 min at 20°C with primary Abs specific for Ki-67 and CD3. After incubation, the slides were washed and incubated with secondary Abs for 30 min before revealing Ab binding with peroxide substrate and counterstaining with hematoxylin.

Results

Synthesis and characterization of peptide vaccines and peptide mimics

The crystal structure of the extracellular domain of EGFR bound to its ligand EGF reveals the specific key residues that are important in binding (13). We selected three peptide epitopes (Table I) that contain at least one region of the binding sequences that make contact with EGF ligand. One of the epitopes (382–410) overlaps with the binding site of cetuximab, which is known to inhibit EGFR ligand binding (9). The EGFR B cell epitopes were synthesized alone in the case of the peptide mimics or co-linearly with a promiscuous Th epitope as in the case of the peptide vaccines. The Th epitope is derived from a modified MVF (288–302) and was linked to the B cell epitope via a four-residue flexible linker for independent folding (23).

Immunogenicity of peptide vaccines in rabbit and binding of vaccine Abs to rhEGFR

We evaluated the immune response to each of the three peptide vaccines in outbred rabbits. The three constructs elicited high amounts of Abs with titers >250,000 (Fig. 1A–C), and high levels were still detected 3 wk after the third immunization. This illustrates that the vaccine constructs were highly immunogenic and able to establish immunological memory in the rabbits. We tested the binding of the Abs to rhEGFR in an ELISA assay using different dilutions, and these Abs were able to bind specifically to the protein in a concentration-dependent manner (Fig. 1D). This suggests that the peptide epitopes were able to mimic the structure of the eEGFR.

Antiproliferative effects of peptide mimics and peptide vaccine Abs

Ligand binding to EGFR results in activation of the receptor that causes formation of dimers with itself and heterodimers with other HER family members. This triggers increase proliferation of the receptor and intracellular signaling. We therefore tested the effects of inhibiting ligand binding with the peptide mimics and peptide vaccine Abs on proliferation using lung and breast cancer cell lines. To measure proliferation, we used the MTT assay, in which the cells were treated with the inhibitors and incubated for 3 d before

Table I. HER-1 peptide vaccines and B cell epitope sequences

| Peptide Designation | Amino Acid Sequence | Molecular Mass (Da) |
|---------------------|---------------------|--------------------|
| MVF-HER-1-347–374   | CH3CONH-KLSSLJKGIVHRLGEGVSLIPVAFRGSFTHTPLPDQEILDILKTV-CONH2 | 5573 |
| HER-1-347–374       | CH3CONH-ILPVAFRGDSFTHTPLDQEILDILKTV-CONH2 | 3160 |
| MVF-HER-1-382–410   | CH3CONH-KLSSLJKGIVHRLGEGVSLIPVAFRGSFTHTPLDQEILDILKTV-CONH2 | 5825 |
| HER-1-382–410       | CH3CONH-ILPVAFRGDSFTHTPLDQEILDILKTV-CONH2 | 3484 |
| MVF-HER-1-418–435   | KLLSLJKGIVHRLGEGVSLIPVAFRGSFTHTPLDQEILDILKTV-CONH2 | 4243 |
| HER-1-418–435       | CH3CONH-SLaTDSLGLRSLKEISDG-CONH2 | 1944 |
adding MTT. Fig. 1E and 1F shows the effect of the vaccine Abs and peptide mimics on the rate of proliferation of breast and lung cancer cells. The results show an increase in the rate of inhibition after treatment, suggesting that the inhibitors are able to block ligand binding and prevent proliferation of EGFR-expressing cancer cells.

**Cross-reactivity of vaccine Abs to EGFR-expressing cells and their ability to cause ADCC**

In the case of assessing binding to human breast (MDA-MB-468) and lung (A549) cancer cells, we used immunofluorescence staining techniques to study the binding affinities. Polyclonal Abs generated to all three peptide constructs showed binding capabilities to both cell lines, with the 382–410 and 418–435 vaccine constructs having the greatest binding affinity compared with preserum (Fig. 2A) and epitope 347–374 showing minimal binding. The vaccine polyclonal Abs did not show any binding to MCF-7 (EGFR-negative) breast cancer cells (results not shown).

One of the main mechanisms of action of humanized Abs is to induce ADCC by stimulating the PBMCs and traffic them to the cancer cells, eventually causing their killing. We tested the effects of our vaccine Abs on breast and lung cancer cells as target cells and using PBMCs as effector cells. We used three different effector to target ratios, and our results showed that the peptide Abs were able to cause ADCC by inducing lysis of the target cells in a concentration-dependent manner. The effects were greater when an E:T ratio of 100:1 was used (Fig. 2B, 2C). These results indicate that the vaccine Abs are able to specifically stimulate human PBMCs to kill cancer cells.

**Peptide mimics and vaccine Abs inhibit EGFR receptor phosphorylation**

We next evaluated the effects of treatment with both types of inhibitors (antipeptide Abs versus peptide mimics) on EGFR receptor phosphorylation. EGFR activation is promoted by ligand binding, which leads to dimerization, triggering phosphorylation and increasing metastasis (5). The levels of phosphorylated EGFR were measured using a very sensitive ELISA kit, and the results showed a similar increase in the levels of inhibition by vaccine Abs and peptide mimics in lung and breast cancer cells (Fig. 3A, 3B). These results indicate that the vaccine Abs and peptide mimics are able to block ligand-induced phosphorylation of the EGFR receptor.

**FIGURE 1.** Immunogenicity of EGFR peptide vaccine in outbred rabbits and antiproliferative effects in rabbit Abs and peptide mimics. (A–C) Relative levels of vaccine Abs measured in an ELISA assay showing immunogenicity of peptide constructs in rabbits (two per each construct). 2y + 3w, for example, indicates the titer of blood drawn 3 wk after the second immunization. (D) Binding of vaccine Abs to rhEGFR. y-axis represents absorbance, which shows levels of binding. MTT cell proliferation assay with breast and lung cancer cells using 50 μg/ml of vaccine Abs (E) and 50 μg/ml of peptide mimics (F) as inhibitors. The percentage inhibition was calculated using the formula (ODUNTREATED – OD TREATED)/ODUNTREATED × 100, and data shown represent an average of three different experiments, with error bars showing SD from the mean.
Apoptosis determination by measuring caspase activity

Cells (A549 and MDA-MB-468) in exponential growing phase were seeded in 96-well plates, and the following day, the cells were treated with peptide mimics and vaccine Abs as inhibitors and incubated for a day. After treatment, apoptosis was evaluated by measuring caspase-3/7 activity using the Caspase-Glo reagent kit (Promega). Results obtained showed a significant increase in the amount of caspase activity in the treated cases when compared with the irrelevant peptide and normal rabbit IgG. Treatment caused a 10-fold increased in caspase activity (Fig. 4), which is indicative of increased apoptosis. These results indicate that the peptide Abs as well as the peptide mimics were able to induce apoptosis in breast and lung cancer cells in vitro.

Antitumor effects of peptide vaccine immunization in FVB/n transplantable mouse model

To evaluate the inhibitory effects of our peptide vaccines in vivo, we used the FVB/n transplantable mouse model in which Met-1 cells from FVB/n background (17) were injected in the mammary fat pad of wild-type FVB/n mice after immunization with our peptides as shown in the immunization schedule (Fig. 5A).

Tumor development in this model is dependent on the HER family receptor overexpression. All mice were immunized at 5 to 6 wk of age and received two boosters at 3-wk intervals. As depicted in Fig. 5B, the vaccine constructs were immunogenic in the mice, and all of the mice had relatively high titers of the Abs (>40,000). Mice were challenged with Met-1 cells and monitored weekly for 6 wk. Results showed that two of the vaccine constructs (382–410 and 418–435) were able to significantly (p < 0.05; Fig. 6A) delay onset of tumor growth and development (Fig. 6A). Tumors in these two groups have a significant delay in onset of tumor development, and the tumors were smaller than the untreated groups. The percentage tumor weight in these two cases was also significantly smaller (p < 0.028, Fig. 6B). The 347–374 construct showed no significant effect on tumor growth and onset of tumor development.

Using immunohistochemical analysis, we evaluated the effects of vaccine immunization on tumor sections by staining for actively dividing cells (Ki-67) and blood vessels (CD31). The number of actively dividing cells was significantly reduced in the case of immunization with the 382–410 and 418–435 vaccine constructs as compared with nonimmunized and the 347 epitope. The staining...
to show the relative number of positive cells was quantified using Image J software (National Institutes of Health), which indicated a great reduction in the amount of dividing cells (*) \( p < 0.05; \) Fig. 6C). The blood vessel staining also showed a significant decrease in microvascular density after treatment with the two vaccine constructs, and quantification also indicated a significant reduction in the vascular density index (*) \( p < 0.005; \) Fig. 6D).

FIGURE 3. Vaccine Abs and peptide mimics decrease EGFR-specific phosphorylation. EGFR-specific phosphorylation was determined using recombinant human phospho-ELISA kit after treatment with vaccine Abs (A) and peptide mimics (B), and percentage inhibition was calculated using the formula \( \frac{(OD_{UNTREATED} - OD_{TREATED})}{OD_{UNTREATED}} \times 100 \). Results shown represent the average of three different experiments with each treatment performed in triplicates, and error bars represent SDs from the mean.

FIGURE 4. Induction of apoptosis by peptide mimics and vaccine Abs. Apoptosis was evaluated by measuring caspase activity after treatment with peptide vaccine Abs (A) and peptide mimics (B). Cells were plated in 96-well plates, treated with inhibitors for 24 h before adding caspase reagent, and read in a luminometer. Normal rabbit IgG and irrelevant peptide were used as negative controls, whereas cetuximab was used as a positive control. Results represent average of three different experiments performed in triplicates, and error bars represent SDs from the mean.
**FIGURE 5.** Immunogenicity of peptide vaccines in FVB/n mice. (A) Immunization scheme for FVB/n mice. Mice (n = 5) were immunized i.m. with 100 μg of EGFR peptide vaccines three times at 3-wk intervals, and 10 d after the third immunization, mice were challenged with Met-1 cells, and tumor growth was monitored for up to 6 wk. (B) Immunogenicity of peptide vaccines in FVB/n mice, and results show relatively high levels of Ab titers (∼40,000) before and after tumor injection.

**Therapy with EGFR peptide mimics prevents tumor growth in vivo**

To test the in vivo effects of the peptide mimics, we used the same FVB/n model as in the case of immunization, but in this experiment, wild-type mice were challenged with Met-1 cells and treated i.v. with the peptide mimics, and results obtained showed a decrease in tumor growth and development with both the 382–410 peptide (**p < 0.005, Fig. 7A**) and 418–435 peptide (**p = 0.014; Fig. 7A**) as compared with untreated and irrelevant peptide. Epitope 347–374 showed a nonsignificant reduction in tumor volume. The effects on percentage tumor weight also correlated with that of tumor growth, with the 382–410 and 418–435 peptides showing greater inhibitory effects (Fig. 7B). In assessing how the peptide inhibitors exert their effects in vivo, we also analyzed the tumor sections after treatment with the peptide mimics for blood vessel density and actively proliferating cells. Tissue sections were stained for Ki-67 and CD31 markers, and results obtained showed a significant reduction in positive cells and vascular density. Tissue sections were stained in vivo with the peptide mimics, and results obtained showed a decrease in tumor growth and development with both the 382–410 and 418–435 constructs (Fig. 7C). There was also a significant reduction in microvascular density with the same two constructs (Fig. 7D).

**Antitumor effects of therapy with peptide mimics and vaccine Abs in lung cancer transplantable SCID mouse model**

We further tested the inhibitory effects of our peptide mimics and vaccine Abs raised in rabbits in a lung cancer transplantable model using BALB/c SCID mice. In this case, we used only the peptide mimics that showed reasonable inhibition in the breast cancer model (382–410 and 418–435 constructs), and results obtained indicated that both peptide mimics just like cetuximab significantly delayed tumor growth and development (Fig. 8A); even more striking was the effects of the Abs to the 418–435 epitope that were better than that of cetuximab (Fig. 8B). Abs to the 382–410 epitope did delay tumor growth, although the effect was not statistically significant. Analysis of the percentage tumor weight per body mass also showed that the Abs to the 418–435 construct was the best inhibitor in this mouse model (Fig. 8C).

**Discussion**

The basic goal of this study was to define biologically relevant EGFR epitopes that could activate the immune system to produce highly specific Abs that will target the tumor cells through vaccination rather than constantly infusing patients with large quantities of Abs such as cetuximab. Additionally, the development of EGFR peptide mimics that could disrupt EGFR signaling pathways by directly preventing ligand binding and interrupting signaling is another goal. The basic hypothesis in the design of EGFR peptide inhibitors is that many proteins exert their biological activity through small regions of their folded surfaces. It is therefore possible to transfer the side chain functional group responsible for binding to a small molecule with the contributions to binding largely intact (24). The overall objective of this study stems from work conducted in our laboratory over the past few decades in developing effective vaccine and therapeutic strategies for HER-2/neu (16, 25), as well as developing novel therapies based on blockade of receptor/ligand interactions such as B7/CD28 (26).

Recent data suggest that cetuximab easily develops resistance and is relatively unstable, with unacceptable safety profiles similar to other humanized mAbs (27–29). The clinical benefit achieved with Abs such as cetuximab and trastuzumab tend to be short-lived. Peptide vaccines can stimulate the immune system to produce more durable cancer-specific Abs and establish immunological memory, which can be important in the case of relapse. In this manuscript, we have further delineated important new biologically active epitopes of EGFR (Table I) that each contain key amino acid residues important for EGFR ligand binding. The peptide constructs were evaluated as therapeutic candidates and also as vaccine candidates in separate sets of experiments. Our approach is to inhibit ligand binding either directly by using the peptide mimics to block binding of the ligand or indirectly by immunization with the peptide vaccine leading to the production of Abs that will be able to bind EGFR and prevent ligand binding. Our approach has been validated with other growth factor proteins such as HER-2 (16, 20, 25) and vascular endothelial growth factor (VEGF) (21, 22), and we are extending this approach to delineate new EGFR epitopes and to other HER family receptors that are expressed in cancer cells.
We showed that all three peptide vaccine constructs were highly immunogenic in rabbits, and the Abs raised against each of the vaccines were able to bind rhEGFR protein in an ELISA assay and also cause inhibition of cell proliferation (Fig. 1). The inhibitory effects of the vaccine Abs greatly rely on their ability to block ligand binding. It is therefore important to test their ability to bind human cancer cells that express EGFR, and this was achieved using immunofluorescence staining. The peptide Abs were able to bind EGFR expressing both breast (MDA-MB468) and lung (A549) cancer cells, and the binding effect was highest in the 382–410 vaccine construct followed by the 418–435 construct (Fig. 2A). The vaccine was not able to bind cells that do not express EGFR (MCF-7) (results not shown). These results point to the fact that we were able to engineer a vaccine that is immunogenic with high binding affinity to the EGFR receptor.

To study the mechanism of action of these inhibitors, we performed several assays. ADCC is a key mechanism of action of most Abs, and we showed that the peptide vaccine polyclonal Abs were able to stimulate PBMCs to cause killing of breast and lung cancer cells (Fig. 2B, 2C). We used human p-EGFR–specific ELISA to measure the levels of p-EGFR after treatment with the inhibitors. Results clearly show a decrease in expression of the phosphorylated proteins by both the peptide mimics and peptide Abs, as illustrated in Fig. 3A and 3B. This confirms that the inhibitors are able to block EGF dimerization. We then used the caspase assay to show that these peptide inhibitors or vaccine Abs were able to cause release of caspase enzymes (Fig. 4A, 4B) equivalent to cetuximab treatment. Thus, these inhibitors were able to cause apoptosis. These studies indicate that either vaccination with the chimeric epitopes and or therapeutic treatment with the peptide mimics would be effective candidates for an EGFR immunotherapeutic approach.

After demonstrating the in vitro antitumor effects of these peptide inhibitors, we evaluated the in vivo effects using two transplantable mouse models, one of which is driven by high expression of the HER family of receptors (Met-1), and the other is highly dependent on EGFR expression (A549). Out of three highly immunogenic peptides (Fig. 5B), only two of the peptide vaccines caused a significant delay in onset of tumor growth and development in the Met-1 breast cancer model (Fig. 6A), and staining

**FIGURE 6.** Effects of peptide vaccine immunization in the FVB/n Met-1 transplantable tumor model. (A) Peptide vaccination caused a delay in onset of tumor growth and development, with the 382–410 and 418–435 vaccine constructs significantly reducing tumor growth (*p < 0.05). (B) Peptide vaccine treatment significantly reduces percentage tumor weight per body mass (*p = 0.027 for 382–410; *p = 0.023 for 418–435 construct). Tumor sections were stained for dividing cells using ki-67 (C) and blood vessels using CD31 (D). Abs and slides were observed under a microscope, and representative photos in each treatment group are shown. Staining was quantified using the Image J software (National Institutes of Health), and results shown below the stained sections represent mean values from three different fields. Error bars represent SD from the mean.
of tumor sections showed a great reduction in actively dividing cells and microvascular density after treatment with the 382–410 and 418–435 construct (Fig. 6C, 6D). In the case of therapy with the peptide mimics, the best peptide constructs were also the 382–410 and 418–435 constructs that showed significant inhibition of tumor growth and great reduction in dividing cells and blood vessel density (Fig. 7A–D). The two peptide epitopes and the vaccine Abs were also tested in the A549 lung cancer model; there was a reasonable delay in onset of tumor development, with the 418–435 epitope showing better results than cetuximab (Fig. 8).

Immunohistochemical staining for blood vessels and actively dividing cells showed no major differences, and this may be because the A549 cells are human cell lines injected into SCID mice, which results to greater tumor heterogeneity as opposed to the mouse Met1 cells that originated from FVB/n mice, and the same FVB/n mice were used as the host. The 382–410 epitope and the vaccine Abs were also tested in the A549 lung cancer model; there was a reasonable delay in onset of tumor development, with the 418–435 epitope showing better results than cetuximab (Fig. 8).

Immunohistochemical staining for blood vessels and actively dividing cells showed no major differences, and this may be because the A549 cells are human cell lines injected into SCID mice, which results to greater tumor heterogeneity as opposed to the mouse Met1 cells that originated from FVB/n mice, and the same FVB/n mice were used as the host. The 382–410 epitope overlapped with the binding residues of cetuximab to EGFR (9), which indicates that the inhibitory effects may be similar to that of cetuximab as they both bind similar regions of the receptor. In contrast, the 418–435 epitope is completely different from cetuximab, and the binding residues do not overlap; this indicates that inhibition by this epitope is independent of the inhibitory effects of cetuximab. This can be a promising epitope in the development of drugs that can target EGFR in tumors that are resistant to cetuximab treatment.

Importantly, combination of the two peptide epitopes may produce enhanced antitumor effects because they both target two separate regions of the receptor, and this can be very important in avoiding resistance. Studies have shown that combining cetuximab with another Ab 425 that has a distinct epitope to cetuximab yielded enhanced inhibitory effects using EGFR-positive breast cancer cells (30). The 382–410 and 418–435 peptide epitopes therefore have the potential to additively inhibit tumor growth and produce better response rates because the epitopes are completely different and do not overlap with each other. An mAb, matuzumab, has been developed that specifically binds EGFR and prevents the conformational rearrangement (31). Both cetuximab and matuzumab are able to block ligand-induced dimerization but via different mechanisms, and this may have potential benefits, especially in cases of cetuximab resistance. Another therapeutic Ab, IMC-11F8, is known to inactivate EGFR by binding to the same domain like cetuximab but using a completely different set of interactions (32). mAb 806 specifically targets a truncated form of EGFR that is commonly found in gliomas, and the same Ab has been shown to also recognize full-length EGFR found on tumor cells and most importantly does not recognize normal EGFR found on normal cells (33). A combined approach using cetux-
imab and another Ab, 425(EMD55900), was shown to produce enhanced inhibitory effects in EGFR-overexpressing breast cancer cells, indicating potential clinical benefits in combining cetuximab with Abs targeting different epitopes of EGFR in the clinic (30). Another group has designed peptides as mimotopes that are recognizable by EGFR Abs like cetuximab and matuzumab and has shown the immunogenicity of these peptide mimotopes in animals with the anti-peptide Abs able to recognize natural EGFR receptor and prevent ligand binding, indicating potentials for EGFR-specific cancer immunotherapy (34).

There is also significant evidence that shows cross-talk between members of the HER family and other receptors such as insulin-like growth factor-1 receptor (35). Similarly, HER activation can result in stimulation of angiogenesis by upregulating VEGF expression (36). To mitigate against multiple resistance mechanisms, we must develop combination therapies within the family and beyond. To address this important issue, we have developed effective inhibitors of VEGF/VEGF receptor 2 (21) aimed at targeting tumor angiogenesis. We have also shown that combination treatment aimed at targeting both the HER-2 and VEGF signaling pathways produced superior antitumor effects in vitro and in vivo (22). Our ongoing goal is to develop additional insulin-like growth factor-1 receptor and HER-3 inhibitors that could be used in a combination approach with HER-1, HER-2, or VEGF peptide inhibitors that we have already developed. Recently, a two-in-one Ab against HER-3 and EGFR has been shown to have superior inhibitory activity compared with monospecific Abs (37), further providing rationale for our peptide approach to effectively treat cancer and avoid drug-resistance mechanisms. A small peptidomimetic molecule targeting HER-2 has been designed to disrupt dimerization not only of EGFR–HER-2, but also of HER-2–HER-3 (38) using biophysical methods.

In conclusion, we have demonstrated that two B cell peptide epitopes that mimic the EGFR ligand binding domain are capable of disrupting ligand binding, and the vaccine constructs are capable of inducing Abs with great antitumor properties that could overcome the limitations associated with cetuximab treatment. The generation of highly specific Abs through active immunization has the potential to establish immunological memory and inhibit development of EGFR-dependent tumors. Additionally, peptide mimics can also be used as therapeutic options for already established tumors. The 382–410 and 418–435 constructs could be used either as a vaccine or a therapeutic candidate that can be translated into clinical trials. Peptides are relatively cheaper, nontoxic, more stable, and easily penetrate tissue barriers, making them preferred candidates for the production of most therapeutic agents. A combination approach using these two epitopes has the potential to induce enhanced antitumor effects. The 418–435 epitope most especially has the potential to overcome resistance to cetuximab treatment because it targets the receptor at a different epitope to that of cetuximab. More recently, EGFR inhibition has been proposed as a therapeutic mechanism in TNBC (39), which represents an important clinical challenge, as there are no effective treatments. Scientific advances relating to the development of new EGFR epitopes is therefore paramount in the battle to treat TNBC that could be translated into the clinic to improve patient outcomes.

**Disclosures**

The authors have no financial conflicts of interest.
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