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Targeted Therapy of Fn14-Positive Breast Tumors Using a TWEAK-Cytotoxin Fusion Protein or Noncovalent Complex

PRINCIPAL INVESTIGATOR:

Jeffrey A. Winkles, Ph.D.

CONTRACTING ORGANIZATION:

University of Maryland-Baltimore

Baltimore, MD 21201-1531

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Jeffrey A. Winkles
Sharron Brown

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Our laboratory research is focused on the potential roles of a TNF-related cytokine named TWEAK and its specific cell surface receptor named Fn14 in tumor biology. We reported previously that the Fn14 gene is highly expressed in many human breast cancers. In this Breast Cancer Concept Award application we proposed to investigate whether we could make Fn14-targeted toxins that would kill Fn14-positive breast cancer cells *in vitro* and *in vivo*. Our most significant findings during the one-year research period are that although TWEAK-based single chain or two chain non-covalently linked toxins do have some cytotoxicity on cancer cells this approach is not ideal because of protein aggregation issues, likely problems with translating these molecules to clinical use, and the recent identification of a second TWEAK-binding protein. A different approach, namely using an Fn14 mAb to deliver toxic cargo, appears to be a better strategy.

15. SUBJECT TERMS
TWEAK, Fn14, breast cancer, immunotoxin

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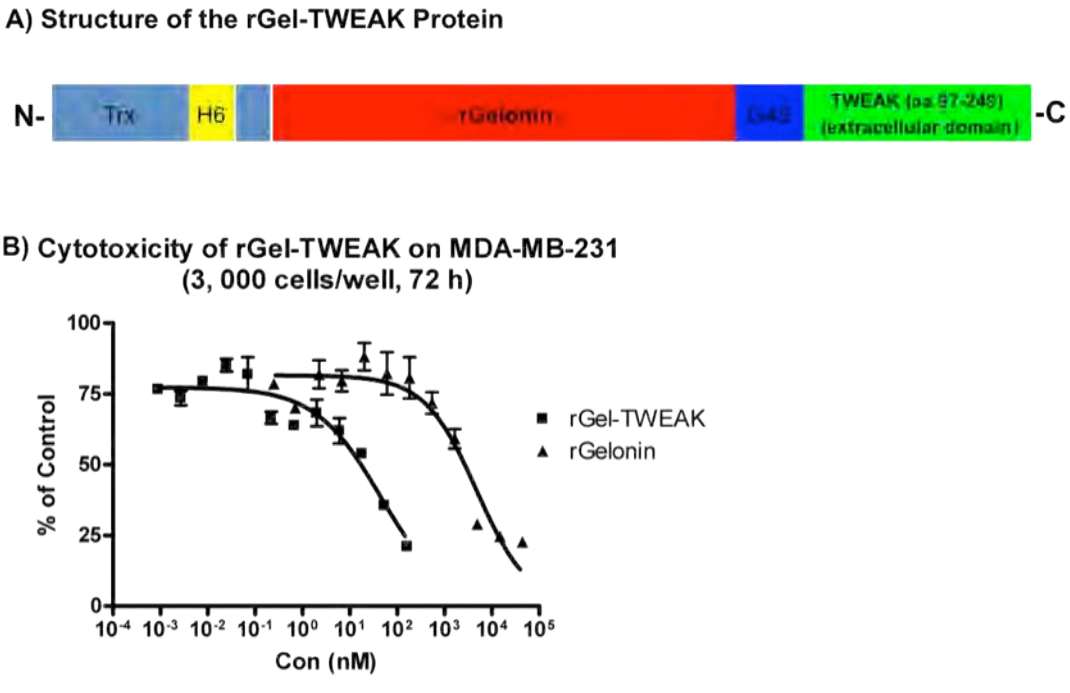
Introduction: Breast cancer kills ~ 570,000 women per year in the USA and thus it is critical that we identify new therapeutic agents. Our laboratory research is focused on the potential roles of a TNF-related cytokine named TWEAK and its specific cell surface receptor named Fn14 in tumor biology (the TWEAK-Fn14 axis is reviewed in reference 1) We reported previously that the Fn14 gene is highly expressed in many human breast cancers (2). In this Breast Cancer Concept Award application we proposed to investigate whether we could make Fn14-targeted toxins that would kill Fn14-positive breast cancer cells *in vitro* and *in vivo*. If so, this could be a new therapeutic approach for certain breast cancer patients.

Body:
Specific Aim 1: To determine whether a TWEAK-cytotoxin fusion protein or noncovalent complex has apoptotic activity on Fn14+ but not Fn14- breast cancer cells cultured *in vitro*.
Task Summary: Construct expression plasmids, purify proteins, test proteins for cytotoxic effects on breast cancer cell lines.

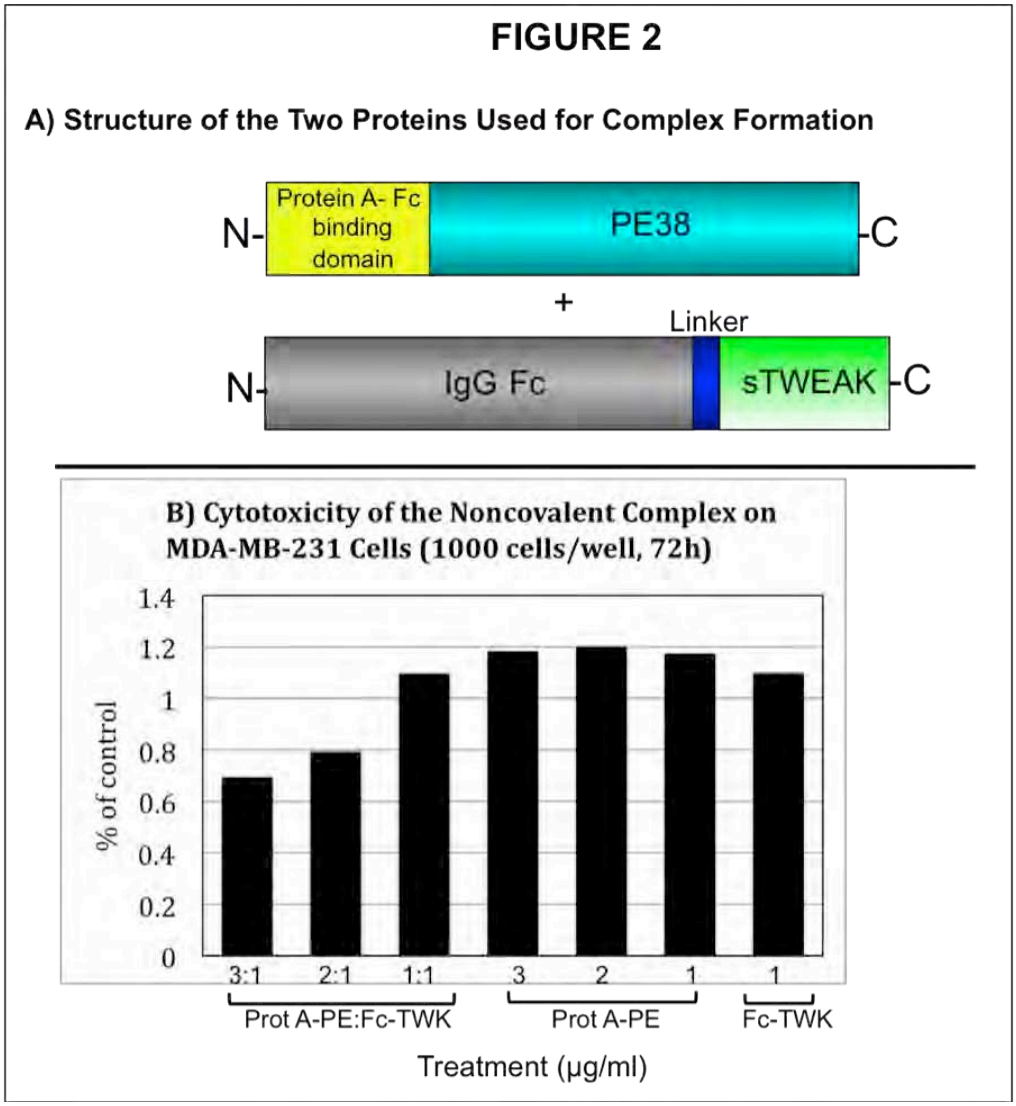
Progress: We initially prepared a TWEAK-cytotoxin fusion protein in which the *Pseudomonas* exotoxin PE38 protein was used as the cytotoxic cargo. It was difficult to isolate this protein from bacterial cells in an unaggregated form and consequently we found it to have low and inconsistent cytotoxic activity on Fn14+ breast cancer cell lines. We then decided to contact an expert in this field, Dr. Michael Rosenblum from MD Anderson Cancer Center, and establish a collaborative project to construct and test additional Fn14-targeted cytotoxins. His laboratory primarily uses a plant toxin named gelonin for their toxin constructs, so they made a recombinant gelonin (rGel)-TWEAK expression plasmid and attempted to express and purify the protein from bacterial cells. The structure of the fusion protein is shown in Figure 1A. Again, the protein was difficult to purify due to aggregation problems; nevertheless, some of the protein was used for cytotoxicity assays. In these experiments, cytotoxicity was measured by treating the cells for three days with the purified proteins and then staining the adherent cells with crystal violet. The stain is solubilized using Sorenson's buffer and then the absorbance is measured at 595 nm. The control is no protein addition. We found that rGel-TWEAK had a weak killing activity on breast cancer cells (only 100-fold better than when gelonin is used alone) (Figure 1B).

FIGURE 1



In regard to the second cell killing strategy listed in this Aim, in which we proposed to express and purify a protein A-Fc binding domain:rGel fusion protein and mix it with an IgG-Fc:TWEAK fusion protein (provided by Dr. Schneider, Univ. of Lausanne) and then add the noncovalent complex to cells, we first did a preliminary experiment using a protein A-Fc binding domain:PE38 fusion protein obtained from a collaborator (Dr. Benhar, Tel-Aviv Univ.) to see how well this general strategy might work. The structure of the two proteins is shown in Figure 2A. In these experiments the two proteins were mixed at three different ratios and each protein was also added alone as negative controls. We did see some cytotoxicity on MDA-MB-231 breast cancer cells when the proteins were added at either a 3:1 or 2:1 ratio (Figure 2B). However, this approach, which requires mixing two proteins together, would probably not be applicable to patient treatment because the non-covalent complex may dissociate during IV administration or during transit in the bloodstream.

FIGURE 2



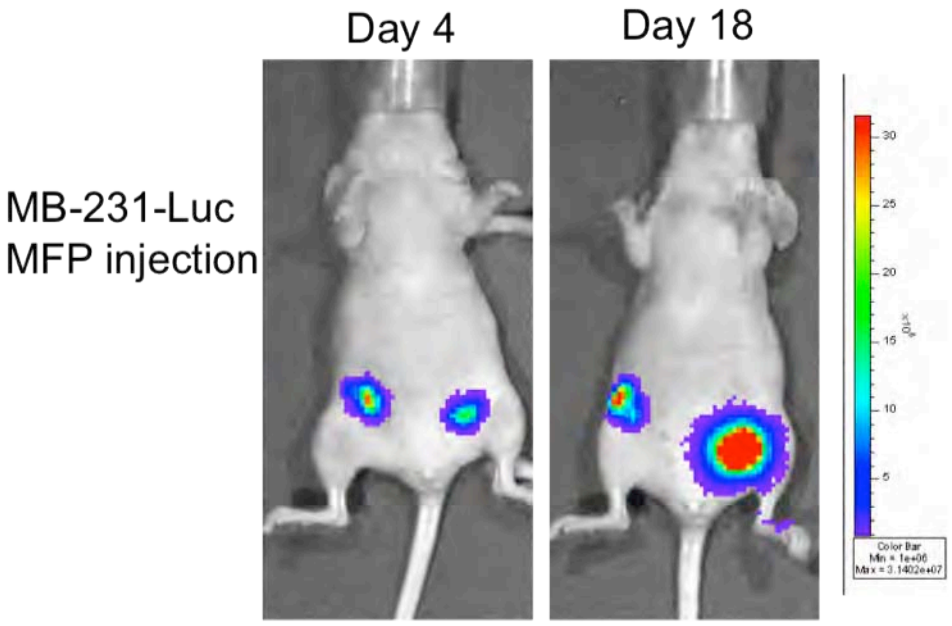
During the course of these studies a manuscript was published indicating that TWEAK can bind a monocyte/macrophage protein named CD163 (3). The identification of a second TWEAK-binding cell surface protein (besides Fn14) suggested to us that using TWEAK to direct toxins to Fn14+ tumor cells may not be the best strategy. Therefore, we have continued to collaborate with the Rosenblum lab on another experimental approach; namely, the use of an anti-Fn14 monoclonal antibody (mAb) to carry the toxin moiety. These types of toxins are called immunotoxins. During the past year we have developed an Fn14-targeting agent consisting of

the Fn14 mAb named ITEM4 (provided by Dr. Yagita, Juntendo Univ.) chemically conjugated to the plant toxin rGel mentioned above. This ITEM4-rGel conjugate binds the Fn14 receptor and has excellent cytotoxic activity on many tumor cell lines, including breast cancer cell lines. This data was included in an Abstract submitted to the Annual AACR Meeting that was held in Washington, DC (4). Our findings are summarized in the attached Abstract and will not be described in detail here. Studies are in progress to develop new single chain Fn14 mAb:rGel fusion proteins that can be used instead of the chemical conjugate to kill Fn14+ tumor cells.

Specific Aim 2: To determine if TWEAK-mediated cytotoxin delivery can inhibit breast tumor xenograft growth in mice.
Task Summary: Generate MDA-MB-231 breast cancer cells that express the firefly luciferase gene, perform xenograft assays in immunodeficient *nu/nu* mice to test if Fn14-targeted toxin reduces tumor growth.

Progress: We obtained MDA-MB-231-luciferase cells from a collaborator (Dr. Martin, Univ. Md. School of Med.) and confirmed that they expressed high Fn14 levels by Western blot analysis. In addition, we have conducted preliminary xenograft assays to ensure that these cells form tumors in nude mice and that we can monitor tumor growth by bioluminescence imaging. An example of our findings is shown in Figure 3. In this experiment, the MDA-MB-231-luciferase cells were combined with Matrigel and injected onto the left and right mammary fat pads (MFP). Mice were imaged using the Xenogen IVIS-200 imaging system located here on our campus. Increased tumor growth is evident during the day 4 to day 18 time period.

FIGURE 3



Studies are in progress to test whether the IV administration of the ITEM4-rGel conjugate can reduce tumor growth using these xenograft assays.

Key Research Accomplishments:

- Construction of TWEAK-toxin fusion protein expression plasmids
- Purification of TWEAK-PE38 and rGel-TWEAK proteins from bacteria
- Measurement of the ability of TWEAK-toxin proteins and TWEAK-toxin noncovalent complexes to kill Fn14+ tumor cells
- Construction of Fn14-targeted immunotoxin expression plasmids
- Purification of Fn14-targeted immunotoxin from bacteria
- Measurement of the ability of Fn14-targeted immunotoxin to kill Fn14+ tumor cells
- Establish MDA-MB-231-luciferase cell xenograft asssay

Reportable Outcomes: Abstract presented at 2010 AACR Annual Meeting Poster Session (attached).

Conclusion: We conclude from this work that the best strategy for therapeutic targeting of Fn14-overexpressing breast cancers will most likely be based on an Fn14 immunotoxin molecule. Future work will be to develop several versions of this immunotoxin and test their efficacy *in vitro* and in mice. This work has increased our scientific knowledge regarding the best way to target Fn14+ cells and in the long-term could lead to human studies.

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4. Zhou, H., Marks, J.W., Winkles, J.A., Yagita, H., and Rosenblum, M.G. (2010).Development and characterization of a potent immunotoxin targeting the Fn14 receptor on solid tumor cells. American Association for Cancer Research Annual Meeting, Washington, DC.

Appendices: AACR Abstract is attached.

Abstract for American Association Cancer Research 2010 Annual Meeting, Washington, DC (Poster Presentation)

Development and characterization of a potent immunotoxin targeting the Fn14 receptor on solid tumor cells

[1]Hong Zhou, [1]John W. Marks, [2]Jeffrey A. Winkles, [3]Hideo Yagita and [1]Michael G. Rosenblum

[1]Immunopharmacology and Targeted Therapy Laboratory, Department of Experimental Therapeutics, M.D. Anderson Cancer Center, Houston, TX, 77030

[2]Departments of Surgery and Physiology, Center for Vascular and Inflammatory Diseases, and the Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD, 21201

[3]Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

TNF-like weak inducer of apoptosis (TWEAK) and FGF-inducible 14 (Fn14) are a TNF superfamily ligand-receptor pair involved in inflammation, oncogenesis, tumor invasion, migration, survival and resistance to chemotherapy. The Fn14 receptor is expressed at relatively low levels in normal tissues, but is known to be dramatically elevated in a number of tumor types, including brain and breast tumors. Thus, the TWEAK/Fn14 axis appears to be an excellent candidate for therapeutic intervention. We have developed an immunoconjugate designated ITEM4-rGel containing a high-affinity anti-Fn14 monoclonal antibody conjugated to recombinant gelonin (rGel), a highly cytotoxic, ribosome-inactivating n-glycosidase. The ITEM4-rGel conjugate was generated and purified and contained no contaminating free antibody or rGel. The final material contained both antibody + 1 rGel (major) and antibody + 2 rGel (minor) species. We analyzed Fn14 expression in human tumor cell lines using flow cytometry and Western blot analysis. Fn14 was expressed in a variety of tumor lines including breast, brain, bladder, skin, lung, ovarian, pancreatic, colon, prostate, and cervical tumor cell lines. Both ITEM4 and ITEM4-rGel were found to bind to cells to an equivalent extent. Confocal immunofluoresence studies showed that ITEM4-rGel specifically and rapidly (within 2 hrs) internalized into MDA-MB-231 breast cancer cells and T-24 bladder cancer cells but not into Fn14-deficient mouse embryonic fibroblasts. Cytotoxicity studies against 22 different tumor cell lines showed that ITEM4-rGel was highly cytotoxic to Fn14-expressing cells (IC_{50} ranged from 0.8 pM-25 nM) and was 50 to 0.5 $x10^6$ fold more potent than free rGel. Minimum contact time studies showed that as little as 12 hr exposure achieved maximal cytotoxic effect. Mechanistic studies showed that ITEM4-rGel induced HMGB1 release following treatment of MDA-MB-231, T-24, AAB 527, and BxPC-3 cells. In addition, target cells showed induction of apoptosis, as measured by Annexin V staining and caspase-3 cleavage. ITEM4-rGel treatment also induced the non-canonical NF-κB pathway, up-regulated the tumor suppressor protein p53 and down-regulated survivin expression. Preliminary mouse xenograft tumor model studies are ongoing. These data suggest that the ITEM4-rGel construct may warrant further development as a novel therapeutic agent against a broad range of solid tumor types. Research conducted, in part, by the Clayton Foundation for Research (MGR), and supported by NIH grant NS55126 (JAW) and DOD Breast Cancer Concept Award BC086135 (JAW).