Award Number: DAMD17-99-1-9259

TITLE: Molecular Engineering to Retarget Human Cytokines

PRINCIPAL INVESTIGATOR: Michael Rosenblum, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
       MD Anderson Cancer Center
       Houston, Texas 77030

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
       Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                     Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
**REPORT DOCUMENTATION PAGE**

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (OIR 074-0188), Washington, DC 20503

<table>
<thead>
<tr>
<th>1. AGENCY USE ONLY (Leave blank)</th>
<th>2. REPORT DATE</th>
<th>3. REPORT TYPE AND DATES COVERED</th>
<th>5. FUNDING NUMBERS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>July 2000</td>
<td>Annual (1 Jul 99 - 30 Jun 00)</td>
<td>DAMD17-99-1-9259</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4. TITLE AND SUBTITLE</th>
<th>6. AUTHOR(S)</th>
<th>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</th>
<th>8. PERFORMING ORGANIZATION REPORT NUMBER</th>
<th>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</th>
<th>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Engineering to Retarget Human Cytokines</td>
<td>Michael Rosenblum, Ph.D.</td>
<td>The University of Texas MD Anderson Cancer Center</td>
<td>Houston, Texas 77030</td>
<td></td>
<td>U.S. Army Medical Research and Materiel Command</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>11. SUPPLEMENTARY NOTES</th>
<th>12a. DISTRIBUTION / AVAILABILITY STATEMENT</th>
<th>12b. DISTRIBUTION CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Approved for public release; distribution unlimited</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>13. ABSTRACT (Maximum 200 Words)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overexpression of HER2/neu in breast cancer is one mechanism responsible for cellular progression of the neoplastic phenotype. Overexpression of HER2/neu appears to result in reduced sensitivity to the cytotoxic effects of tumor necrosis factor (TNF) and reduced sensitivity to immune effector killing. The single-chain antibody scfv23 recognizes the cell-surface domain of HER2/neu. The cDNA was fused to the cDNA encoding human TNF and cloned into an expression plasmid. The fusion protein was expressed and purified by ion exchange chromatography. SDS-PAGE demonstrated a single band at the expected molecular weight (43 kDa). Western analysis confirmed the presence of both the antibody component and the TNF component. The construct was shown to have biological activity similar to that of authentic TNF (S.A. 420 nM) against L-929 cells. The scfv23/TNF construct bound to SKBR-3 (HER2 positive) but not to A-375 human melanoma (HER2 negative) cells. Cytotoxicity studies against breast carcinoma cells (SKBR-3-HP) overexpressing HER2/neu demonstrate that the scfv23/TNF fusion construct was 1000-fold more active than free TNF. These studies suggest that fusion constructs targeting the HER2/neu surface domain and containing TNF are more effective cytotoxic agents in vitro than native TNF and may be effective against tumor cells expressing intermediate levels of HER2/neu.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14. SUBJECT TERMS</th>
<th>15. NUMBER OF PAGES</th>
<th>16. PRICE CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion Construct, TNF, Her2/neu, cytokines, signal transduction</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>17. SECURITY CLASSIFICATION OF REPORT</th>
<th>18. SECURITY CLASSIFICATION OF THIS PAGE</th>
<th>19. SECURITY CLASSIFICATION OF ABSTRACT</th>
<th>20. LIMITATION OF ABSTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unclassified</td>
<td>Unlimited</td>
</tr>
</tbody>
</table>

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89) Prescribed by ANSI Std. Z39-18 298-102
Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.
TABLE OF CONTENTS

Cover ..................................................................................................................... 1
SF 298 ................................................................................................................... 2
Foreword .............................................................................................................. 3
Table of Contents ............................................................................................... 4
Introduction ......................................................................................................... 5
Body ..................................................................................................................... 5-6
Key Research Accomplishments ....................................................................... 7
Reportable Outcomes ......................................................................................... 7
Conclusions ......................................................................................................... 8
References .......................................................................................................... 8
Appendices .......................................................................................................... 8
Abstract .............................................................................................................. 9
Publication ..........................................................................................................10-16
INTRODUCTION:

The goal of this research is to understand how overexpression of the HER2/neu protooncogene can modulate cellular response to the cytotoxic effects of the cytokine TNF. In this context, the chimeric fusion construct scfv23/TNF which binds to HER2/neu and contains TNF appears able to overcome HER2/neu mediated resistance to this cytokine. Therefore, this research project focuses on both in vitro and in vivo development of the scfv23/TNF as a potential therapeutic agent and on understanding the signal transduction events responsible for these observations in breast cancer.

BODY:

Below are reprinted the first two Objectives from the approved Statement of Work:

Technical Objective 1

We propose to develop genetic fusion constructs of TNF for the purpose of targeting cytotoxic cytokines to human tumors.

Task 1- months 1-5

Construction of scfv23/TNF in two possible configurations. Confirm by DNA sequence analysis.

Task 2- months 6-9

Express and purify the constructs. Analyze TNF and antibody biological activity.
Technical Objective 2

We next propose to examine the in vitro tumor cell binding ability and the antitumor activity of scfv23/TNF against target human tumor cell lines compared to native TNF.

Task 3- months 10-15

Assess scfv23/TNF cytotoxic activity against TNF sensitive and TNF resistant breast tumor cell lines.

The Appendix contains a galley proof of a manuscript entitled, “A Novel Recombinant Fusion Toxin Targeting HER2/NEU-Over-Expressing Cells and Containing Human Tumor Necrosis Factor”. This article is in press in the *International Journal of Cancer* and describes the PCR construction of a chimeric gene encoding scfv23/TNF and insertion of this gene into a bacterial expression vector. The completed vector (designated pCFR-09, Figure 1) was then transfected into bacterial host cells and the recombinant protein was expressed as shown in the manuscript (Figure 2). Western analysis demonstrated the presence of both TNF and the scfv23 antibody components in the construct(Figure 3). The scfv23 component binding HER2 on the surface of breast cancer cells was shown to be intact (Figures 4 and 5). The biological activity of the TNF component was assessed using L-929 cells and was also found to be intact compared to native TNF. Another chimeric construct of scfv23/TNF in an alternate configuration was generated and tested; however, no significant differences were found with this material and a decision was made to focus on one product. Against cells expressing HER2/neu, the construct was shown to be 2,000 fold more active than native TNF. Against cells expressing much higher TNF levels, the construct did not appear to be more active than TNF itself possibly suggesting a difference in the signaling events as a result of HER2 overexpression.
As described, these studies complete Objective 1. Significant progress has also been made towards completion of Objective 2. Thus far, there have been no significant problems of note. Production of the scfv23/TNF at high levels is still a limitation, but progress has been made towards improving both the purification process and the bacterial yield of material.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Generated a scfv23/TNF chimeric construct.
- Expressed and purified the construct.
- Demonstrated that the construct is capable of binding Her2/neu and contains active TNF.
- Demonstrated that the chimeric scfv23/TNF is 2,000 fold more active than TNF on Her2/neu expressing cells.

**REPORTABLE OUTCOMES:**

Publications:


Abstracts:

Funding Applied For Based on this Work:

1. Grant Number: DAMD17-99-1-9259
   Investigator: Michael G. Rosenblum, Ph.D.
   Amount of Award: $263,461
   Award Period: July 1, 1999 through July 31, 2002

CONCLUSIONS:

In summary, we have generated a unique chimeric fusion construct designated scfv23/TNF which appears to be capable of binding specifically to breast tumor cells expressing HER2/neu. This fusion construct appears to be specifically cytotoxic to HER2/neu expressing tumor cells although the mechanism of action by which this occurs has not been elucidated. This novel construct should be considered for development as a new therapeutic agent for the treatment of breast cancer.

REFERENCES:

Listed under Reportable Outcomes.

APPENDICES:

Attached manuscript and abstract.
IMMUNOLOGY/ PRECLINICAL AND CLINICAL 6

#1237 TNF FUSION CONSTRUCTS TARGETED TO HUMAN MELANOMA CELLS CAN OVERCOME RESISTANCE TO TNFα. Yuying Liu, Lawrence Cheung, and Michael Rosenblum, The Univ of Texas M D Anderson Cancer Ctr, Houston, TX.

Fusion constructs targeting tumor cells have significant potential for directed therapeutic applications in both solid and hematological malignancies. We have developed a fusion construct of TNFα and a single-chain antibody (scFvMEL) recognizing the surface domain of the gp240 antigen present on human melanoma cells. This construct was expressed in bacterial cells and purified to homogeneity using IMAC. The construct (45 kDa) was found to bind specifically to antigen-positive but not antigen-negative cells in culture. The TNF component was biologically active as assessed by the L-929 assay. The fusion construct was more cytotoxic to antigen-positive human melanoma cells than TNFα alone, and more directly cytotoxic to antigen-negative, TNF-resistant human melanoma cells. Minimal contact time studies indicate a difference between the scFvMEL/TNF construct and TNFα itself. The augmented cytotoxicity of scFvMEL/TNF was mediated by antibody-specific binding to the cell surface since competition with free 2MED reduced cytotoxicity. Studies also suggest that the scFvMEL/TNF construct self-associates into a trimeric structure. The scFvMEL/TNF molecule also activated the NF-κB pathway via phosphorylation and proteolytic degradation of IκBα in both TNF-sensitive and TNF-resistant human melanoma cell lines. This study demonstrates that TNF is a potent mediator of the tumor microenvironment and may represent a new target for the development of novel therapeutic strategies.

#1238 CLINICAL PHARMACOLOGY STUDIES OF RHI-12 ADMINISTERED BY THE INTRAPERITONEAL ROUTE. Weihe (Wendy) Zhang, Renato Lemos, Robert Dissman, Sally S Freedman, James L Abbruzzese, and Michael G Rosenblum, M D Anderson Cancer Ctr, Houston, TX, and Temple Univ Sch of Medicine, Philadelphia, PA.

Recombinant human IL-12 (rhl-12) is a cytokine of approximately 75 kDa composed of heterodimeric chains of 35 and 40 kDa. The biological properties of rhl-12 include immunomodulatory and antitumor effects. The antitumor effects of rhl-12 are proposed to occur as a result of the anti-angiogenic properties of this molecule. As a part of Phase I clinical trials in patients with ovarian cancer, patients received intraperitoneal doses of rhl-12 via an intraperitoneal catheter. Samples of blood and peritoneal fluid (pf) were obtained at various time points after administration and frozen until analysis. Levels of rhl-12, TNF, and IL-1α were assessed by ELISA assay. Values were subjected to nonlinear regression analysis to obtain standard pharmacokinetic parameters. Immediately after administration, peritoneal fluid concentrations of rhl-12 were found to range from 100-500 μg/ml and persisted for up to 48 hrs in most patients. Initial post-treatment plasma levels of rhl-12 were 10-100 fold lower than of and ranged from 0.1-10 μg/ml. At lower doses, levels in plasma and pf followed a variable pattern of clearance over time. Plasma and pf from initial trials studies were not detectable in 2/4 patients, 1/16 patients and IFN-γ was found elevated (0.1-1000 pg/ml) in 4/8 patients studied. These studies suggest that the increased concentrations of IFN-γ and TNFα were the most frequently detected after IL-12 administration and may play a role in the host response mechanism. Further studies on the role of these cytokines in the host response in vivo appear to be warranted.

#1239 DIFFERENT INFLUENCE OF INTERLEUKIN-15 ON CYTOTOXIC T-LYMPHOCYTES AND LAK CELLS ON CELL-MEDIATED CYTOTOXICITY IN PATIENTS WITH PANCREATIC CANCER. Matthias Herbert Fesper, Thomas E Langwiler, Hakim Jaballah, Wolfram T Kwolek, and Jakob R Ibsch, Univ Hosp Eppendorf, Hamburg, Germany.

Interleukin-15 shares no homology with IL-2, but requires parts of the IL-2 receptor complex for binding and signaling. Its possible role for use in novel immunotherapy approaches remains unclear. This study was performed to evaluate the influence of IL-15 on tumor-specific CTL and LAK cells in pancreatic cancer. LAK cells were generated from buffy coats and high-dose rhl-2, while CTLs were generated from HLA-2 positive tumor-associated lymphocytes, activated on anti-CD3, and used in vitro using autologous tumor cell stimulation. Prior to functional assays using the LDH-method, CTL and LAK cells were incubated with IL-15 (100 ng/ml) for 1-3 days. Target cells were autologous and allogeneic pancreatic cancer cells with/without MAb anti-HLA I (W02) or MAb anti-HLA-2 (BB7.2). Normal LAK cells lysed pancreatic cancer cells to a high amount (>55%) at 40:1 target/effector ratio (T/E). The lysis was not HLA-2 restricted (P>0.05). Incubation with IL-15 did not enhance or lower cytotoxicity in LAK cells. TAL-derived CTL lysed autologous and allogeneic tumor cells HLA-2 restricted with a median of 45% at 40:1 T/E for HLA-2 positive and 17% for HLA-2 negative, resp. (P<0.001). Incubation with IL-15 enhanced lysis of autologous tumor cells time- and dose-dependent (P<0.05). Lysis of autologous tumor cells was significant higher than of allogeneic tumor cells (P<0.05). Lysis was inhibited in all experiments by incubation with MAb anti-HLA I (P<0.05) and - -A (P<0.05). Our data demonstrate that there is a different influence of IL-15 on HLA-2 restricted and tumor-specific CTL vs LAK cells. Further studies on the functional role of IL-15 are necessary to evaluate its distinct mechanisms of action.

#1240 ABANTY-GULKIRKIN FUSION PROTEIN IMMUNOTHERAPY FOR PULMONARY METASTASES IN A SYNGENIC MURINE CELLS. KINCHIN, My M Zimko, Peishen Huang, A. Khattab, A. Epstein, Keck Sch of Medicine, Univ of Southern CA, Los Angeles, CA.

Interleukin-2L-2), tumor necrosis factor-α(TNF-α), and interferon-γ( IFN-γ), important cytokines which may be involved in the natural rejection of their efficacy in clinical trials. However, this was only achieved by doses of the toxicities associated with systemic administration. Genetic engineering body/cytotoxicity fusion proteins represent a new therapeutic approach which the targeted delivery of potent immunomodulatory mediators to tumor minimizing systemic toxicity. Our laboratory has developed a novel tumor system known as Tumor Necrosis Therapy, in which monomolecular antibody intracellular antigens accessible only in degrading and necrotic regions tumors. In our current study, the efficacy of chimeric TNCy toxin fusion proteins were examined in a murine model of colon carcinoma. BALB/c mice were injected Intravenously with 2x105 RENCA cells day one and received daily treatment with either the antibody fusion protein relevant control on days five through nine. On day 21, the mice were sacrificed and the number of tumor nodules in the lungs were enumerated after staining. Results demonstrated that non-toxic doses of these fusion proteins significantly decreased the number of lung metastasis compared to controls treated with antibody alone. Targeted immunotherapy of melanoma through the use of antibody-cytotoxic fusion proteins represents a major improvement in the delivery of potent immune modulators directed to the site of metastasis in this syngeneic mouse tumor model.

#1241 BIDIRECTIONAL ACTIVATION OF DENDRITIC CELLS AND L-LEUCINE EXPRESSING, ACTIVATED T CELLS. M Terai, R Yadausa, K Hasun, N Wada, M Ishido, T Osaka, Jpn, and Jefferson Med Coll of Thomas Jefferson Univ, Philadelphia, PA.

There is accumulating evidence that mutual interaction between dendritic (DC) and T cells are critically important for the establishment of an effective immune response. Interleukin-12 (IL-12) produced by DC triggers the development of Th1 responses and induces production of cytokines such as IFN-γ (Pha- ma). On the other hand, stimulation of DC with CD40 ligand (CD40L) promotes maturation of DC and induces IL-12 production. We investigated in vitro interaction between DC and CD40L-expressing, activated T cells (AT). DC were obtained from peripheral blood monocytes cultured and matured for 4-5 days. AT consisted of peripheral blood mononuclear cells activated by PHA and then stimulated with calcium ionophore. The AT produced IFN-γ and highly expressed CD4 on the surface. The DC expressed CD40, CD11c, CD43, MHC I, and IL-12 co-culture of DC and AT induced significant production of IL-12, which was seen with DC alone. IL-12 production was maximum at a DC:AT ratio of 1:4. IL-12 production and IFN-γ production continued increases through 1 hours of co-culture (1,000 pg/ml, 2,000 pg/ml at 37 hours, respectively) contrast, the IL-12 level reached a plateau at 18 hours (maximum 700 pg/ml control: DC stimulated with LPS without AT. The IFN-γ level reached plateau at 3 hours (maximum 600 pg/ml) with AT alone. These results confirm, in vivo in vivo in vivo in vivo the importance of mutual interaction between DC and AT and support clinical application of DC and AT therapy.

#1242 DEVELOPMENT OF AN ASSAY TO DETECT ANTI-HU14.18 RESPONSE FOLLOWING IN VIVO THERAPY WITH ANTI-GD2 FUSION PROTEIN, HU14.18-BL2. Amy M Ostendorf, Mark R Albertini, Jacek Gan, Jacoby Dean, Stephen D Gillies, and Paul M Soldner, Lexicon Pharmaceuticals and Children’s Hospital of Wisconsin, Milwaukee, MA, and Lexicon, Inc., Scottsdale, AZ.

The Hu14.18-BL2 fusion protein (HuFp) consists of a humanized monoclonal antibody (Ab), hu14.18, genetically linked to IL2. The Ab portion of HuFp recognizes GD2, a cell surface disialoganglioside overexpressed in melanomas, certain other tumors. The University of Wisconsin is conducting a Phase I/II Trial of HuFp in patients with GD2+ malignancies. We have created an ELISA order to detect anti-HuFp responses in patients receiving HuFp. In this ELISA system, we measure the ability of 12.5ng/well of HuFp to bind GD2-coated ELISA plates. HuFp is administered through use of a biotinylated goat-human IL-2 Ab followed by addition of an avidin-HRP conjugate and colorimetric detection. This system has been tested using an anti-idiotypic (anti-Id) murine that recognizes the antigen binding portion of the HuFp, in order to stimulate production of murine anti-idiotypic Abs in patient studied. Anti-Id Abs have been mixed with human serum and tested in this ELISA. Dose dependent inhibition of HuFp binding to 96 was observed using this murine anti-Id Ab. We intend to use this assay to evaluate sera from patients receiving HuFp in our clinical trial. Work that confirms the humanized antibody can be detectable because they inhibit or reduce this interaction. This assay should provide a reproducible means of evaluating patient response to HuFp, specific molecule nature of any response detected, and its influence on HuFp pharmacokinetics will require further investigation.

#1243 PHASE I/II TRIAL OF INTERLEUKIN-2 (IL-2) AND 13-CIS RETINOL ACID (RA) AS MAINTENANCE THERAPY IN ADVANCED CANCER. F Rech, S De Filippis, M Rosselli, G Saggio, P L Pompili, A Cesta, and S Rea, E F T I, Oncology, Avesina, Italy, Surg Oncology Univ L’Aquila, Italy.

Proceedings of the American Association for Cancer Research • Volume 41 • March 2020
A NOVEL RECOMBINANT FUSION TOXIN TARGETING HER-2/NEU-OVER-EXPRESSING CELLS AND CONTAINING HUMAN TUMOR NECROSIS FACTOR

Michael G. Rosenblum*, Shirley A. Horn and Lawrence H. Cheung

Immunopharmacology and Targeted Therapy Section, Department of Bioimmunotherapy, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Over-expression of the proto-oncogene HER2/neu in breast cancer and certain other tumors appears to be a central mechanism that may be partly responsible for cellular progression of the neoplastic phenotype. Transfection of mammalian cells and over-expression of HER2/neu appears to result in reduced sensitivity to the cytotoxic effects of tumor necrosis factor (TNF) and reduced sensitivity to immune effector killing. The single-chain recombinant antibody scFv23 recognizes the cell-surface receptor of HER2/neu. The cDNA for this antibody was fused to the cDNA encoding human TNF, and this scFv23/TNF fusion construct was cloned into a plasmid for expression in Escherichia coli. The fusion protein was expressed and purified by ion-exchange chromato- graphy. SDSPAGE demonstrated a single band at the expected m.w. (43 kDa). Western analysis confirmed the presence of both the antibody component and the TNF component in the final fusion product. The fusion construct was tested for TNF activity against L-929 cells and found to have biological activity similar to that of authentic TNF (SA 429 nm). The scFv23/TNF fusion construct bound to SKBR-3 (HER2-positive) but not to A-275 human melanoma (HER2-negative) cells. Cytotoxicity studies against log-phase human breast carcinoma cells (SKBR-3-HP) over-expressing HER2/neu demonstrated that the scFv23/TNF fusion construct was 1000-fold more active than free TNF. Tumor cells expressing higher levels of HER2/neu (SKBR-3-HP) were relatively resistant to both the fusion construct and native TNF. These studies suggest that fusion constructs targeting the HER2/neu surface domain and containing TNF are more effective cytotoxic agents in vitro than native TNF and may be effective against tumor cells expressing intermediate, but not high, levels of HER2/neu.

The HER2/neu proto-oncogene encodes a 185 kDa transmem- brane glycoprotein kinase (gp 185) with extensive homology to the epidermal growth factor (EGF) receptor (Shepard et al., 1991; Jardines et al., 1993; King et al., 1985; Schechter et al., 1985; Yamamoto et al., 1986). Transfection studies suggest that HER2/neu over-expression may play a direct role in cellular transformation to neoplastic phenotype (DiFiore et al., 1987; Heddle et al., 1987). Amplification of the gene and over-expression of the gp 185 protein product have been described in a number of human cancers, including mammary and ovarian carcinomas, gastric tumors, and colon and salivary gland adenocarcinomas (Sema et al., 1985; Yokota et al., 1988). Slamov et al. (1987) found HER2/neu over-expressed in approximately 30% of 189 primary breast cancer specimens. Their study demonstrated that over-expression of HER2/neu was correlated with poor disease prognosis. Follow-up studies have also suggested that HER2/neu cellular expression is associated with a shortened disease-free survival (DFS) (Seshadi et al., 1993; Mansour et al., 1994). Thus, the clinical observations of the importance of HER2/neu as a negative prognostic indicator in vivo have been repeatedly confirmed by molecular studies demoting the central role of this oncogene in promotion of the growth of transformed cells and in increasing their metastatic potential.

One of the key roles this oncogene appears to play is in mod- ulation of the cellular response to cytotoxic cytokines such as tumor necrosis factor (TNF) (Tang et al., 1994; Lichtenstein et al., 1991). A variety of research groups have demonstrated that HER2/neu-transformed cells are resistant to the cytotoxic effects of TNF.

In addition, NIH 3T3 cells transfected with HER2/neu appear to be resistant to immune effector cell killing mediated by membrane-bound TNF. Since TNF plays a central role in immune surveillance functions (Saks and Rosenblum, 1992), resistance to its cytotoxic effects mediated by HER2/neu over-expression in breast cancer may allow transformed cells a growth advantage by escaping host defense mechanisms. To date, there have been few studies aimed at elucidating the biochemical events linking HER2/neu signal transduction with TNF signal transduction in breast cancer cells.

Monoclonal antibodies (MAbs) have the potential to serve as targeting vehicles for various classes of therapeutic agents and to be utilized for targeting proteinaceous therapeutic agents such as interleukins, lymphokines, and cytokines (Theuer and Pastan, 1993; Reifeld et al., 1997). However, there are demonstrated pitfalls of this approach, including heterogeneous tumor antigen expression in vivo; pharmacological barriers resulting in poor tumor penetration by the antibody, due in part to antibody size; antigenicity of antibodies and antibody constructs, leading to reduced utility. Molecular approaches to address these concerns have provided numerous options to reconfigure natural antibodies while simultaneously incorporating effector or toxin functions within the same molecule (Colnaghi et al., 1993; Hand et al., 1994). Studies by our group and by others initially demonstrated the utility of chemical constructs of antibodies and cytokines such as s-FN (Ozello et al., 1994; Zuckereman et al., 1987). Reifeld et al. (1997) have studied fusion constructs of full-length antibodies in which framework domains are replaced by human cytokines (described as immunocytokines). Clinical trials of these recombinant constructs are now in progress (Gillies et al., 1991; Reifeld and Gillies, 1996a,b).

Single-chain antibodies (scFvs or sFvs), incorporating the binding characteristics of the parent immunoglobulin, consist of the antibody VL and VH domains (the Fv fragment) linked by a designed flexible peptide tether. Compared to intact IgGs, scFvs have the advantages of smaller size (approx. 30 kDa) and structural simplicity (single-chain vs. 4 chains) with comparable antigen-binding affinities, and they are more stable than the analogous 2-chain Fab fragments (Pantolino et al., 1991). It has been proposed that the smaller size of scFvs provides better penetration into tumor tissue, improved pharmacokinetics, and a reduction in the immunogenicity observed with i.v. administered Fabs (Savage et al., 1993) compared to that of intact murine antibodies. Numerous recombinant antibodies fused to plant or bacterial toxins such as Pseudomonas exotoxin, ricin, and gelonin have been reported

*Correspondence to: Michael G. Rosenblum, Immunopharmacology and Targeted Therapy Section, Box 044, Department of Bioimmunotherapy, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA. Fax: 713-794-4261. E-mail: mrosenbl@notes.mdacc.tmc.edu

Grant sponsor: National Institutes of Health; Grant number: CA 16672; Grant sponsor: Clayton Foundation for Research.

Received 17 January 2000; Revised 2 March 2000; Accepted 17 March 2000.

ARTN0: 30055
(Pretjes, 1993) and are currently undergoing pre-clinical and phase I trials (Theuer and Parast, 1993; Uckan and Frankel, 1993).

TNF is a cytokine polypeptide secreted primarily by activated macrophages (Carswell et al., 1975; Aggarwal et al., 1985), which shares some sequence homology (30%) with another peptide hormone, lymphotixin (LT or TNF-B) secreted by activated lymphocytes (Rosenblum and Donato, 1989). Purified recombinant human TNF is a single-chain, non-glycosylated polypeptide of m.w. 17.1 kDa. In vitro, TNF is cytostatic or cytotoxic to a number of human tumor cells, including SKBR-3 breast carcinoma and A-375 human melanoma. Several groups have demonstrated that human tumor cells can display between 100 and 5,000 TNF receptor sites per cell (Theuer and Parast, 1985; Sugarman et al., 1985). However, no apparent correlations have been observed between receptor number (or affinity) and the cellular response to the cytotoxic effects of TNF, suggesting that post-receptor signaling events are primarily modulate TNF biochemical effects (Nisis et al., 1985). Cloning and sequencing of the genes encoding the TNF receptor have shown that 2 separate gene products encode TNF-binding activity and appear to be homologous in portions of their extracellular binding domains (Stoiber et al., 1985; Hohmann et al., 1989; Loetscher et al., 1990; Schall et al., 1990). This family of molecules appears distinct when compared with sequences defining characteristics of other cytokine or growth factor receptors.

Previous studies in our laboratory have demonstrated that chemical comparisons of human TNF and MAbs display significant cytotoxic properties against tumor cells in culture that appear to be far superior to those of native TNF (Rosenblum et al., 1989, 1991, 1995). In addition, studies in xenograft models suggest that these immunoconjugates readily accumulate specifically in tumor tissues and demonstrate superior in vivo anti-tumor activity compared with native TNF. The purpose of the current study was to extend these original observations by developing a second-generation monoclonal antibody to a recombinant single-chain antibody fused to the TNF molecule, thereby incorporating both antibody and TNF functions within the same molecule. We therefore designed and constructed a recombinant fusion toxin composed of a single-chain antibody targeting the HBR2/new protooncogene and containing human TNF as a cytotoxic effector molecule. Further studies were performed to evaluate properties of the fusion construct and its potential for overcoming HBR2-mediated resistance to the cytotoxic effects of TNF.

**Materials and Methods**

The mammalian cell lines SKBR-3 and L-929 were obtained from the ATCC (Rockville, MD). Trypsin and yeast extract were purchased from Difco (Detroit, MI). L-Arabinosone was purchased from Sigma (St. Louis, MO). Rabbit polyclonal anti-sFv23 antibody was a generous gift from Oncolix (Gaithersburg, MD). Goat anti-rabbit IgG peroxidase conjugate was obtained from Boehringer-Mannheim (Indianapolis, IN). DTE (dithiothreitol) was purchased from Sigma. Tween-20 came from Fisher Scientific (Pittsburgh, PA). FBS was purchased from Atlanta Biologicals (Norcross, GA).

**sFv23/TNF gene construction**

The cDNA encoding the single-chain anti-HBR2/new antibody designated sFv23 was obtained from Oncolix, and the cDNA encoding mature human TNF was a generous gift from Dr. J. Kloosterbergard (M.D. Anderson Cancer Center, Houston, TX). The sFv23/TNF cDNA was constructed by 2-step PCR. The first step consisted of separate PCR amplification of the antibody and TNF coding sequences, utilizing forward and reverse primers for each sequence. The second step consisted of PCR of the sequences, utilizing overlap primers additionally incorporating a flexible tethers (G4S) between the antibody and TNF (Fig. 1).

**Expression of fusion protein sFv23/TNF in Escherichia coli**

Bacterial colonies transfected with the plasmid carrying the sFv23/TNF insert were agitated in a bacterial shaker (Innova 4330).
Tweens). After successive washing with TBS/Tween-20, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase (1:5,000 dilution in TBS). The membrane was developed using ECL reagents (Amersham, Arlington Heights, IL) and exposed to X-ray film (Kodak, Rochester, NY).

**L-929 TNF bioassay**

The cytotoxic activity of TNF was determined based on cytotoxicity to the transformed murine fibroblast cell line L-929. Log-phase cells in culture media (RPMI-1640 with 1.5 mM glutamine and 10% FBS) were plated in a 96-well tissue culture plates (Falcon, Lincoln Park, NJ) at a density of 2 × 10^4 cells/well and incubated overnight at 37°C in a 5% CO₂ atmosphere. Then, 200 µl of TNF in PBS starting at 100 units/ml and serial dilutions were added in the presence of actinomycin D (0.5 µg/ml, Sigma). Likewise, serial dilutions of sFv23/TNF were added, and the plate was incubated for 24 hr. Surviving adherent cells were then stained by adding 100 µl of crystal violet (0.5% w/v in ethanol). The stain was incubated on the plates for 0.5 hr, excess stain was washed off with PBS (0.1 M sodium citrate, pH 4.2). Plates were read on a microplate ELISA reader at 540 nm.

**Binding studies of sFv23/TNF**

Binding of sFv23/TNF to SKBR-3 cells was also assessed by ELISA. Log-phase SKBR-3 cells were washed in PBS, and 50,000 cells/50 µl PBS were added to each well of a 96-well tissue culture plate and dried overnight in a 37°C oven. Plates were blocked by addition of 100 µl 1% BSA in PBS. A 50 µl aliquot of sFv23/TNF fusion protein and serial 2-fold dilutions were then added to appropriate wells and incubated for 1 hr at room temperature. After 3 washes (PBS/Tween-20), anti-sFv23 rabbit polyclonal antibody (1 µg/ml in PBSA/Tween-20) was added and incubated for 1 hr. Wells were washed, and 100 µl of horseradish peroxidase-conjugated goat anti-rabbit antibody (Boehringer-Mannheim) were added. Plates were developed by addition of ABTS substrate in 0.1 M citrate buffer (pH 4.2, Sigma) and incubated for 1 hr. Optical density was measured at 405 nm on a Bio-Tek Autoreader.

**Cytotoxicity of TNF and sFv23/TNF against human breast tumor SKBR-3 cells**

Log-phase SKBR-3 cells were diluted to 8,000 cells/100 µl medium. Aliquots (100 µl) were added to 96-well, flat-bottomed tissue culture plates and incubated for 24 hr at 37°C with 5% CO₂.

Purified sFv23/TNF or recombinant human TNF was diluted 1:8 in culture medium (McCoY's 5a with 1.5 mM glutamine and 10% FBS). Aliquots of each sample (200 µl) were added to the plate in 2-fold serial dilutions. Plates were further incubated at 37°C, 5% CO₂ for 72 hr. Remaining adherent cells were stained by adding 150 µl crystal violet (Fisher, 0.5% in 20% methanol). Dye-stained cells were solubilized by addition of 150 µl of Sorenson's buffer (0.1 M sodium citrate, pH 4.2) in 50% ethanol, and the plates were read at 540 nm in an ELISA plate reader.

**RESULTS**

PCR products were cloned into a vector for bacterial expression of the recombinant insert. The complete insert was submitted for dideoxynucleotide sequencing (M.D. Anderson Cancer Center Core Sequencing Facility), and the final gene product sequence was confirmed. We utilized a flexible 14–amino acid linker to join the VH and VL regions and a smaller linker (G4S) to link the TNF to the antibody (Fig. 1).

Bacterial expression of the scFv23/TNF fusion construct is shown in Figure 2. After growth and induction with arabinose at 37°C, production of the construct was approximately 5% to 10% of total protein, as assessed by SDS-PAGE. Production of the target fusion construct was estimated to be 25 to 50 mg/l, as assessed by Western analysis. Purification of the soluble protein utilizing ion-exchange chromatography resulted in essentially homogeneous material, as assessed by SDS-PAGE after elution from the exchange resin. Yield of final purified material was approximately 100 µg/l of bacterial culture. Western analysis of the product utilizing antibodies to either TNF or sFv23 (Fig. 3) demonstrated an immunoreactive species with both antibodies at the expected m.w. (43 kDa).

Binding of the sFv23/TNF fusion toxin to adherent SKBR-3 cells was assessed by ELISA using an anti-TNF antibody. Binding of both the native sFv23 single-chain antibody and the sFv23/TNF fusion construct is shown in Figure 4. The binding of both agents was similar and appeared to be dose-dependant. A slightly higher binding of the fusion construct compared to the antibody was noted at the highest concentrations tested. Optimal binding to target cells occurred after incubation with 0.75 µM of the fusion construct (Fig. 5). There was no apparent binding of the construct

**LANE SAMPLE**

- **A** TNF(17kD) Standard
- **B** Uninduced sFv23 bacterial lysate
- **C** Induced sFv23 soluble lysate
- **D** Affinity (IMAC) resin prior to elution
- **E** sFv23 eluate from affinity resin
- **F** Uninduced sFv23-TNF bacterial lysate
- **G** Induced sFv23-TNF soluble lysate
- **H** Affinity (IMAC) resin prior to elution
- **I** sFv23-TNF conjugate from affinity resin
- **J** Molecular weight markers

**Figure 2** - Expression and purification of both the sFv23 antibody as well as the sFv23/TNF fusion construct from E. coli, utilizing an immobilized metal affinity column (IMAC).
Figure 3 - SDS-PAGE and Coomassie staining profiles of bacteria prior to induction (left lane) and following induction (right lane) by addition of IPTG. The position of the scFv23/TNF fusion construct is indicated by the arrow. Western analysis using anti-sFv23 antibody demonstrates reactivity against purified scFv23 antibody (left lane) and the scFv23/TNF fusion construct (right lane); probing the same Western blot with an anti-TNF antibody demonstrates reactivity only with the scFv23/TNF construct.

Figure 4 - ELISA binding of either sFv23 or scFv23/TNF to SKBR-3-immobilized cells.

to antigen-negative (A-375) cells tested under identical conditions (Fig. 5).

The cytotoxicity of TNF and scFv23/TNF fusion toxin was assessed against murine L-929 cells (data not shown). IC50 values were 1 and 100 pM for native TNF and the antibody/TNF fusion toxin, respectively. This demonstrated approximately a 100-fold decrease in the apparent specific activity for the construct compared to native TNF. This decrease in specific activity could be due to steric hindrance of the antibody interfering with interaction of TNF with its receptor site. Alternatively, since TNF operates optimally as a compact trimer in solution (Rosenblum et al., 1991), the antibody component could also interfere with optimal aggregation of the TNF component in solution.

Two breast carcinoma cell lines, SKBR-3-LP and SKBR-3-HP, were assessed for their relative expression of HER2/new and relative sensitivity to TNF-induced cytotoxicity. The LP variant cell line contained relatively higher levels of HER2/new compared to the HP line (Fig. 6). In addition, the LP line demonstrated much greater resistance to TNF-induced cytotoxic effects. The cytotoxicity of TNF and scFv23/TNF was assessed against log-phase SKBR-3-HP cells; as demonstrated in Figure 7, the IC50 for TNF exceeded 40,000 units/ml, while the scFv23/TNF fusion toxin demonstrated an IC50 value 2,000-fold lower, at approximately 20 units/ml. In contrast, the activity of both TNF and scFv23/TNF was assessed against TNF-resistant cells expressing higher levels of HER2/new. These cells were relatively resistant to the cytotoxic effects of both the scFv23/TNF construct and TNF (Fig. 8). However, the IC50 of TNF against these cells was 5 x 10^5 units/ml, while that of the scFv23/TNF construct was approximately 20-fold lower (2 x 10^5 units/ml).
Figure 6 – Western analysis of 2 variants of SKBR-3 cells (insert) demonstrates that SKBR-3-LP cells express approximately 5-fold lower HER2 protein than SKBR-3-HP cells. Direct comparison of the cytotoxicity of continuous exposure of various concentrations of TNF demonstrates that the cell line expressing higher levels of HER2 was effectively resistant to TNF while the cells expressing low levels of HER2 were sensitive to the cytotoxic effects of TNF.

Figure 7 – Cytotoxicity of either TNF or purified sFlv23/TNF against log-phase SKBR-3-HP cells in culture. The IC_{50} of the sFlv23/TNF construct (20 units/ml) was approximately 2,000-fold lower than that of free TNF (4,000 units/ml).

DISCUSSION

Numerous groups have examined the biological activity of antibodies chemically conjugated with or genetically fused to numerous human cytokines (Oozello et al., 1993; Kim et al., 1998). We initially described a novel construct containing recombinant human TNF-α chemically conjugated to the murine anti-melanoma MAh ME-018 (Rosenblum et al., 1989, 1991, Rosenblum et al., 1995). Against TNF-sensitive melanoma cells, the construct was several-fold more cytotoxic than free TNF. In addition, this ME TNF construct was extremely cytotoxic to human melanoma target cells virtually resistant to TNF itself. Follow-up studies demonstrated that the augmented cytotoxicity of the ME/TNF construct was primarily due to the antibody component of the construct binding to target cells. Studies of the ME/TNF construct in xenograft tumor models demonstrated efficient localization within solid tumors after systemic administration, similar to that of the native antibody. Finally, this construct demonstrated impressive cytotoxicity against s.c. xenografts in nude mice. These studies clearly demonstrated that antibody-mediated delivery of agents such as cytotoxic cytokines is feasible and has the potential to modify and enhance the in vivo and in vitro biological properties of the original cytokine. Since our initial report of antibody/TNF conjugates, other groups have also reported antibody/cytokine constructs with agents such as GM-CSF, IL-2, and lymphokine (Reisfeld and Gillies, 1996a,b; Reisfeld et al., 1996; Dreier et al., 1998).

Over-expression of the HER2/neu oncogene appears to suppress the cellular cytotoxic response to TNF (Hudziak et al., 1990; Lichtenstein et al., 1990, 1992). However, the cellular mechanisms that could account for this effect have not been elucidated. In a study by Hudziak et al. (1990), transformation of NIH 3T3 cells with HER2/neu provided cellular resistance to the cytotoxic effects of TNF without appreciably affecting TNF receptor function in the transfectants. The current study, examining 2 SKBR-3-selected variant cell lines expressing high and intermediate levels of HER2, demonstrates that the IC_{50} values for TNF were >10 and 0.2 nM, respectively (Fig. 5), thus confirming previous observations. Studies by Lichtenstein et al. (1990, 1992), utilizing ovarian and breast tumor cell lines over-expressing HER2 to various degrees, also found that resistance to TNF was associated with HER2/neu expression. The TNF resistance was not due to reduction in TNF receptor number or signal-transduction events such as MHC expression or ADP-ribosyl polymerase activity. In another study, these authors demonstrated that increased HER2/neu expression also correlated with resistance to killing by lymphokine-activated killer cells (Fady et al., 1993). Therefore, over-expression of HER2 in breast cancer cells appears to provide a distinct growth advantage since the transformed phenotype can effectively escape growth control by soluble cytokine mediators and by immune effector cells empowered to maintain homeostasis.
The scFv23/TNF construct characterized in our study represents a design improvement over our initial chemical construct of a full-length antibody conjugated to recombinant TNF. Compared to the original construct, the fusion toxin is smaller (50 vs. 190 kDa) and therefore better able to penetrate into solid tumors. In addition, the scFv23/TNF construct is simpler to produce and may be more stable in vivo than the original chemical version.

Against TNF-resistant, log-phase SKBR-3 cells, the scFv23/TNF construct was 2,000-fold more active than TNF itself (Fig. 7). These data suggest that the scFv23/TNF fusion construct targeting HER2 may have the ability to overcome cellular resistance mechanisms to TNF induced by intermediate levels of HER2/Neu but not in the presence of extremely high cellular levels of HER2/Neu. Against the SKBR-3-LP cell line, which expresses much higher levels of HER2/Neu, TNF and the scFv23/TNF fusion construct demonstrated similar cytotoxic effects at doses below 10^6 unit/ml. This suggests that at very high cellular levels of HER2/Neu, critical signaling pathways for TNF-mediated cytotoxicity may be blocked beyond the ability of this targeted fusion construct to overcome.

Activation of the signaling pathway transduced by scFv23/TNF is associated with rapid changes in phosphorylation of several substrates, phospholipid metabolism, and interaction with guanine nucleotide binding proteins (G proteins) (Darnay and Aggarwal, 1997). As with other hormone-responsive tissues, the induction of a biological response may be regulated cellulary by expression of appropriate molecules or substrates capable of interaction with a common receptor signaling complex. Therefore, each of these signaling components may play a role in TNF signal transduction in an appropriate target tissue. Studies in our laboratory (Donato et al., 1991, 1992, 1993) suggest that VEGF receptor phosphorylation status as well as modulation of c-myc and ornithine decarboxylase activity play a major role in the early events leading to cytotoxic TNF signal transduction. The ability of HER2/neu overexpression to modulate TNF intracellular signaling through any of the biochemical pathways described above and the ability of the scFv23/TNF complex to affect these pathways are not known at this time. It is possible that the ability of the initial 2MU2/TNF conjugate to overcome resistance to TNF in melanoma cells may be associated with mechanisms underlying the improved cytotoxicity of the scFv23/TNF fusion construct against breast carcinoma cells. Further in vitro mechanistic studies are ongoing to examine these potential mechanisms and cytotoxic pathways for the fusion constructs and chemical conjugates compared to native TNF. These studies may provide an additional rationale for the utilization of these agents in a clinical therapeutic setting as well as insight into possible control mechanisms in the TNF cytotoxicity pathway.

ACKNOWLEDGEMENTS

We thank Ms. J. Merchant for excellent assistance in the preparation of this report. In addition, we acknowledge NIH Core Grant CA 16672, for support of the DNA Sequencing Facility and USAMRCm.

REFERENCES


HEIDEN, R.M., SCHLIESINGER, J., and ULLRICH, A. Increased expression of the putative growth factor receptor PDGFR2 in HER2 and HER2 promotes the growth of the growth factor receptor (EGFR) in breast cancer. Pathobiology, 61, 268-282 (1993).


OZELLIO, L., DE ROSA, C.M., BLANK, R., GANTZ, K., CHERIAN, R.L., and HABER, D.V., Sr. The use of tumor-targeted interferon alpha conjugated to a


