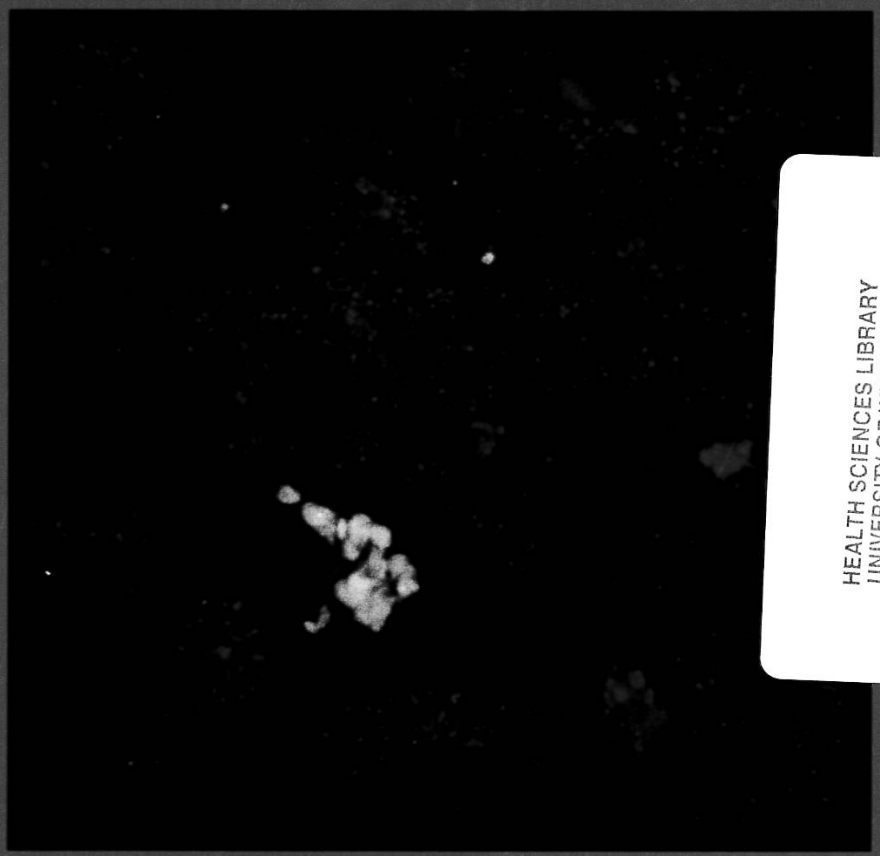
15 October 2000

Vol. 88 No. 2

INTERNATIONAL Journal of Cancer



Publication of the International Union Against Cancer
Published for the UICC by Wiley-Liss, Inc.

www.interscience.wiley.com

ISSN 0020-7136

Int. J. Cancer: **88,** 267–273 (2000)

Publication of the International Union Against Cancer

A NOVEL RECOMBINANT FUSION TOXIN TARGETING HER-2/*NEU*–OVER-EXPRESSING CELLS AND CONTAINING HUMAN TUMOR NECROSIS FACTOR

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ERRATUM

ROSENBLUM, MICHAEL G., HORN, SHIRLEY A., and CHEUNG, LAWRENCE H., A novel recombinant fusion toxin targeting HER-2/*neu*–over-expressing cells and containing human tumor necrosis factor. *Int. J. Cancer,* **88,** 267–273 (2000).

Due to the authors' error, a mistake appeared in the legend to Figure 6. The figure and correct legend are printed below.

The authors regret this error.

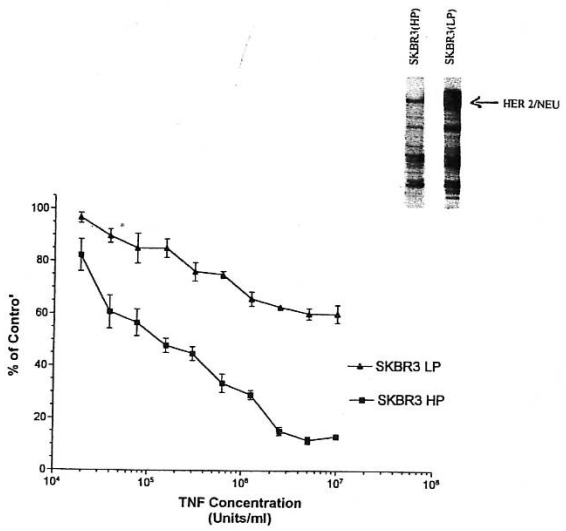

FIGURE 6 – Western analysis of 2 variants of SKBR-3 cells (insert) demonstrates that SKBR-3-LP cells express approximately 5-fold higher HER2 protein than SKBR-3-HP cells. Direct comparison of the cytotoxic effects of continuous exposure of various concentrations of TNF demonstrates that the cell line 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.

and colon and salivary gland adenocarcinomas (Semba *et al.,* 1985, Yokota *et al.,* 1988). Slamon *et al.* (1987) found HER2/*neu* over-expressed in approximately 30% of 189 primary breast carcinomas examined. 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) (Seshadri *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 demonstrating 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 modulation 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/

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

Grant sponsor: National Institutes of Health; Grant number: CA 16672; research conducted, in part, by the Clayton Foundation for Research.

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Received 17 January 2000; Revised 13 March 2000; Accepted 17 March 2000.

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A NOVEL RECOMBINANT FUSION TOXIN TARGETING HER-2/*NEU*–OVER-EXPRESSING CELLS AND CONTAINING HUMAN TUMOR NECROSIS FACTOR

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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 sFv23 recognizes the cell-surface domain of HER2/*neu*. The cDNA for this antibody was fused to the cDNA encoding human TNF, and this sFv23/TNF fusion construct was cloned into a plasmid for expression in *Escherichia coli*. The fusion protein was expressed and purified by ion-exchange chromatography. SDS-PAGE 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 420 nM). The scFv23/TNF construct bound to SKBR-3 (HER2-positive) but not to A-375 human melanoma (HER2-negative) cells. Cytotoxicity studies against log-phase human breast carcinoma cells (SKBR-3-HP) over-expressing HER2/*neu* demonstrate that the sFv23/TNF fusion construct was 1,000-fold more active than free TNF. Tumor cells expressing higher levels of HER2/*neu* (SKBR-3-LP) 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*. *Int. J. Cancer* 88:267–273, 2000.
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The HER2/*neu* proto-oncogene encodes a 185 kDa transmembrane 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 a neoplastic phenotype (DiFiore *et al.,* 1987; Hudziak *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 (Semba *et al.,* 1985; Yokota *et al.,* 1988). Slamon *et al.* (1987) found HER2/*neu* over-expressed in approximately 30% of 189 primary breast carcinomas examined. 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) (Seshadri *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 demonstrating 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 modulation 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; Reisfeld *et al.,* 1997). However, there are demonstrated pitfalls of this approach, including heterogenous 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 α-IFN (Ozzello *et al.,* 1994; Zuckerman *et al.,* 1987). Reisfeld *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; Reisfeld and Gillies, 1996*a,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

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(Preijers, 1993) and are currently undergoing pre-clinical and phase I trials (Theuer and Pastan, 1993; Uckun and Frankel, 1993).

TNF is a cytotoxic polypeptide secreted primarily by activated macrophages (Carswell *et al.,* 1975; Aggarwal *et al.,* 1985), which shares some sequence homology (30%) with another peptide hormone, lymphotoxin (LT or TNF-β) 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 (Tsujimoto *et al.,* 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 primarily modulate TNF biochemical effects (Nitsu *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 (Stauber *et al.,* 1988; 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 conjugates of human TNF and MAbs display significant targeted 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 immunocytokines 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 molecular construct of 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 HER2/*neu* protooncogene and containing human TNF as a cytotoxic effector molecule. Further studies were designed to examine the biological properties of the fusion construct and its potential for overcoming HER2-mediated resistance to the cytotoxic effects of TNF.

MATERIAL AND METHODS

The mammalian cell lines SKBR-3 and L-929 were obtained from the ATCC (Rockville, MD). Tryptone and yeast extract were purchased from Difco (Detroit, MI). L-Arabinose was purchased from Sigma (St. Louis, MO). Rabbit polyclonal anti-scFv23 antibody was a generous gift from Oncologix (Gaithersburg, MD). Goat anti-rabbit IgG peroxidase conjugate was obtained from Boehringer-Mannheim (Indianapolis, IN). DTE (dithioerythritol) 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-HER2/*neu* antibody designated sFv23 was obtained from Oncologix, and the cDNA encoding mature human TNF was a generous gift from Dr. J. Klostergaard (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 final step consisted of PCR of the sequences, utilizing overlap primers additionally incorporating a flexible tether (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;

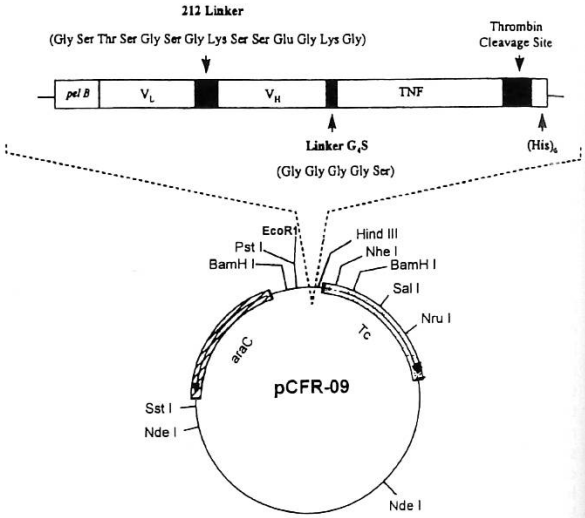

FIGURE 1 – Design of the sFv23/TNF fusion construct and assembly of the expression vector.

New Brunswick Scientific, Edison, NJ) in 1 l of TYE medium (15 g tryptone, 10 g yeast extract, 5 g NaCl) containing 50 μg/ml tetracycline (Sigma) overnight at 37°C at 250 rpm. Bacterial cells were harvested by centrifugation, and the pellet was dispersed into 2 l of fresh TYE. Expression of the target protein was induced by addition of arabinose to a final concentration of 0.1%. The culture was further incubated at 37°C for 2 to 3 h. Cells were collected by centrifugation, resuspended in 80 ml extraction buffer [50 mM Tris-HCl (pH 8), 20 mM EDTA, 0.25 mg/ml lysozyme (Sigma)], and incubated with shaking for 1 hr at room temperature. Triton X-100 and sodium chloride were added to the sample at a final concentration of 2% and 0.5 M, respectively, then incubated for an additional 30 min. After centrifugation, the insoluble fraction of the inclusion bodies was resuspended in 160 ml of 50 mM Tris-HCl (pH 8.0), 20 mM EDTA and sonicated (6 × 20 sec) using a Vir Sonic 300 sonicator (Virtis, Gardiner, NY). Inclusion bodies were then harvested by centrifugation, washed 3 times in the same buffer, and stored at −80°C.

Protein solubilization, refolding, and purification

Insoluble inclusion bodies were denatured by addition of 6 M guanidine, 100 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 50 mM DTE to make a final concentration of 3 mg/ml protein (as assessed by Bradford protein determination). After a minimum of 2 hr agitation at room temperature, solubilized proteins were diluted 100-fold in refolding buffer [100 mM Tris-HCl (pH 7.5), 2 mM EDTA] and incubated at 12°C for 48 hr. The protein was subsequently bound to a small column containing SP Sepharose Fast Flow Resin (Pharmacia, Gaithersburg, MD). The bound fraction was eluted from the column by addition of 2 M NaCl in 100 mM Tris-HCl (pH 7.5), 2 mM EDTA. Eluted protein fractions were pooled and dialyzed against TBS, and the final product was then further characterized as described below.

Western methods

Protein samples from the crude extracts of *E. coli,* the purified inclusion bodies, sFv23, and r-TNF were analyzed by SDS-PAGE under reducing conditions. The gel was electrophoretically transferred overnight onto a nitrocellulose transfer and immobilization membrane (Protran; VWR, Sugar Land, TX). The membrane was incubated in 5% BSA/TBS [20 mM Tris-HCl, 137 mM NaCl, and 0.5% Tween 20 (pH 7.6)] and then incubated for 1 hr with anti-sFv23 rabbit polyclonal antibody (1:10,000 dilution in TBS/

Tween). 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% CO_2 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 ht, excess stain was removed, the plates were washed with water and allowed to air-dry, and the remaining dye was solubilized by addition of 150 μl of Sorenson's buffer (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 5% 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 tapped dry, 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% CO_2. Purified sFv 23/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% CO_2 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 (BioTek, Inc., Winooski, VT).

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 tether (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 scFv23 (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

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).

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