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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Overexpression of the HER2/neu oncogene in breast cancer cells blocks the biological activity of the cytokine TNF and allows these cells to escape immune surveillance mechanisms. We have developed a fusion construct composed of a single-chain antibody recognizing HER2/neu and TNF. Against cells over-expressing HER2/neu and resistant to TNF, this scfv23/TNF fusion construct was shown to have impressive cytotoxic properties (I.C.50 = 35-50 nM). Signal transduction studies indicate that the cytotoxic effects of the scfv23/TNF construct are not mediated by changes in TNFR-1, TRADD, TRAF2, NfkB, AKT, or caspase 3 or 7. In contrast, we found activation of caspase 8 and 6 to be the primary pathway for activation of apoptosis by scfv23/TNF. We also found that treatment of SKBR3 cells with scfv23/TNF resulted in inhibition of MMP-9. This is in contrast to treatment of cells with TNF, which results in activation of MMP-9. In preparation for animal model studies, we have also developed a bacterial expression system for generation of the requisite large amounts of protein needed for the planned in vivo studies.

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Introduction:

The overexpression of the HER2/neu oncogene occurs in approximately 30 % of patients with breast cancer and is a negative prognostic indicator associated with a poor prognosis, lowered response to therapy and a shortened disease free interval. Studies have demonstrated that overexpression of the HER2/neu oncogene results in a cellular phenotype resistant to the pro-apoptotic effects of TNF. Studies in our lab have also demonstrated a link between response of tumor cells to TNF and HER2/neu expression. We have developed a unique fusion construct composed of a single-chain anti-HER2/neu antibody(scfv23) fused to TNF. This scfv23/TNF construct was found to be highly cytotoxic to cells expressing the HER2/neu oncogene which are resistant to native TNF. In the current progress report, we present further developmental studies examining the in vitro cytotoxic effects of the scfv23/TNF fusion construct compared to free TNF. In addition, we provide studies in support of a biochemical rationale for the development of this agent in eventual clinical trials to treat poor-prognosis patients whose tumors overexpress HER2/neu.

Second Annual Progress Report:

The past 12 months, we have primarily focused on in vitro studies of the scfv23/TNF fusion construct in support of our Approved Statement of Work Plan as follows:

Technical Objective 2

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

Task 3 - Assess scfv23/TNF cytotoxic activity against TNF sensitive and TNF resistant breast tumor cells. Evaluate possibility of antibody mediation of improved cellular toxicity.

Task 4 - Examine TNF related mechanisms which may be related to improvements in scfv23/TNF cytotoxic mechanism of action.

Second Annual Report of Progress in Support of the Approved Statement of Work:

Bioactivity and Specific Activity of scfv23/TNF and TNF on L-929 Cells

Various concentrations of the scfv23/TNF fusion construct and TNF were applied to log-phase L-929 cells. As shown in Figure 1, the $I.C._{.50}$ of TNF was calculated to be 4.4 pM (SA = 230 U/nmole). In contrast, the biological activity of the scfv23/TNF fusion construct on L-929 cells was found to be higher with an $I.C._{.50}$ of 0.76 pM (SA = 13,200 U/nmole).

Figure 1

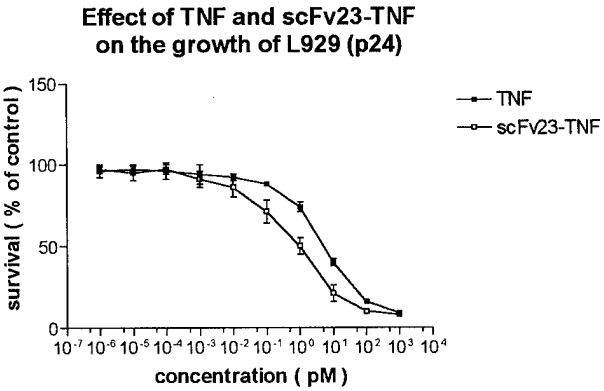
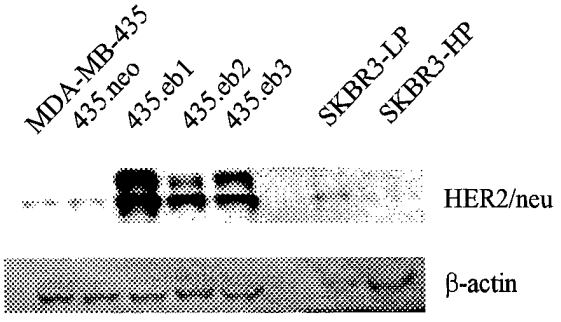


Figure 2

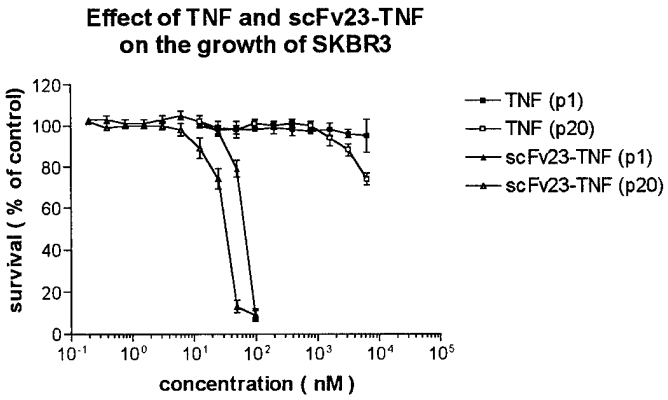


Biological Activity of scfv23/TNF and TNF on Cells Expressing HER2/neu

The cell line SKBR3 has previously been demonstrated in our lab and others to down-regulate HER2/neu expression after prolonged passage in vitro. Western analysis of HER2/neu expression in the SKBR3 high(HP, passage 20) and low passage (LP, passage 1) cell lines is shown in Figure 2. There was approximately a 15 fold

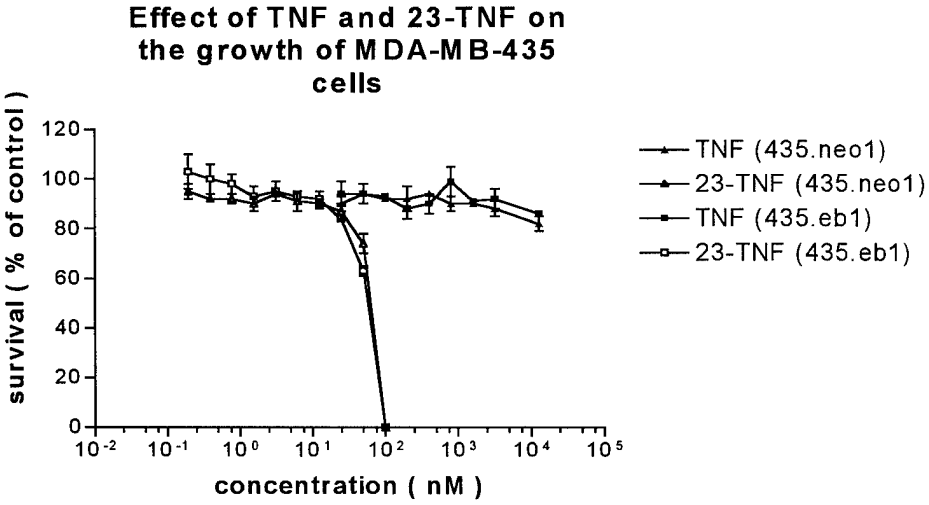
decrease in the relative HER2/neu expression levels in the high passage cells (HP) compared to the lower passage lines (LP). Both TNF and scfv23/TNF were added to SKBR3 cells from low passage(p1) expressing high levels of HER2/neu and cells from high passage(p20) expressing lower levels of HER2/neu. As shown in Figure 3, cells expressing high levels of HER2/neu (p1) were resistant to cytotoxic effects of TNF itself. On the other hand, cells expressing lower levels of HER2/neu (p20) demonstrated some sensitivity to TNF, but only at the highest dose levels tested. SKBR3 cells expressing high levels of HER2/neu (p1) showed remarkable sensitivity to the cytotoxic effects of the scfv23/TNF fusion construct with an $I.C._{50}$ of approximately 80 nM. In contrast, cells expressing lower levels of HER2/neu(SKBR3 p20) demonstrated an $I.C._{50}$ of approximately 40 nM..

Figure 3



Studies were also performed on human breast tumor cells which were transfected with the HER2/neu oncogene. As shown in in Figure 2, the MDA-MB-435 cells express various levels of HER2/neu depending on the clonal line selected. The parental MDA-MB-435 cells express low, detectable levels of HER2/neu while the transfected clones designated eb3, eb2 and eb1 over-express increasing levels of HER2/neu. As shown in Figure 4, TNF itself had no demonstratable cytotoxic effects on cells expressing low and high levels of HER2/neu. In contrast, both cell lines appeared to be almost equally sensitive to the cytotoxic effects of

Figure 4



the scfv23/TNF fusion construct with an I.C.$_{50}$ of approximately 50 nM.. This I.C. $_{50}$ was similar to that found for SKBR3 cells.

Differential Effects of TNF and scfv23/TNF on Signal Transduction Events

In order to detail the intracellular events responsible for the observed biological properties of the scfv23/TNF fusion construct compared to native TNF, we next examined various biochemical events associated with TNF mediation of its cytotoxic effects.We examined the effects of the two agents on TNFR-1, TRADD, TRAF2. As shown in Figure 5, we treated SKBR3 (passage1) cells with 50 uM of either TNF or scfv23/TNF at a dose of 50 uM for various times as shown. The cells were harvested and subjected to Western analysis for the various proteins. We found no changes to the levels of the various proteins after treatement with either agent.

Figure 5

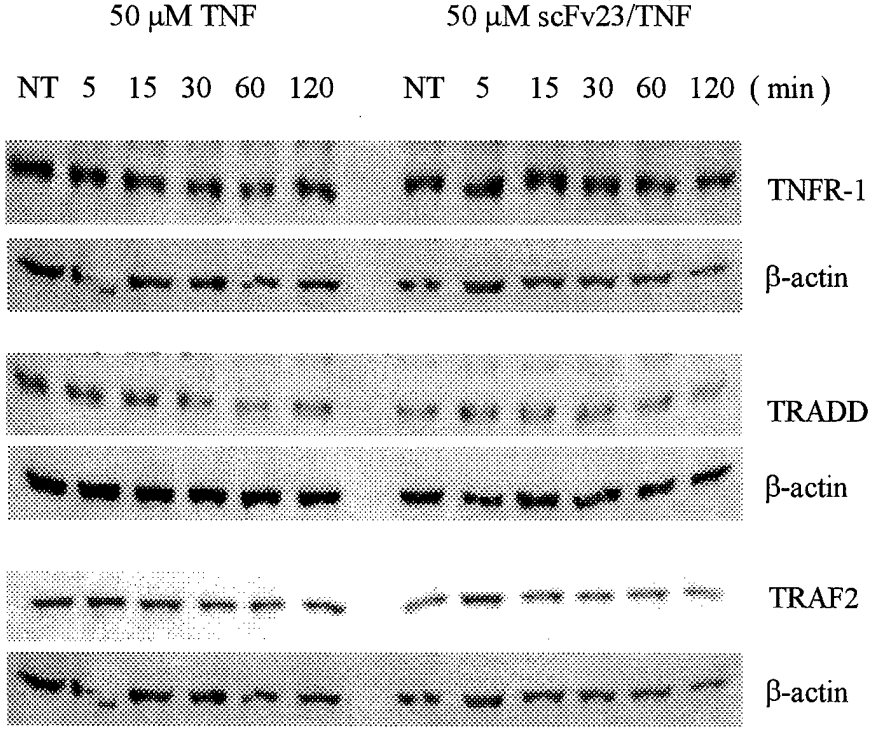
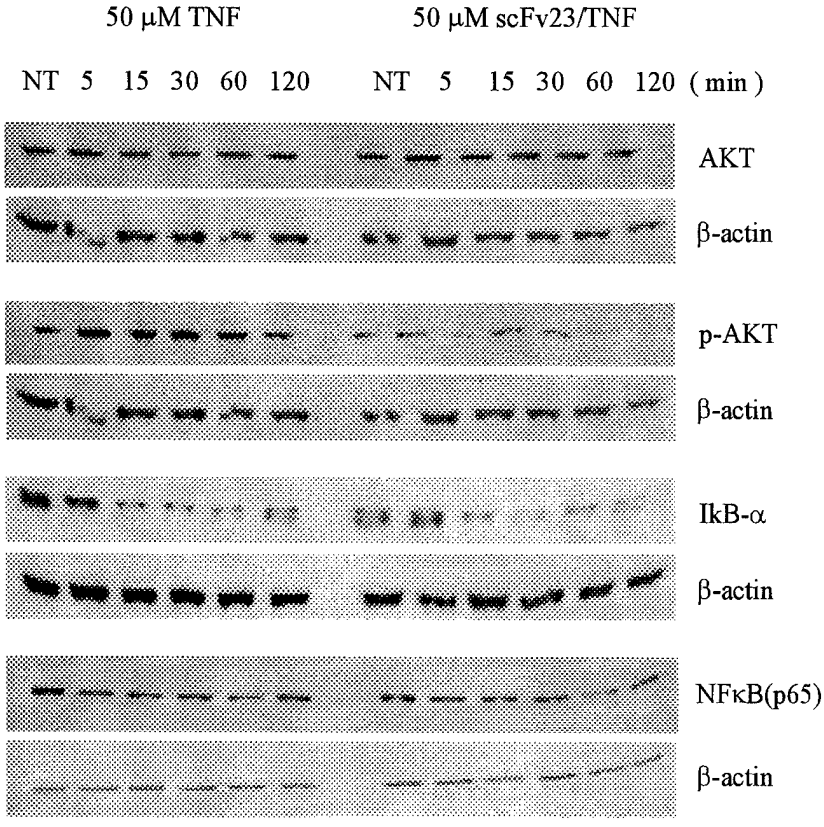


Figure 6

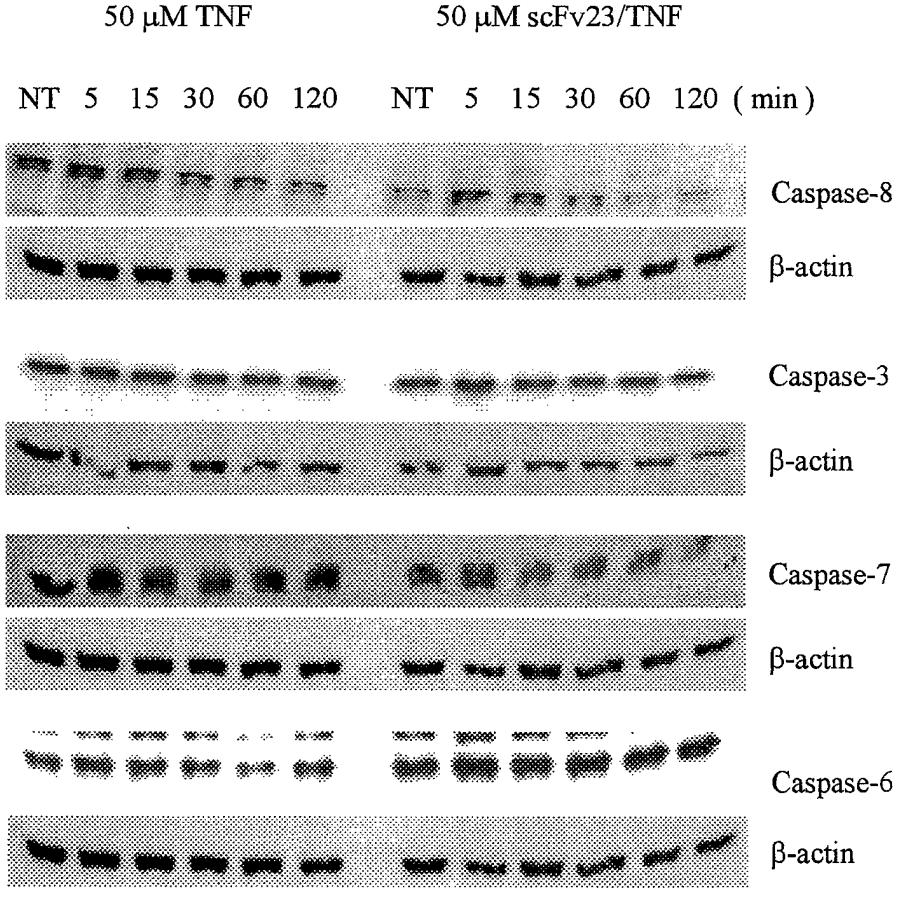


We next examined the effects of these agents on the AKT signaling pathway which

is an alternative pathway capable of impacting TNF signal transduction events. This pathway and its interactions with the TNF signaling pathway are detailed in Figure 10. As shown in Figure 6, treatment of cells with TNF alone resulted in a 2 fold increase in the basal levels of phosphorylated AKT within 5 minutes after drug addition.By 60 minutes, the levels of p-AKT declined to baseline levels. In contrast, treatment with scfv23/TNF had no effect on (or slightly reduced) the levels of p-AKT. After 5 minutes, treatment with TNF was shown to increase the phosphorylation of IkB-a. After 15 minutes, levels of IkB declined in treated cells.One hour after addition of TNF, levels of IkB increased in treated cells. Treatment of cells with scfv23/TNF resulted in no significant change in Ikb levels over time.

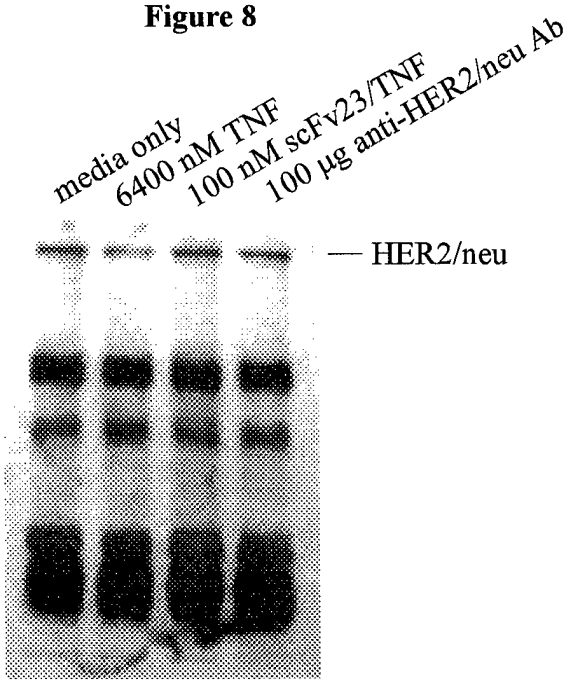
The caspace series of proteins is also a central mediator of the apoptotic effects of TNF and other cytokines. We examined the effects of treatment of SKBR3 cells with TNF and scfv23/TNF on caspace 3,6, 7 and 8. As shown in Figure 7, there was a slight increase in caspace-8 activation 5 minutes after treatment with scfv23/TNF and no discernable increase in caspace-8 after treatment with TNF. Caspace-8 activation declined over time after treatment with both agents .There was no change in caspace-3 or caspace-7 activity after treatment with either agent. Treatment of cells with TNF caused no significant change in caspace-6 activation. However, 5 minutes after treatment with scfv23/TNF, cells demonstrated a

Figure 7



significant (4 fold)increase in caspace-6 concentration within 5 minutes after administration of the scfv23/TNF. This increase persisted until 1 hr after administration when caspace levels declined beck to baseline levels

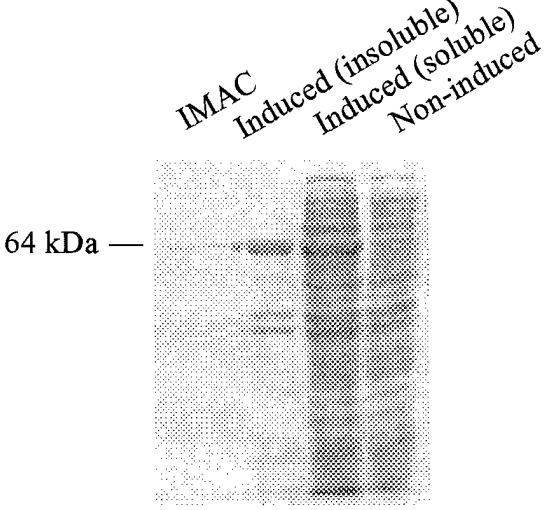
Figure 8



In order to examine the effects of the scfv23/TNF fusion construct on the HER2/neu signaling cascade, SKBR3 (LP) cells were treated with either TNF or scfv23/TNF. Cells were harvested 10 minutes after drug administration and the HER2/neu phosphorylation levels were examined by Western analysis. As shown in figure 8, treatment with TNF reduced phosphorylation of HER 2 by almost 30%. Treatment with either scfv23/TNF or an anti-HER2 antibody demonstrated only a slight increase in phosphorylation levels of HER2/neu compared to untreated controls.. We concluded from these studies that treatment with the scfv23/TNF fusion construct had little direct impact on phosphorylation and signal transduction of HER2/neu itself.

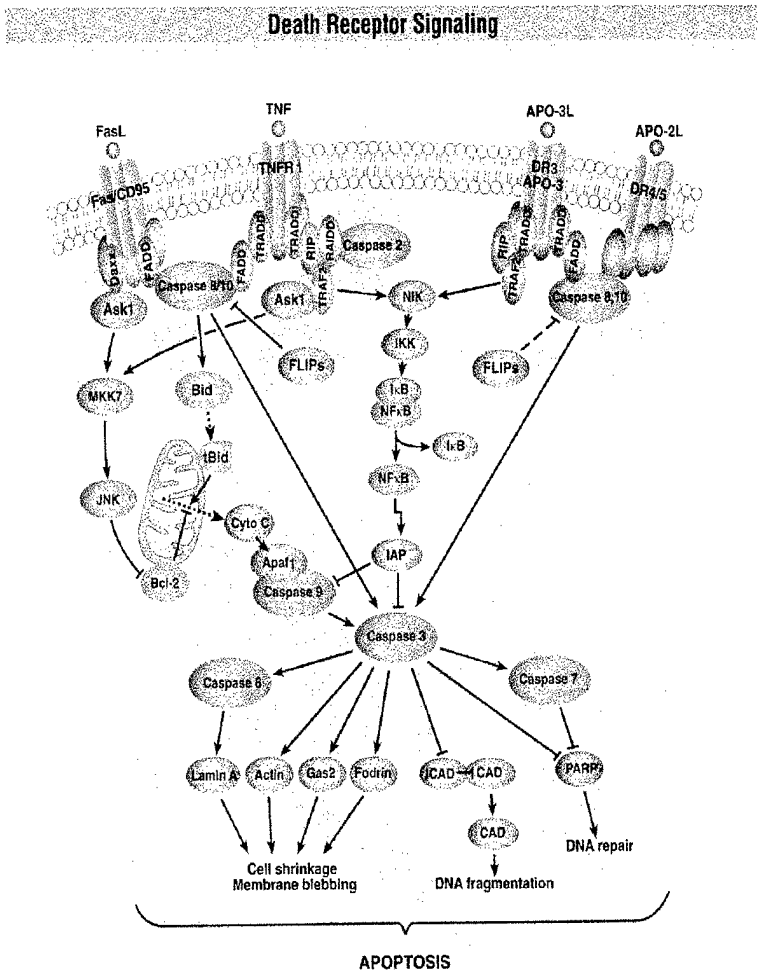
The next phase of the study involving Technical Objectives 3 and 4 of the Approved Statement of Work will require substantial amounts of purified scfv23/TNF for in vivo pharmacokinetic and efficacy studies. To that end, we have developed a high-level bacterial expression of soluble fusion construct. Efforts to re-fold insoluble scfv23/TNF leading to biologically active material have been unsuccessful to date. As shown in Figure 9, high levels of both soluble and insoluble fusion construct are generated by this process. The yield of purified target protein using immobilized metal affinity chromatography (IMAC) was 1.25 ug/ml of culture.

Figure 9



This procedure will allow generation of significant amounts of purified, soluble, biologically active material as shown in Figure 1 (above).

Figure 10



Key Research Accomplishments:

- Examined the biological activity of the TNF component in the highly purified scfv23/TNF construct. Specific actvity against L-929 cells approximately 5 fold greater than that of TNF.

- Examined cytotoxic activity of TNF and scfv23/TNF against SKOV3 high and low passage cells. Both lines were insensitive to TNF, but sensitive to the cytotoxic effects of scfv23/TNF ($I.C._{50}$ ~ 50 nM)

- Cytotoxic mechanism of action of scfv23/TNF does not involve effects on TNFR1, TRADD or TRAF2

- Cytotoxic mechanism of action of scfv23/TNF does not involve signaling through AKT, p-AKT, IkB-a or NfkB. In contrast, TNF activated AKT phosphorylation.

- Cytotoxic mechanism of scfv23/TNF action does not involve signaling through caspase 3 or caspase-7

- Treatment of cells with scfv23/TNF does result in transient activation of caspase-8 followed by degradation.

- Treatment of cells with scfv23/TNF results in significant activation of caspase-6. Treatment with TNF showed no activation of caspase-6.

- Treatment of SKBR3 cells with TNF resulted in increased expression of MMP-9. Treatment with equivalent amounts of scfv23/TNF showed slight activation at the lowest dose and complete inhibition at the higher doses.

- These signaling studies demonstrate that the effects of scfv23/TNF are distinct from that of TNF and appear to be mediated through activation of caspase-6.

- Treatment of HER2/neu positive SKBR3 cells with TNF resulted in a slight decrease in HER2/neu phosphorylation, wheras treatment with scfv23/TNF construct did resulted in a slight increase in phosphorylation of the HER2/neu protein.

- The bacterial expression of the scfv23/TNF has been limiting. We have succeeded in improving the bacterial expression levels of the fusion construct and have developed a purification method to generate high levels of protein for animal model studies to commence.

Reportable Outcomes:

See attached manuscript (in preparation)

Conclusions:

Although previous studies have demonstrated that overexpression of the HER2/neu oncogene results in a phenotypic effect rendering breast tumor cells resistant to the cytotoxic effects of free TNF, we have found that these breast tumor cells appear to be extremely sensitive to the cytotoxic effects of TNF when delivered to tumor cells using an antibody recognizing the external domain of the HER2/neu growth factor receptor. Studies have suggested that overexpression of the

HER2/neu oncogene can suppresses the TNF cytotoxic signal by endogenous activation and blockade of the AKT and NfkB signaling pathway leading to apoptosis. Our studies have demonstrated that the scfv23/TNF fusion construct generates a cytotoxic signal by activation of the caspase-6 pathway and thereby circumvents the HER2/neu generated blockade of the TNF signal transduction pathway. These studies are important in the pre-clinical development of the scfv23/TNF fusion construct because they demonstrate that this agent may have a significant therapeutic impact in vivo on breast tumor cells over expressing the HER2/neu oncogene. These studies provide a further proof of concept and rationale for the clinical development of this agent for breast cancer patients.

The next Technical Objectives to be performed in the Approved Statement of Work require significant quantities of purified scfv23/TNF fusion construct. As described, we have completed efforts to scale up bacterial production of the material and have completed studies to provide a more highly purified biologically active product for the next steps in the pre-clinical assessment of this novel therapeutic agent.

References:

None

Appendices:

None

Intracellular Cytotoxic Signaling Events Generated by an Antibody/TNF fusion Construct in Target Cells: Identification of Unique Events Compared to TNF

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ABSTRACT

Tumor necrosis factor (TNF) is a cytotoxic cytokine which operates through a specific cell-surface receptors creating cytotoxic signals through interaction with caspace-dependant pathways leading to apoptosis. We have previously developed a novel fusion construct of a single-chain antibody (scfv23) recognizing the external domain of the HER2/neu protooncogene (gp 185) linked by a flexible tether to TNF. Preliminary data demonstrated that the scfv23/TNF fusion construct displayed cytotoxic activity specifically to cells expressing the HER2/neu cell-surface domain. Current studies have clearly demonstrated that the biological activity of the TNF component of the scfv23/TNF remains intact compared to that of free TNF against the murine fibroblast standard cell line L-929. ELISA assays have also demonstrated previously that the scfv23/TNF construct is also capable of binding specifically to cells expressing the HER2/neu proto-oncogene. Cytotoxic effects of the scfv23/TNF were examined against cells expressing various levels of HER2/neu. Human breast (SKBR 3) cells expressing lower levels of HER2/neu were most sensitive to the cytotoxic effects of the fusion construct ($I.C._{50}$: 35 nM) compared to cells expressing high levels of HER2/neu ($I.C._{50}$: 65 nM). Studies of human breast tumor MDA-MB-435) cells transfected with the HER2/neu gene and selected for high expression (435.eb1) compared to low expression (435.neo1) demonstrated that both cell lines were resistant to the cytotoxic effects of TNF treatment at doses up to 1 uM. In contrast, the scfv23/TNF fusion construct demonstrated an $I.C._{50}$ of approximately 50 nM. Surprisingly, there was no significant difference in the cytotoxicity curves between the cell lines

expressing low and high-levels of HER2/neu. Treatment of SKBR3 cells with either TNF or the scfv23/TNF fusion construct had no detectable effects on the cellular levels of TNFR-1, TRADD or TRAF2. There were also no significant differences in the effects of TNF and scfv23/TNF on the levels of AKT. There were also no significant differences in the levels of caspace8, 3 and caspase 7 after treatment of cells with TNF or scfv23/TNF. Treatment of cells with scfv23/TNF was found to result in a significant (~4-fold) increase in the levels of caspase 6 within 5 minutes after administration of scfv23/TNF. Treatment with TNF alone had no significant effect on caspase-6. Treatment of cells with TNF also was found to result in a significant increase in the levels of MMP-9 compared to untreated controls. On the other hand, treatment with the scfv23/TNF fusion construct showed only a slight augmentation of MMP-9 at the lowest dose of the fusion construct tested.

INTRODUCTION

The HER2/neu is a 185-kDa transmembrane receptor tyrosine kinase that belongs to the epidermal growth factor family (1-3). Over-expression of HER2/neu is found in 20-30% of human breast cancers and also seen in ovarian, lung, and gastric adenocarcinomas (4-8). Their study demonstrated that over-expression of HER2/neu was correlated with poor disease prognosis. One of the key roles this oncogene appears to play in modulation of cellular response to cytotoxic cytokines such as tumor necrosis factor (TNF) (9-10). A variety of research groups have demonstrated that HER2/neu-transfected cells are resistant to the cytotoxic effects of TNF. Since TNF plays a central role in immune surveillance functions (11), 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. One mechanism allowing escape from apoptosis is activation of survival signal transduction pathways in breast cancer cells. Recently, several survival signaling pathways have been described. The best characterized pathway is signaling via activation of phosphatidylinositol 3'-kinase (PI3K) and the proteinase kinase Akt. When activated, Akt can directly phosphorylate and inactivate the proapoptotic molecule Bad (12) or signal through the transcription factors Forkhead (13) and nuclear factor NFκB (14). Thus, the Akt signaling pathway has a critical role in anti-apoptosis that may be contribute to the pathogenesis of cancer (15-16).

In dominant-negative (DN)-Akt transfected MDA-MB-453 breast cancer cells and HER2/neu-transformed NIH 3T3 cells, blocking of the Akt pathway by a DN-Akt sensitizes the

HER2/neu-overexpressing cells to TNF-induced apoptosis and inhibits IκB kinase, IκB phosphorylation, and NF-B activation (17). This result suggests that HER2/neu constitutively activates the Akt/NFB anti-apoptotic cascade to confer resistance to TNF on cancer cells.

We previously developed a novel fusion construct of a single-chain antibody (scFv23) recognizing the external domain of the HER2/neu proto-oncogene linked by a flexible tether to TNF. This construct displayed cytotoxic activity specifically to HER2/neu over-expressing SKBR3 breast cancer cells and remains intact TNF activity. Binding of TNF-α to TNF receptor-1 (TNF-R1) can induce the formation of signaling complexes, TNF-R1-TRADD-FADD-pro-caspase-8 with subsequent release of activated protease caspase-8 (18). The activation of caspase-8 is thought to result in proteolytic activation of other caspase proteases (19), which in turn mediate characteristic morphological and biochemical changes of death receptor-triggered apoptosis (20-21). We therefore proposed that the scFv23/TNF-mediated cytotoxic signal pathway may be different from that of native TNF.

In this study, we examined the Akt survival pathway and death receptor signaling pathway in SKBR3 breast cells (LP) and found that scFv23-TNF-induced cytotoxic signaling pathway is mediated by caspase-6 activation.

MATERIALS AND METHODS

Cell Lines and Cultures. Murine L929 fibroblasts and human SKBR-3 breast carcinoma cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc., Rockville, MA) and McCoy's 5A modified medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml

streptomycin. HER-2/neu transfected MDA-MB-435 cells were grown in Dulbecco's modified Eagle Medium Nutrient Mixture F-12(DMEM/F-12) containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 500 μg/ml of G418 (Stratagene, La Jolla, CA).

ScFv23/TNF Gene Construction. The cDNA encoding the single-chain anti-HER2/neu antibody designated scFv23 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 scFv23-TNF cDNA was constructed by 2-step polymerase chain reaction (PCR). The first step consisted of separate PCR amplification of the scFv23 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 (G_4S) between the scFv23 and TNF.

Expression and Purification of scFv23-TNF Fusion Proteins. The plasmid pET32-23-TNF carrying the sFv23-TNF insert was transformed into Escherichia coli AD494 (DE3) pLysS strain. Single colonies were grown overnight at 37^{0}C in Luria-Bertani broth (Sigma Co., St. Louis, MO) containing 100 μg/ml of ampicillin (Roche Diagnostics Co., Indianapolis, IN), 15 μg/ml of kanamycin (Roche), and 34 μg/ml of chloramphenicol (Roche). Cultures were diluted 25-fold in the same medium and grown at 37^{0}C to an OD_{600} of 0.5. Expression of the target fusion protein was induced with 100 μM isoprophy-β-$_D$-thiogalactopyranoside (IPTG) for 6 hr at 23^{0}C. Cells were collected by centrifugation, resuspended in 100 ml of extraction buffer [10 mM Tris-HCl (pH 8) and 100 μg/ml lysozyme], and incubated with shaking for 30 minutes at 4^{0}C. After

sonication using a Vir Sonic 300 sonicator (Virtus, Gardiner, NY), soluble proteins were harvested by ultracentrifugation (Beckman Coulter, Inc., Fullerton, CA) and loaded onto a TALON metal affinity column (Clontech Laboratories, Palo Alto, CA) equilibrated with 40 mM Tris-HCl (pH 8). Unbound proteins were removed with washing buffer (40 mM Tris-HCl, pH 8, 500 mM NaCl, and 15 mM imidazole). The bound fraction containing scFv23-TNF fusion protein was eluted from the column by addition of elution buffer (40 mM Tris-HCl, pH 8, 500 mM NaCl, and 500 mM imidazole). Eluted protein fractions were pooled and dialyzed against dialysis buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl). To remove tag protein, recombinant enterokinase (rEk; Novage, Madison, WI) was added and incubated overnight at room temperature. After enterokinase treatment, rEk cut scFv 23-TNF was further purified using Q-sepharose resin (Pharmacia Biotech, Uppsala, Sweden) by elution buffer containing 800 mM sodium chloride.

Cytotoxicity Assay. Cells were seeded (1 x 10^4/well) in flat-bottom 96-well microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ) and 24 hr later scFv23-TNF and TNF were added in triplicate wells. After 72 hr, 50 μl of XTT labeling mixture was added to each well, after which the cells incubated for another 4 hr. The spectrophotometrical absorbance was measured at 450 nm using the ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT).

Western Blot Analysis. Cells were washed two times with phosphate buffered saline (PBS) and lysed on ice for 20 min in 0.3 ml of lysis buffer (10 mM Tris-HCl, pH 8, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2% NP-40). Cell lysates were fractionated by 10% SDS-PAGE and transferred on protran nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH).

Membranes were blocked 2 hours in Tris-buffered saline (TBS) containing 3% bovine serum albumin. After incubation at room temperature with either mAb Caspase-3, rabbit anti-caspase-6, rabbit anti-caspase-7, rabbit anti-caspase-8, rabbit anti-TRADD, rabbit anti-TRAF2, rabbit anti-TNFR-1, rabbit anti-IκBα, mAb NFκB(p65), rabbit anti-PARP(Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-p-Akt, and rabbit anti-Akt (Cell signaling Technology, Beverly, MA), membranes were washed in Tris-buffered saline containing 0.2% (v/v) Tween-20 (TBST) and incubated with 1:4000 dilution of goat anti-mouse/goat anti-rabbit or swain anti-goat horseradish peroxidase conjucate (Bio-Rad Laboratories, Hercules, CA) in TBS containing 3% BSA. Membranes were washed four times with TBST and developed with ECL detection reagent (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

RESULTS

Bioactivity of scfv23/TNF and TNF on L-929 Cells. Various concentrations of the scfv23/TNF fusion construct and TNF were applied to log-phase L-929 cells. As shown in Fig. 1, the $I.C._{50}$ of TNF was calculated to be 4.4 pM (SA = 230 U/nmole). In contrast, the biological activity of the scfv23/TNF fusion construct on L-929 cells was found to be lower with an $I.C._{50}$ of 0.76 pM (SA = 13,200 U/nmole).

Biological Activity of scfv23/TNF and TNF on Cells Expressing HER2/neu. The cell line SKBR3 has previously been demonstrated in our lab and others to down-regulate HER2/neu expression after prolonged passage in vitro. Western analysis of HER2/neu expression in the SKBR3 high and low passage cell lines is shown in Fig. 2. There was approximately a 15-fold

decrease in the relative HER2/neu expression levels in the high passage cells (HP) compared to the lower passage lines (LP). Both TNF and scfv23/TNF were added to SKBR3 cells from low passage expressing high levels of HER2/neu and cells from high passage expressing lower levels of HER2/neu. As shown in Fig. 3, cells expressing high levels of HER2/neu were resistant to cytotoxic effects of TNF itself. On the other hand, cells expressing lower levels of HER2/neu demonstrated some sensitivity to TNF, but only at the highest dose levels tested. SKBR3 cells expressing high levels of HER2/neu showed remarkable sensitivity to the cytotoxic effects of the scfv23/TNF fusion construct with an $I.C._{50}$ of approximately 80 nM. In contrast, cells expressing lower levels of HER2/neu(SKBR3) demonstrated an $I.C._{50}$ of approximately 40 nM..

Studies were also performed on human breast tumor cells which were transfected with the HER2/neu oncogene. As shown in Fig. 2, the MDA-MB-435 cells express various levels of HER2/neu depending on the clonal line selected. The parental MDA-MB-435 cells express low, detectable levels of HER2/neu while the transfected clones designated eb3, eb2 and eb1 over-express increasing levels of HER2/neu. As shown in Fig. 4, TNF itself had no demonstratable cytotoxic effects on cells expressing low and high levels of HER2/neu. In contrast, both cell lines appeared to be almost equally sensitive to the cytotoxic effects of the scfv23/TNF fusion construct with an $I.C._{50}$ of approximately 50 nM.. This $I.C._{50}$ was similar to that found for SKBR3 cells.

Differential Effects of TNF and scfv23/TNF on Signal Transduction Events. In order to detail the intracellular events responsible for the observed biological properties of the scfv23/TNF fusion construct compared to native TNF, we next examined various biochemical

events associated with TNF mediation of its cytotoxic effects. We examined the effects of the two agents on TNFR-1, TRADD, TRAF2. As shown in Fig. 5, we treated SKBR3 cells with 50 uM of either TNF or scfv23/TNF at a dose of 50 uM for various times as shown. The cells were harvested and subjected to Western analysis for the various proteins. We found no changes to the levels of the various proteins after treatment with either agent.

We next examined the effects of these agents on the AKT signaling pathway which is an alternative pathway capable of impacting TNF signal transduction events. This pathway and its interactions with the TNF signaling pathway is detailed in Fig. 11. As shown in Fig. 6, treatment of cells with TNF alone resulted in a 2-fold increase in the basal levels of phosphorylated AKT within 5 minutes after drug addition. By 60 minutes, the levels of p-AKT declined to baseline levels. In contrast, treatment with scfv23/TNF had no effect on (or slightly reduced) the levels of p-AKT. After 5 minutes, treatment with TNF was shown to increase the phosphorylation of IkB-a. After 15 minutes, levels of IkB declined in treated cells. One hour after addition of TNF, levels of IkB increased in treated cells. Treatment of cells with scfv23/TNF resulted in no significant change in Ikb levels over time.

The caspase series of proteins is also a central mediator of the apoptotic effects of TNF and other cytokines. We examined the effects of treatment of SKBR3 cells with TNF and scfv23/TNF on caspase 3,6, 7 and 8. As shown in Fig. 7, there was a slight increase in caspase-8 activation 5 minutes after treatment with scfv23/TNF and no discernable increase in caspase-8 after treatment with TNF. Caspase-8 activation declined over time after treatment with both agents. There was no change in caspase-3 or caspase-7 activity after treatment with either agent.

Treatment of cells with TNF caused no significant change in caspase-6 activation. However, 5 minutes after treatment with scfv23/TNF, cells demonstrated a significant (4-fold) increase in caspase-6 concentration within 5 minutes after administration of the scfv23/TNF. This increase persisted until 1 hr after administration when caspase levels declined beck to baseline levels.

DISCUSSION

The impact of HER2 expression on various pro-apoptotic pathways has been carefully examined in several studies ( ). Zhou et al (17) have recently suggested that overexpression of HER2/neu in transfected cells results in endogenous activation of the AKT and NF-kB pathways without extracellular stimulation. The constitutive activation of these pathways appears to result in a blockade in the pro-apoptotic cascade mechanisms responsible for mediation of the cellular cytotoxic response to TNF. In the current study, we did find evidence that the SKBR3 cells which overexpress HER2/neu also had endogenous levels of p-AKT, AKT and Ik-B. This could explain the lack of response we have noted to the cytotoxic effects of TNF. However, since the cytotoxic effects of the scfv23/TNF fusion construct should be mediated through TNF signaling events, we had assumed that cellular resistance to TNF should also manifest in resistance to the scfv23/TNF fusion construct. Our studies clearly demonstrate that both SKBR3 and MDA-MB-435 cells which overexpress Her2/neu and which are resistant to TNF are sensitive to the cytotoxic effects of the scfv23/TNF fusion construct. We therefore proposed that the scfv23/TNF construct demonstrated cytotoxic signal transduction effects which may be different from that of native TNF.

We found that the primary signal transduction event induced by the scfv23/TNF construct different from that of TNF itself appeared to be activation of caspase-6 signaling. TNF alone was incapable of activating this signal. Whether the activation of caspase-6 is the sole difference in signaling between these two agents is unclear. Additionally, it is also unclear whether the activation of caspase-6 is alone responsible for the cytotoxic effects of the scfv23/TNF fusion construct. Vento et al ( ) have suggested that apoptotic induction in retinoblastoma cells by administration of arachadonic acid may occur through caspase-6 activation as a result of caspase-3 activation. Furthermore, studies of apoptosis generated by administration of toxins such as ricin, diphtheria and pseudomonas toxins have demonstrated a role for caspase-3 and caspase-6 mediation of cytotoxic effects. The current study also demonstrated a potential role for caspase-6 in the induction of cytotoxic signaling events of the scfv23/TNF construct. However, we were unable to demonstrate a concordant activation of caspase-3 which is upstream of caspase-6. This may suggest that the scfv23/TNF construct is capable of activating caspase-6 through a caspase-3 independent mechanism.

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FIGURE LEGENDS

Fig. 1: Effects of TNF and scfv23/TNF on the growth of murine fibroblast (L-929) cells.

Fig. 2: Western analysis of HER2/neu expression on various human breast carcinoma cell lines.

Fig. 3: Cytotoxic effects of TNF and scfv23/TNF on log-phase SKBR3 low passage (p1) and high passage (p20) cells

Fig. 4: Cytotoxic effects of TNF and scfv23/TNF on MDA-MB-435 cells transfected with HER2/neu and expressing low (435.neo1) and high (435.eb1) levels of HER2/neu.

Fig. 5: Western analysis of the effects of TNF and scfv23/TNF on the levels of TNFR-1, TRADD and TRAF2 various times after treatment of SKBR3 cells.

Fig. 6: Western analysis of the effects of TNF and scfv23/TNF on the levels of AKT, p-AKT, IkB-a and NfkB various times after treatment of SKBR3 cells.

Fig. 7: Western analysis of the effects of TNF and scfv23/TNF on the levels of caspase-8, caspase-3, caspase-7 and caspase-6 various times after treatment of SKBR3 cells.

Fig. 8: Western analysis of the effects of TNF and scfv23/TNF on expression levels of MMP-9 in SKBR3 cells.

Fig. 9: SDS-PAGE analysis of production of scfv23/TNF in a bacterial expression system.

Fig. 10: Western analysis of the effects of TNF and scfv23/TNF treatment on phosphorylation of HER2/neu in SKBR3 cells.

Fig. 11: Intracellular signal transduction cascade leading to apoptosis.

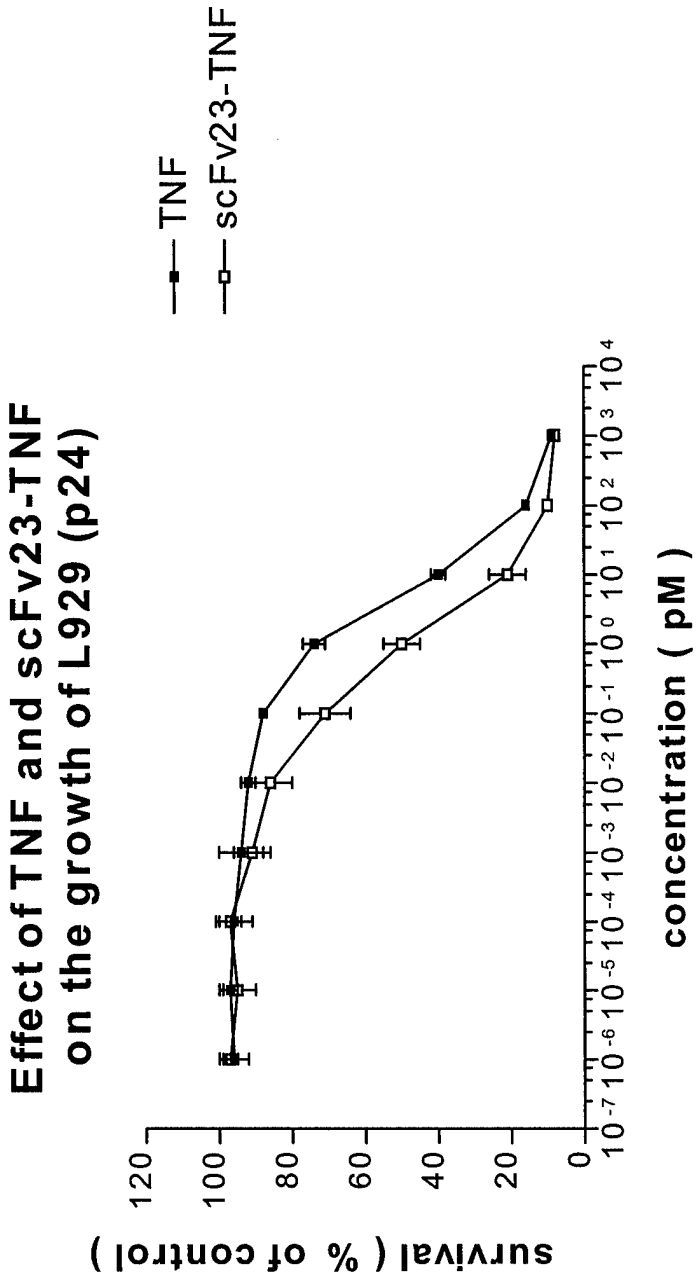

Effect of TNF and scFv23-TNF
on the growth of L929 (p24)
TNF
scFv23-TNF
survival (% of control)
120
100
80
60
40
20
0
concentration (pM)
10-7 10-6 10-5 10-4 10-3 10-2 10-1 100 101 102 103 104

Fig. 1

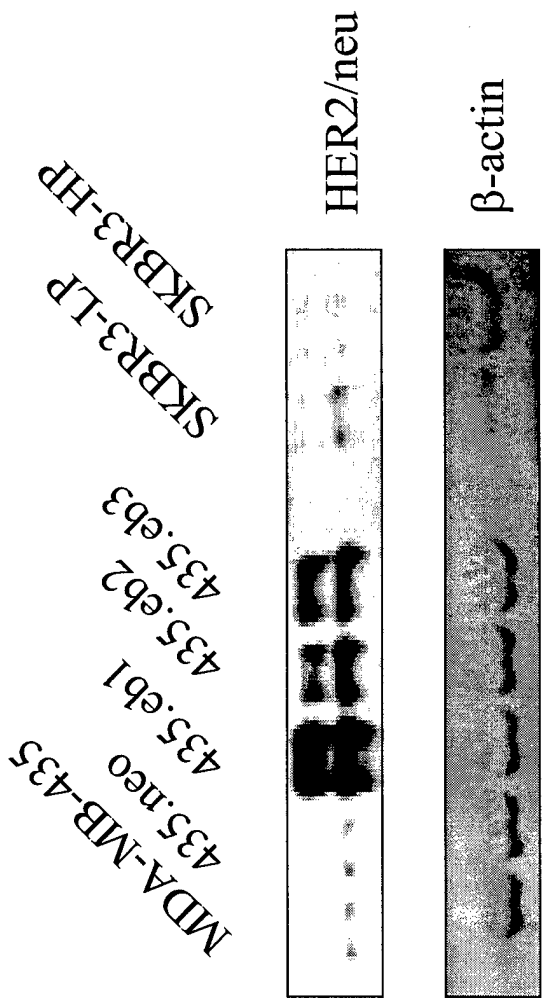

HER2/neu
β-actin
MDA-MB-435
435.neo
435.eb1
435.eb2
435.eb3
SKBR3-LP
SKBR3-HP

Fig. 2

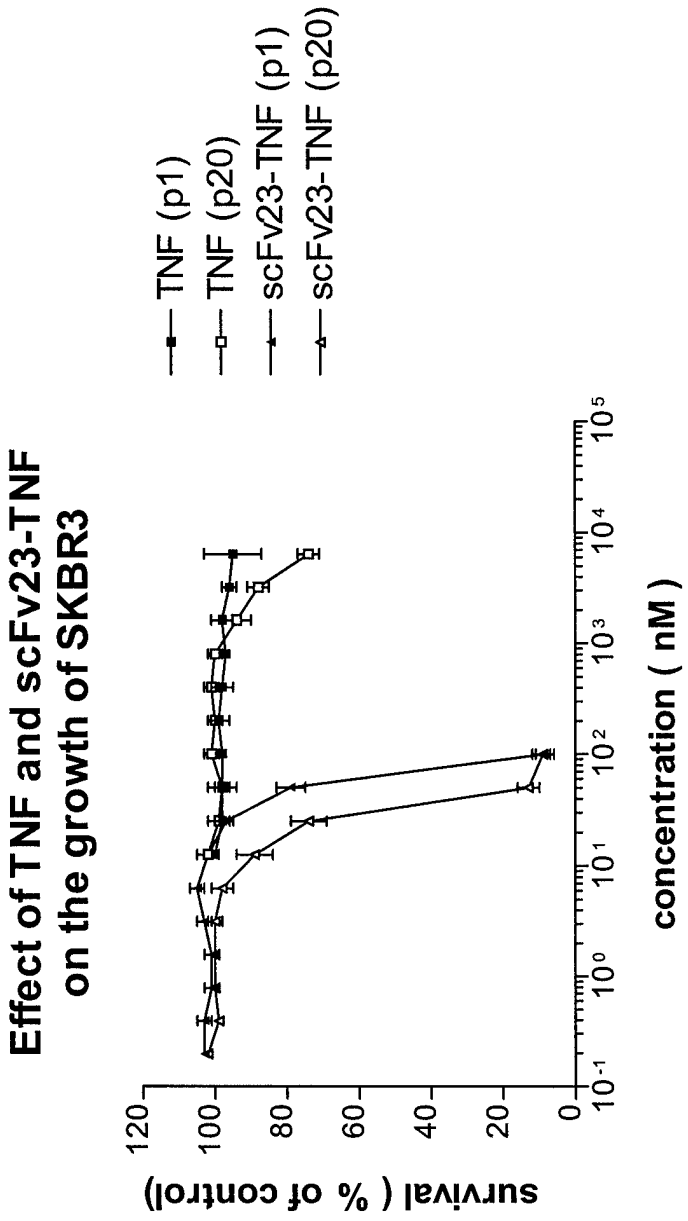

Effect of TNF and scFv23-TNF
on the growth of SKBR3
TNF (p1)
TNF (p20)
scFv23-TNF (p1)
scFv23-TNF (p20)
survival (% of control)
120
100
80
60
40
20
0
10-1
100
101
102
103
104
105
concentration (nM)

Fig. 3

Fig. 4

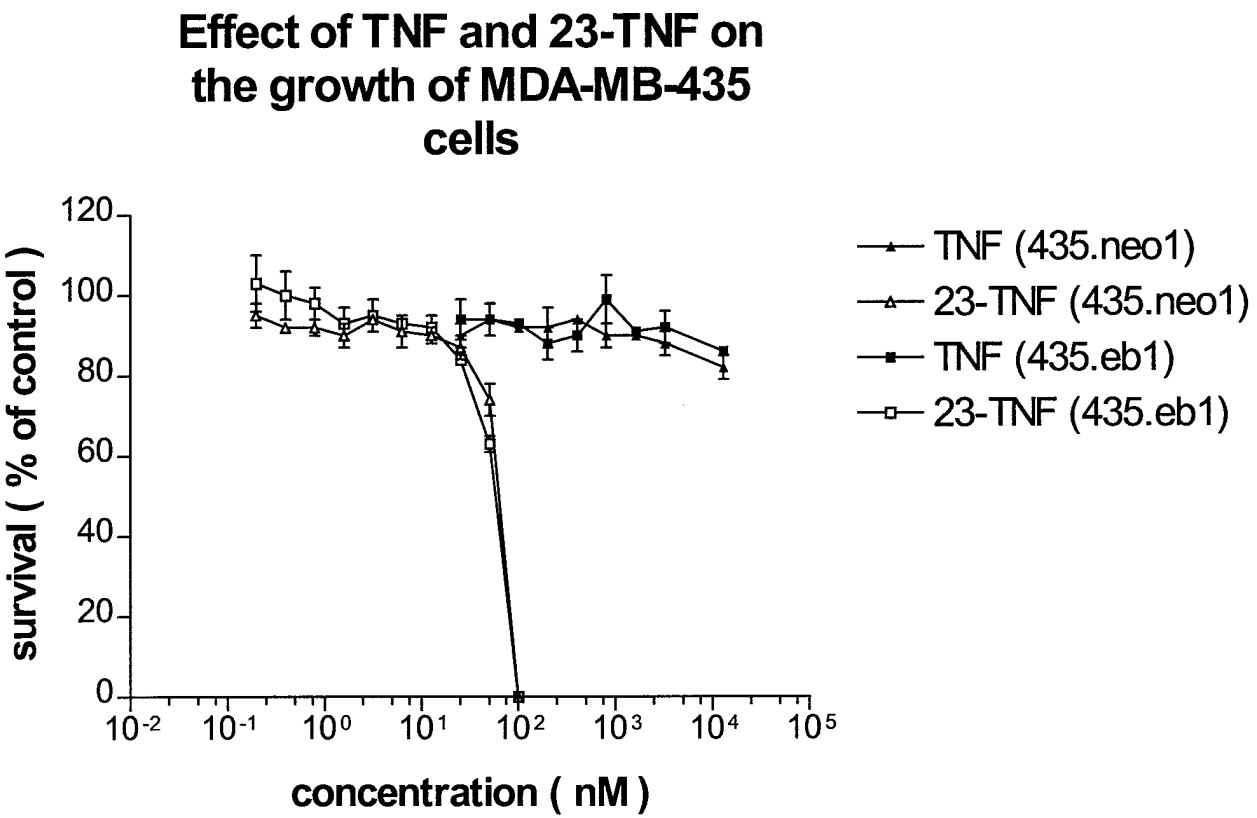

Effect of TNF and 23-TNF on the growth of MDA-MB-435 cells
survival (% of control)
120
100
80
60
40
20
0
10-2
10-1
100
101
102
103
104
105
concentration (nM)
TNF (435.neo1)
23-TNF (435.neo1)
TNF (435.eb1)
23-TNF (435.eb1)

Fig. 5

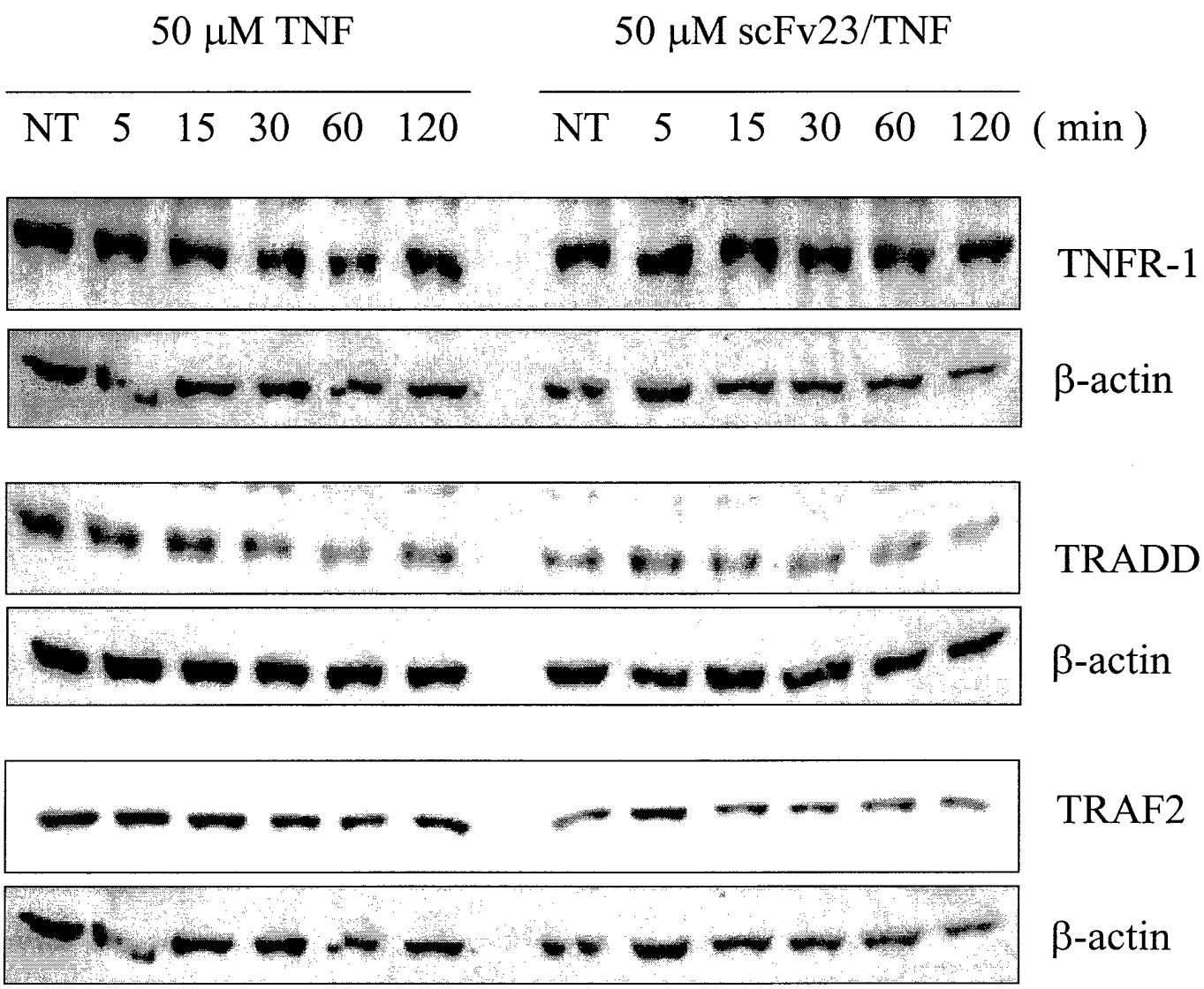

50 μM TNF
50 μM scFv23/TNF
NT 5 15 30 60 120
NT 5 15 30 60 120 (min)
TNFR-1
β-actin
TRADD
β-actin
TRAF2
β-actin

Fig. 6

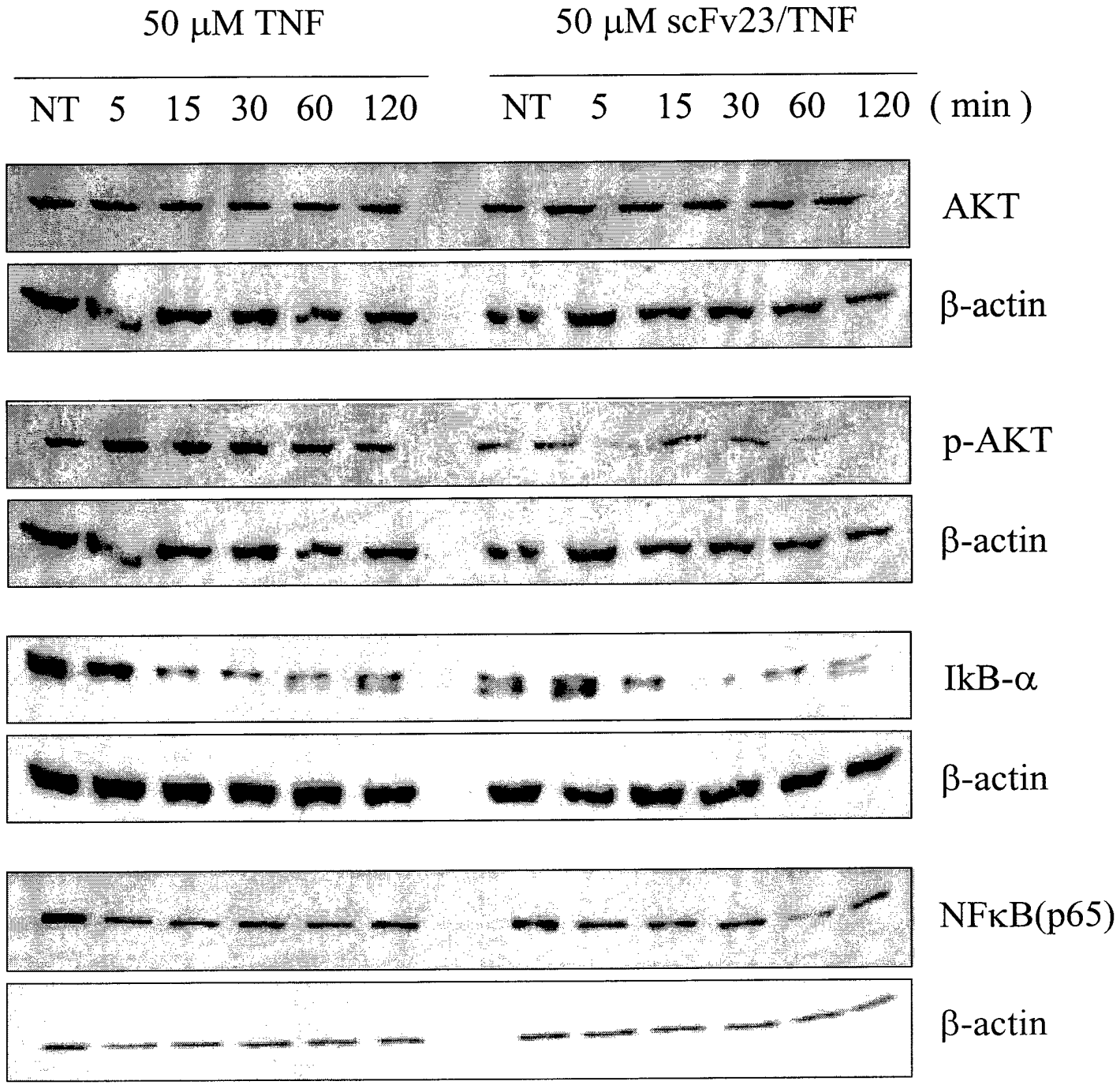
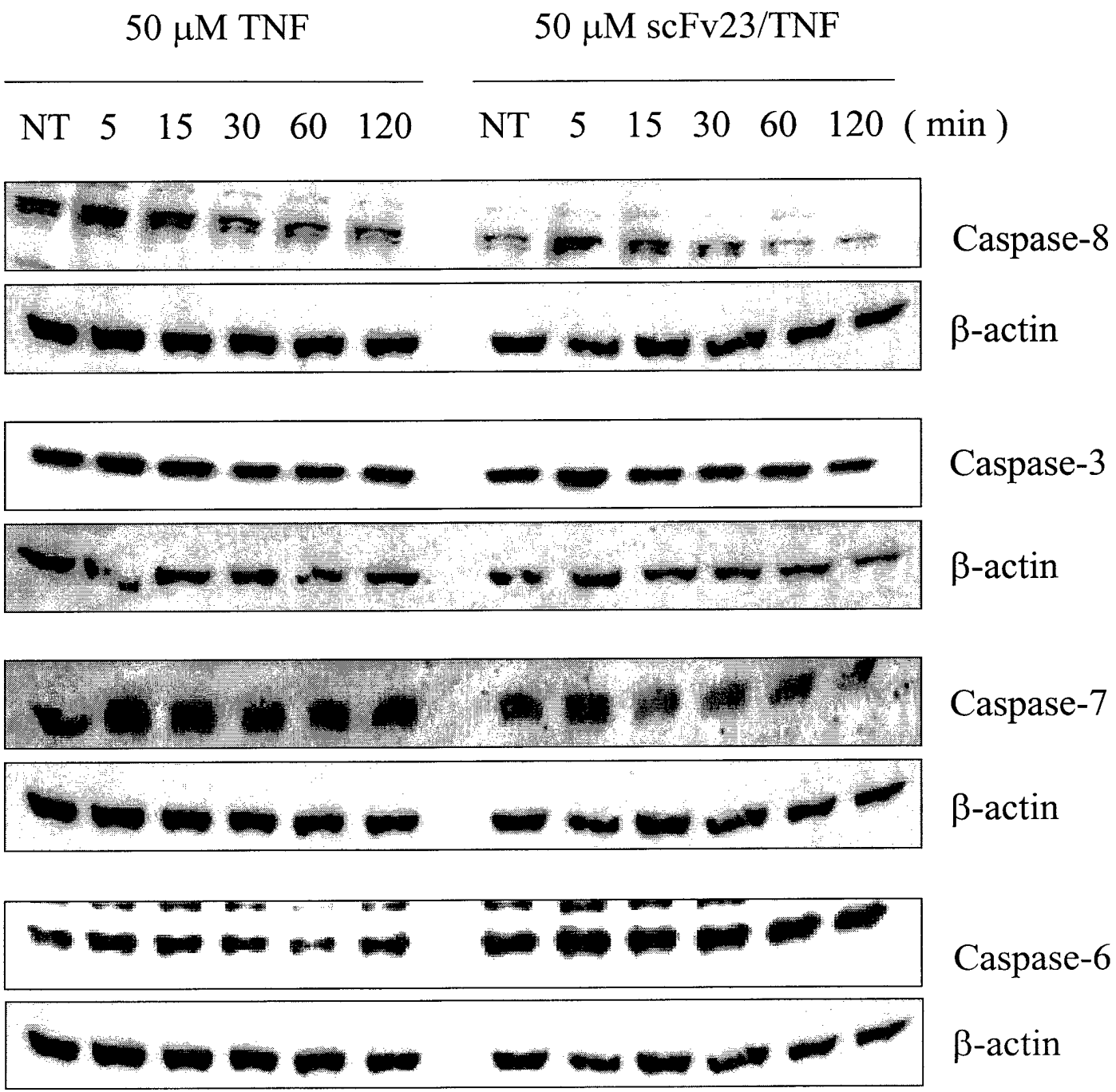

50 μM TNF
50 μM scFv23/TNF
NT 5 15 30 60 120
NT 5 15 30 60 120 (min)
AKT
β-actin
p-AKT
β-actin
IkB-α
β-actin
NFκB(p65)
β-actin

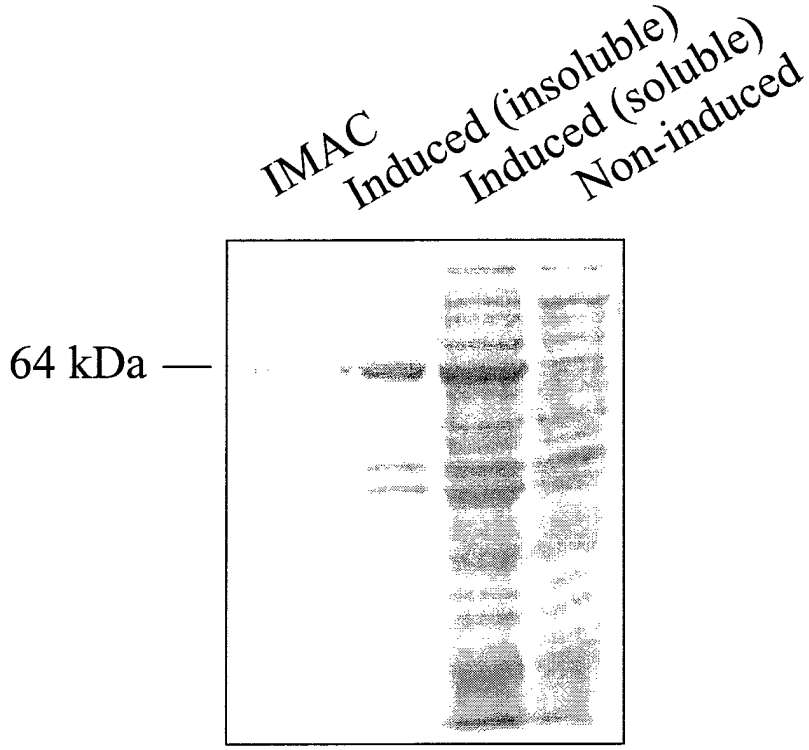
Fig. 7


50 μM TNF
50 μM scFv23/TNF
NT 5 15 30 60 120
NT 5 15 30 60 120 (min)
Caspase-8
β-actin
Caspase-3
β-actin
Caspase-7
β-actin
Caspase-6
β-actin


MMP-9
control
100 nM TNF
50 nM TNF
25 nM TNF
100 nM 23-TNF
50 nM 23-TNF
25 nM 23-TNF

Fig. 8

Fig. 9



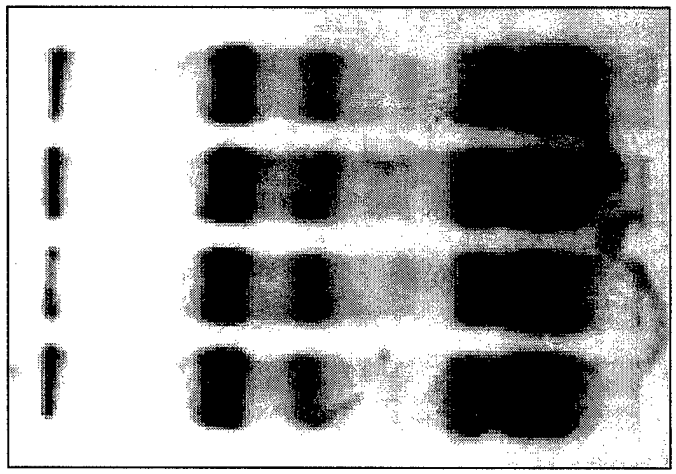
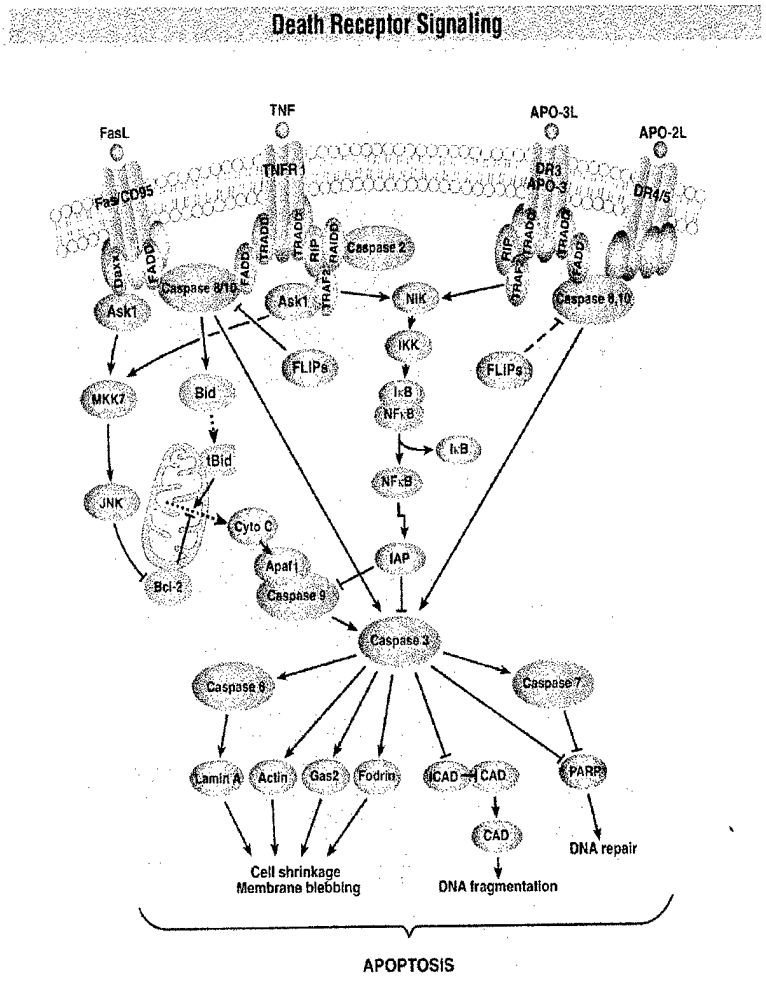

HER2/neu
media only
6400 nM TNT
100 nM scFv23/TNF
100 µg anti-HER2/neu Ab

Fig. 10

Figure 11


Death Receptor Signaling
FasL
TNF
APO-3L
APO-2L
Fas/CD95
TNFR1
DR3
APO-3
DR4/5
DAXX
FADD
TRADD
RIP
TRAF2
RAIDD
Caspase 2
Caspase 8/10
Ask1
NIK
IKK
FLIPs
MKK7
Bid
tBid
IκB
NFκB
JNK
Cyto C
Apaf 1
Bcl-2
IAP
Caspase 9
Caspase 3
Caspase 6
Caspase 7
Lamin A
Actin
Gas2
Fodrin
ICAD
CAD
PARP
DNA repair
Cell shrinkage
Membrane blebbing
DNA fragmentation
APOPTOSIS