The immunocytokine scFv23/TNF sensitizes HER-2/neu–overexpressing SKBR-3 cells to tumor necrosis factor (TNF) via up-regulation of TNF receptor-1

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Abstract

Overexpression of HER-2/neu confers cellular resistance to tumor necrosis factor (TNF)–mediated cytotoxicity to SKBR-3 breast cancer cell lines. To understand the correlation between HER-2/neu expression and TNF resistance, we examined the unique signaling pathways associated with the cytotoxic effects of the immunocytokine scFv23/TNF, recombinant single-chain antibody fusion constructs containing TNF and targeting HER-2/neu, in TNF-resistant SKBR-3-LP cells. We found that treatment of HER-2/neu–overexpressing SKBR-3-LP cells with scFv23/TNF resulted in a 5- to 7-fold higher level of TNF receptor-1 expression 48 hours after exposure. In addition, treatment of SKBR-3-LP cells with scFv23/TNF resulted in down-regulation of Akt phosphorylation and induced apoptosis through cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase. ScFv23/TNF-induced cytotoxicity was inhibited by blocking of the binding of the TNF component of scFv23/TNF to TNF receptor-1 and was dependent on activation of caspase-8 and caspase-3. These results indicate that the immunocytokine scFv23/TNF sensitizes TNF-resistant HER-2/neu–overexpressing SKBR-3-LP cells to TNF-induced apoptosis via the overexpression of TNF receptor-1 and suggest that the overexpression of TNF receptor-1 plays a crucial role in TNF sensitivity in HER-2/neu–overexpressing cancer cells. ScFv23/TNF targeting the HER-2/neu may be an effective cytotoxic agent against HER-2/neu–overexpressing cancer cells, which are inherently resistant to TNF. [Mol Cancer Ther 2005;4(8):1205–13]

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Introduction

The HER-2/neu proto-oncogene is a 185 kDa transmembrane receptor tyrosine kinase, which belongs to the epidermal growth factor family (1–3) and is overexpressed in 20% to 30% of human breast cancers and ovarian cancers (4, 5). HER-2/neu overexpression has been shown to enhance proliferative, prosurvival, and metastatic signals in breast cancer cell lines (6–8) and has been associated with poor prognosis in ovarian, node-positive, and node-negative breast carcinomas (9–12). One of the key roles this oncogene seems to play is in modulation of cellular response to cytotoxic cytokines, such as tumor necrosis factor (TNF; refs. 13, 14). Numerous research groups have shown that both naturally HER-2/neu–overexpressing cells and HER-2/neu–transfected cells are resistant to the cytotoxic effects of TNF (15, 16). Because TNF plays a central role in immune surveillance functions (17), resistance to its cytotoxic effects mediated by HER-2/neu overexpression in breast cancer may allow transformed cells a growth advantage by escaping host defense mechanisms.

The use of antibodies for the specific delivery of cytokines to tumor cells has been shown by numerous groups using TNF, IFN, interleukin 2, and lymphotoxin (18–20). Several of these studies have shown that the antibody-targeted cytokine was more effective than the original cytokine. With the use of an antibody construct containing TNF, we initially showed that delivery of TNF to tumor cells, using first chemical conjugates and then recombinant single-chain antibody fusion constructs containing TNF and targeting gp240 (ZME/TNF) and HER-2/neu (scFv23/TNF), could overcome resistance of tumor cells to TNF *in vitro* (18, 21, 22). Previous studies showed the production, purification, and biological characterization of the scFv23/TNF construct against HER-2/neu–overexpressing breast SKBR-3 (22). However, the underlying mechanisms which may account for these observations have remained largely unknown. In this report, we examined mechanisms accounting for the differences in cytotoxicity of TNF itself and the scFv23/TNF immunocytokine by analyzing differences in the cellular response and signal transduction properties of these actions on SKBR-3-LP cells expressing high levels of HER-2/neu. These studies suggest that scFv23/TNF can sensitize TNF-resistant SKBR-3-LP cells to the cytotoxic effect of TNF primarily through modulation of TNF receptor-1 (TNF-R1).

Materials and Methods

Materials

Monoclonal anti–HER-2/neu antibody, rabbit polyclonal anti–TNF-R1 antibody, rabbit polyclonal anti–TNF receptor-2

(TNF-R2) antibody, rabbit polyclonal anti–caspase-8 antibody, monoclonal anti–caspase-3 antibody, monoclonal anti–poly(ADP-ribose) polymerase antibody, rabbit polyclonal anti–TNF receptor-associated death domain (TRADD) antibody, rabbit polyclonal anti–TNF-receptor-associated factor 2 (TRAF2) antibody, and rabbit polyclonal anti–IκB-α antibody were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti–phospho-Akt antibody and rabbit polyclonal anti-Akt antibody (Cell Signaling Technology, Beverly, MA) were used for Western blot analysis. For inhibition assays, recombinant human TNF-R1:Fc fusion protein was purchased from Alexis (San Diego, CA). The general caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-3 inhibitor (z-DEVD-fmk) were purchased from R&D Systems (Minneapolis, MN). Herceptin was purchased form Genentech (South San Francisco, CA). The sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) cell growth assay kit was purchased from Roche Diagnostics Co. (Indianapolis, IN).

Cell Lines and Culture

SKBR-3 cells were grown in McCoy's 5A modified medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. SKBR-3 low passage cells expressing high amounts of HER-2/neu (SKBR-3-LP) used in our study were between passage 5 and 8 whereas the SKBR-3 high passage cells used were between passage 40 and 45 and displayed comparatively lower levels of HER-2/neu (SKBR-3-HP). The L3.6pl human pancreatic cancer cell line was kindly provided by Dr. Killian (M.D. Anderson Cancer Center, Houston, TX) and was grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

In vitro Cytotoxicity Assays

SKBR-3 cells were seeded (1×10^4/well) in flat-bottomed 96-well microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ) and 24 hours later, scFv23, TNF, scFv23/TNF, or Herceptin (Genentech) was added in triplicate wells. To examine the effect of caspase inhibitor on the cytotoxicity of scFv23/TNF, SKBR-3-LP cells were pretreated with or without 200 μmol/L general caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), or caspase-3 inhibitor (z-DEVD-fmk; R&D Systems) for 2 hours and then treated with various concentrations of scFv23/TNF. After 72 hours, 50 μL of XTT labeling mixture (Roche) were added to each well; after which the cells were incubated for another 4 hours. The spectrophotometric absorbance was measured at 450 nm using an ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT).

Assessment of the Role of TNF-R1 in scFv23/TNF Cytotoxicity

SKBR-3 cells were seeded (1×10^4/well) in flat-bottomed 96-well microtiter plates (Becton Dickinson Labware) and 24 hours later were pretreated with recombinant human TNF-R1:Fc fusion protein (Alexis) for 2 hours, and then treated with TNF, Herceptin (Genentech), or scFv23/TNF added in triplicate wells. After incubation for 72 hours, cell viability was detected by XTT assay (Roche).

Detection of Apoptosis

The development of apoptotic cell death was detected by DNA fragmentation and by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay. To evaluate DNA fragmentation, SKBR-3-LP cells were seeded at 5×10^5 cells/60 mm Petri dish, allowed to adhere overnight, and then treated with 200 nmol/L TNF or 200 nmol/L scFv23/TNF. After 24 and 48 hours of exposure, cells were washed with PBS, resuspended in a DNA extraction buffer [5 mmol/L Tris-HCl (pH 8), 50 mmol/L EDTA, 10 μg/mL RNase, 0.25% SDS], and then incubated for 1 hour at 37°C. To remove protein, resuspended cell lysates were treated with 100 μg/mL proteinase K for 3 hours at 50°C. DNA was extracted using phenol and chloroform followed by ethanol precipitation. The genomic DNA was resuspended in Tris-EDTA (pH 8) and was fractionated by electrophoresis on a 1% agarose gel containing ethidium bromide.

To assess apoptosis using terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay, SKBR-3-LP cells were plated on glass coverslips, allowed to adhere overnight, and then treated with 200 nmol/L TNF or 200 nmol/L scFv23/TNF for 24 and 48 hours. The cells were washed with PBS, permeabilized (0.1% Triton X-100, 0.1% sodium citrate), and then fixed in 4% paraformaldehyde. Fixed cells were stained with an *in situ* cell death detection kit (Roche). Cells undergoing apoptosis were identified by fluorescence microscopy (Nikon, Tokyo, Japan).

Western Blot Analysis

SKBR-3 and L3.6pl cell lines were seeded at 5×10^5 cells/60 mm Petri dish, allowed to grow overnight, and then treated with 200 nmol/L scFv23, 200 nmol/L TNF, 200 nmol/L scFv23/TNF, or 10 mg/mL of Herceptin. After treatment, cells were washed twice with PBS and lysed on ice for 20 minutes in 0.3 mL lysis buffer [10 mmol/L Tris-HCl (pH 8), 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 0.2% NP40]. Cell lysates (50 μg) were fractionated by 8% to 15% SDS-PAGE and electrophoretically transferred to Immobilon-P nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). Membranes were blocked for 2 hours in TBS containing 3% bovine serum albumin and then probed with various antibodies [monoclonal anti–HER-2/neu antibody, rabbit polyclonal anti–TNF-R1 antibody, rabbit polyclonal anti–TNF-R2 antibody, rabbit polyclonal anti–caspase-8 antibody, monoclonal anti–caspase-3 antibody, monoclonal anti–poly (ADP-ribose) polymerase (PARP) antibody, rabbit polyclonal anti-TRADD antibody, rabbit polyclonal anti-TRAF2 antibody, rabbit polyclonal anti–IκB-α antibody, rabbit polyclonal anti–phospho-Akt antibody, and rabbit polyclonal anti-Akt antibody]. Goat anti-mouse,

goat anti-rabbit, or swain anti-goat antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) were used to visualize immunoreactive proteins at 1:4,000 dilution using enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Data are presented as the relative density of protein bands normalized to β-actin. The intensity of the bands was quantified with the use of histogram.

Results

Sensitivity to scFv23/TNF and Correlation with HER-2/neu, TNF-R1, and TNF-R2 Expression

Studies in our lab have previously shown that the human breast cancer cell line SKBR-3 seems to down-regulate HER-2/neu cellular expression after prolonged passage *in vitro*. Western blot analysis (Fig. 1A) confirms that high passage cells (SKBR-3-HP, passage >40) express 6-fold lower levels of HER-2/neu compared with low passage cells (SKBR-3-LP, passage <10). In addition, SKBR-3-HP cells also expressed 2.3-fold higher levels of TNF-R2 but equivalent levels of TNF-R1. We next evaluated the response of these two cell lines to the cytotoxic effects of Herceptin, scFv23/TNF, or TNF. Compared with SKBR-3-HP cell lines expressing low levels of HER-2/neu, SKBR-3-LP cells expressing high levels of HER-2/neu were more sensitive to the cytotoxic effects of Herceptin. On the other hand, SKBR-3-HP cells were more sensitive to the cytotoxic effects of TNF compared with SKBR-3-LP cells, thus confirming results from previous studies that HER-2/neu overexpression correlates with resistance to TNF. In contrast, both SKBR-3 cell lines showed virtually identical sensitivity to scFv23/TNF (Fig. 1B). These results suggest that continual culture of the SKBR-3 cell line results in down-regulation of HER-2/neu and a concomitant up-regulation of the TNF-R2. Studies by other groups (23, 24) indicate that TNF-R1 is primarily responsible for mediating a TNF cytotoxic signal. It is unclear whether these observations are causally related or correlated with cellular resistance to the cytotoxic effects of TNF in SKBR-3 cells. However, these data suggest that the scFv23/TNF immunocytokine can overcome TNF cellular resistance associated with HER-2/neu overexpression. Furthermore, the significant differences we observed in biological activity between scFv23/TNF and TNF itself on SKBR-3-LP cells afforded us an opportunity to compare mechanistic pathways which may be responsible for these observations.

The Role of TNF-R1 in scFv23/TNF-Induced Growth Inhibition

To determine whether the cytotoxic effects of scFv23/TNF were mediated through interaction with the cell-surface TNF-R1, we specifically blocked the binding of the TNF component of the scFv23/TNF fusion construct to TNF-R1 using TNF-R1:Fc fusion protein. As shown in Fig. 2, addition of TNF-R1:Fc was able to abrogate scFv23/TNF or TNF-induced cytotoxicity, but not Herceptin-induced cytotoxicity, on SKBR-3-LP or SKBR-3-HP cells. Inhibition of the cytotoxic effects of scFv23/TNF was directly

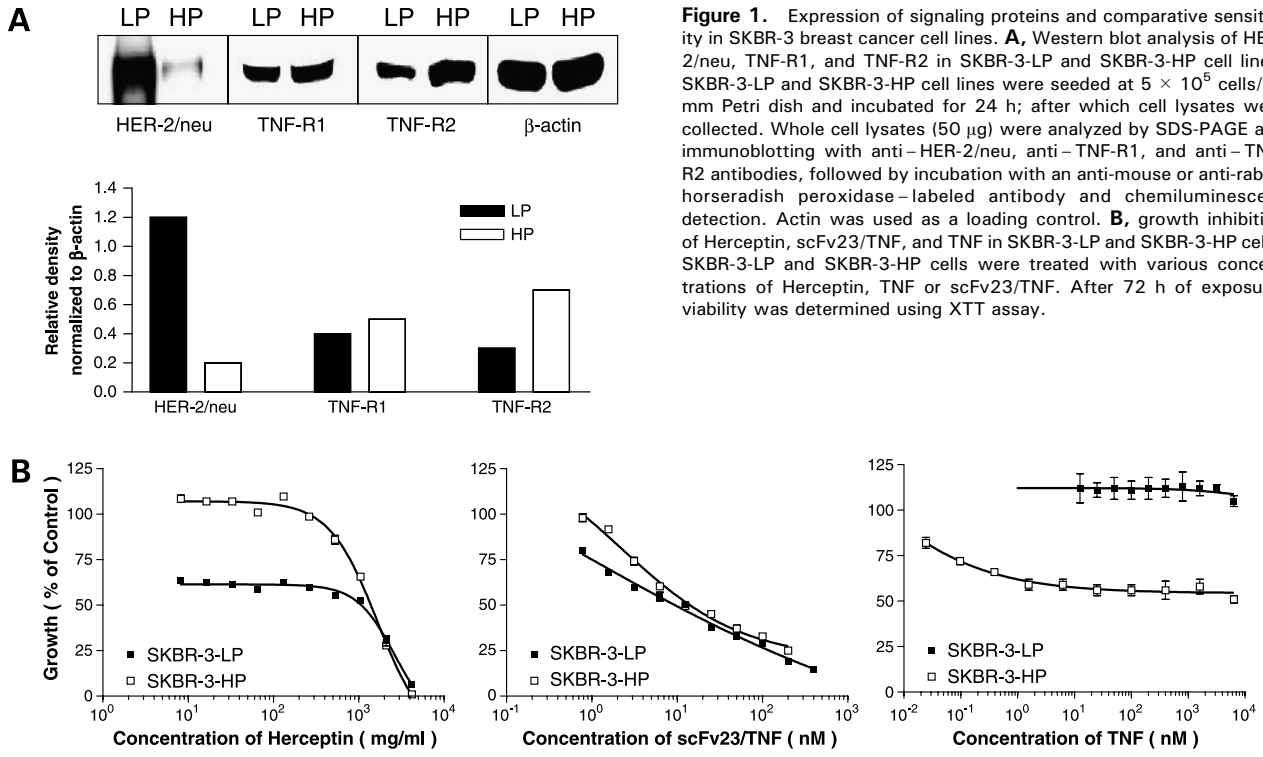

Figure 1. Expression of signaling proteins and comparative sensitivity in SKBR-3 breast cancer cell lines. **A,** Western blot analysis of HER-2/neu, TNF-R1, and TNF-R2 in SKBR-3-LP and SKBR-3-HP cell lines. SKBR-3-LP and SKBR-3-HP cell lines were seeded at 5×10^5 cells/60 mm Petri dish and incubated for 24 h; after which cell lysates were collected. Whole cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti–HER-2/neu, anti–TNF-R1, and anti–TNF-R2 antibodies, followed by incubation with an anti-mouse or anti-rabbit horseradish peroxidase–labeled antibody and chemiluminescent detection. Actin was used as a loading control. **B,** growth inhibition of Herceptin, scFv23/TNF, and TNF in SKBR-3-LP and SKBR-3-HP cells. SKBR-3-LP and SKBR-3-HP cells were treated with various concentrations of Herceptin, TNF or scFv23/TNF. After 72 h of exposure, viability was determined using XTT assay.

dependent on the concentration of TNF-R1:Fc fusion protein added. Our results clearly suggest that scFv23/TNF-induced cytotoxicity is principally mediated by interaction with the cell surface TNF-R1.

Effect of scFv23/TNF on TNF-R Expression

We next examined whether the immunocytokine scFv23/TNF can modulate the cellular expression of TNF-R1. We treated SKBR-3-LP cells with scFv23, TNF, scFv23/TNF, or Herceptin. Treatment of SKBR-3-LP cells with either scFv23/TNF or scFv23 antibody alone induced up-regulation of TNF-R1 expression in a time-dependent fashion. This seemed to be an effect of the scFv23 component because TNF treatment reduced the levels of TNF-R1. Because scFv23/TNF treatment was found to induce a 5-fold increase in TNF-R1 expression in SKBR-3-LP cells, we next investigated whether the scFv23/TNF-mediated up-regulation of TNF-R1 was restricted to a particular type of tumor cell. We examined the effect of scFv23/TNF on TNF-resistant, HER-2/neu–overexpressing L3.6pl human pancreatic cancer cells. Treatment of L3.6pl cells with scFv23/TNF was also found to dramatically induce up-regulation of TNF-R1 in a manner identical to that found for SKBR-3-LP cells (Fig. 3A). These results suggest that the cellular expression level of TNF-R1 may be directly correlated with the cytotoxic effect of scFv23/TNF in TNF-resistant, HER-2/neu–overexpressing tumor cell lines.

In addition to the use of scFv23/TNF, we next examined whether other HER-2/neu targeting molecules such as Herceptin can modulate the expression of TNF-R1 or TNF-R2 on HER-2/neu–overexpressing SKBR-3-LP cells. As shown in Fig. 3B, we found that treatment with scFv23/TNF or Herceptin had no effect on the expression of TNF-R2 whereas Herceptin induced 1.8-fold and scFv23/TNF induced 7.3-fold higher expression of TNF-R1 compared with controls. This result clearly suggests that the expression and function of TNF-R1, but not of TNF-R2, are involved in TNF resistance in HER-2/neu–overexpressing SKBR-3-LP cells.

With the observation that the anti–HER-2/neu single-chain antibody (scFv23) can induce up-regulation of TNF-R1, we next examined the effect of scFv23 on cell growth. We treated SKBR-3-LP cells with scFv23 alone or in combination with TNF and compared this with TNF or scFv23/TNF cytotoxicity. We found that the combination of scFv23 and TNF was much more cytotoxic than TNF alone and was similar to the effect of the scFv23/TNF fusion construct against TNF-resistant SKBR-3-LP (Fig. 4). Therefore, our results suggest that the induction of TNF-R1 expression by treatment with either scFv23 or scFv23/TNF plays a crucial role in regulating TNF sensitivity in HER-2/neu–overexpressing cancer cells.

Effect of scFv23/TNF on Survival Pathways

After binding to TNF-R1, TNF exerts dualistic biological functions by activating both survival pathways and apoptotic pathways. Activation of TNF-R1 results in activation of nuclear factor κB (degradation of IκB-α) and induction of nuclear factor κB–regulated antiapoptotic factors by a pathway including TRADD and TRAF2 (25). To determine whether the TNF component of scFv23/TNF can activate antiapoptotic pathways compared with native TNF, we treated HER-2/neu–overexpressing SKBR-3-LP cells with 200 nmol/L of scFv23, TNF, or scFv23/TNF for various times, harvested cells, and subjected cell lysates to Western blot analysis. As shown in Fig. 5, treatment with

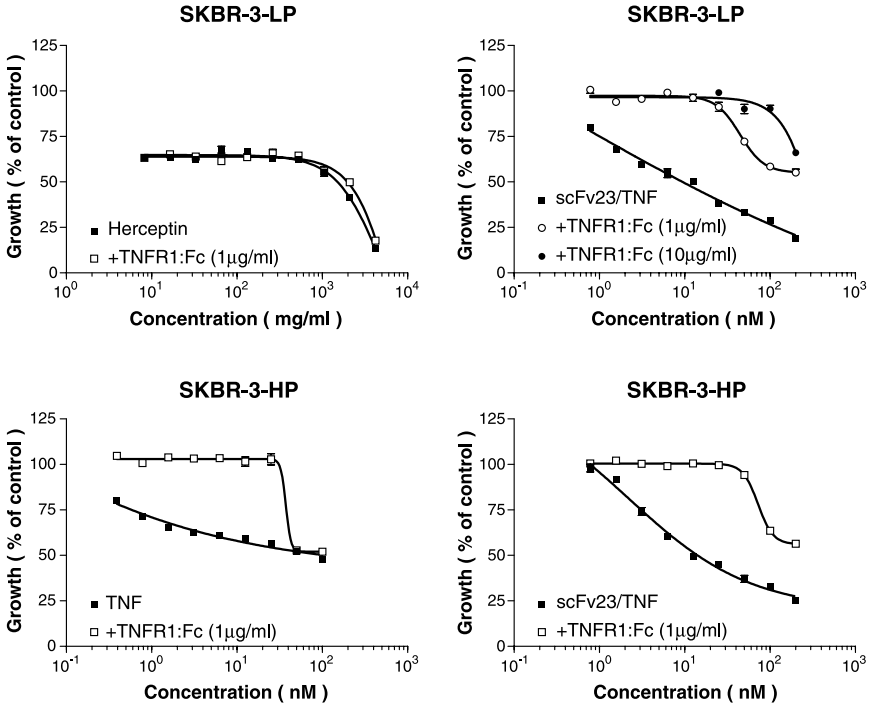

Figure 2. Role of TNF receptor in scFv23/TNF-induced growth inhibition. To determine whether the cytotoxic effects of scFv23/TNF, Herceptin, or TNF were mediated entirely through interaction with cell surface TNF-R1, we blocked the binding of scFv23/TNF to TNF-R1 using TNF-R1:Fc fusion protein (1 and 10 μg/mL). After 72 h of exposure, viability was determined using an XTT assay.

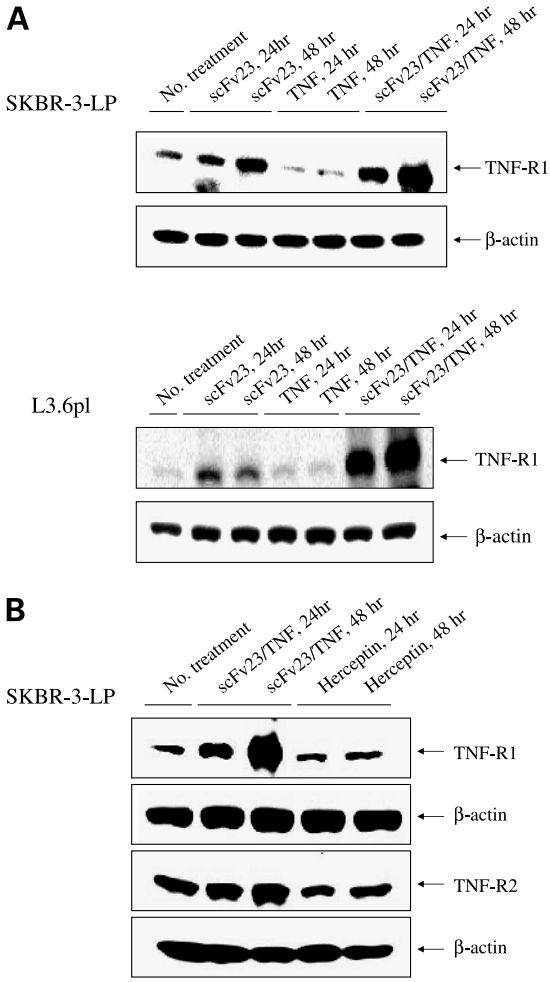

Figure 3. Effect of scFv23/TNF on modulation of TNF-R1 expression. To determine whether scFv23/TNF can modulate the expression of TNF-R1, we treated SKBR-3-LP and L3.6pl cells with scFv23, TNF, scFv23/TNF (**A**), or Herceptin (**B**). Whole cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti–TNF-R1 and anti–TNF-R2 antibodies, followed by incubation with an antimouse or antirabbit horseradish peroxidase–labeled antibody and chemiluminescent detection. Actin was used as a loading control.

scFv23/TNF for 180 minutes resulted in a modest decrease in TRADD. Treatment of cells with scFv23, TNF, or scFv23/TNF had no effect on the levels of TRAF2. On the other hand, after 30 minutes of treatment, IκB-α was degraded in both TNF- and scFv23/TNF-treated cells; treatment with scFv23 had no effect on IκB-α degradation. This suggests an effect related to the TNF component of the scFv23/TNF construct. Three hours after addition of either TNF or scFv23/TNF, levels of IκB-α seemed to increase back to basal levels. These results suggest that the IκB-α pathway may be involved in scFv23/TNF-mediated signaling transduction and this effect did not seem to be an effect of the scFv23 component of the scFv23/TNF construct.

Overexpression of HER-2/neu has been shown to result in activation of different downstream pathways such as Akt kinase pathway, which leads to cell proliferation and

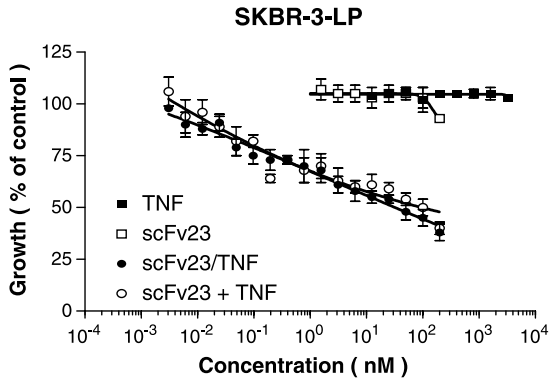

Figure 4. Modulation of TNF sensitivity in SKBR-3-LP cells. To determine whether the overexpression of TNF-R1 can modulate the TNF sensitivity in HER-2/neu–overexpressing SKBR-3-LP cells, SKBR-3-LP cells were treated with TNF, scFv23, scFv23/TNF, or TNF in combination with scFv23. After 72 h of exposure, viability was determined using XTT assay.

cell survival. To determine whether scFv23/TNF treatment affects the Akt survival pathway, SKBR-3-LP cells were treated with scFv23, TNF, or scFv23/TNF. The activation of Akt kinase was then assessed by Western blot analysis using antibodies to Akt and phospho-Akt. As shown in Fig. 5, treatment with either scFv23 or TNF had no effect on the total cellular content or the phosphorylation of Akt. On the other hand, treatment of cells with scFv23/TNF resulted in down-regulation of phosphorylated Akt after 30 minutes of drug administration. This suggests that scFv23/TNF can apparently modulate this survival pathway. This seems to be specific for the scFv23/TNF construct because neither scFv23 nor TNF treatment alone had any significant effect.

Effects of scFv23/TNF on Apoptotic Pathways

After binding to TNF receptors on the tumor cell surface, the cytotoxic effects of TNF-α can be directly mediated by

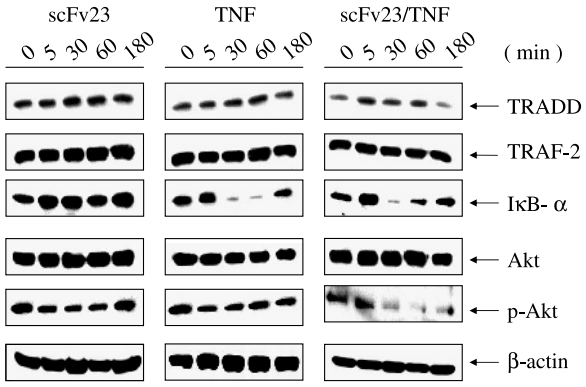

Figure 5. Effects of scFv23, TNF, and scFv23/TNF on the expression of TRADD, TRAF2, IκB, Akt, and phospho-Akt. SKBR-3-LP cells were treated at the indicated times with 200 nmol/L scFv23, 200 nmol/L TNF, or 200 nmol/L scFv23/TNF. After treatment, cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti-TRADD, anti-TRAF2, anti-IκB, anti-Akt, or anti–phospho-Akt antibodies, followed by incubation with an antirabbit horseradish peroxidase–labeled antibody and chemiluminescent detection. Actin was used as a loading control.

activating signaling pathways that initiate programmed cell death (26, 27). To determine whether the cytotoxic effect of scFv23/TNF was associated with apoptosis compared with native TNF, DNA was extracted from SKBR-3-LP cells at 24 and 48 hours after exposure to either 200 nmol/L TNF or scFv23/TNF. The DNA was subjected to electrophoresis on a 1% agarose gel. A DNA fragmentation pattern characteristic of apoptosis was detected in scFv23/TNF-treated but not in TNF-treated SKBR-3-LP cells (Fig. 6A). SKBR-3-LP cells were also assayed for apoptosis by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end-labeling staining. As shown in Fig. 6B, scFv23/TNF-treated cells showed DNA fragmentation as well as nuclear condensation typical of apoptotic cell death at 48 hours of exposure.

The caspase series of proteins is known to be a central mediator of the apoptotic effects of TNF and other cytokines. To determine whether caspase-8 and caspase-3 were activated in SKBR-3-LP cells during scFv23/TNF-induced cell death, we investigated the cleavage of caspase-8, caspase-3, and its substrate poly(ADP-ribose) polymerase. Treatment with TNF had no effect on caspase-8, caspase-3, and PARP cleavage. In contrast, treatment with scFv23/TNF resulted in cleavage of caspase-8, caspase-3, and PARP at 48 hours (Fig. 7). To determine whether scFv23/TNF-induced apoptosis was dependent on activation of the caspase-8 and caspase-3 pathway, we examined the effect of caspase inhibitors on the cytotoxicity of scFv23/TNF against SKBR-3-LP cells. Figure 8 shows that scFv23/TNF-induced cytotoxicity was inhibited by general caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk), and caspase-3 inhibitor (z-DEVD-fmk). This result illustrates that scFv23/TNF elicits an apoptotic response which seems to be mediated, at least in part, through a caspase-8 and caspase-3–dependent cascade.

Discussion

Overexpression of HER-2/neu seems to be associated with a survival advantage and with TNF resistance in breast, ovarian, and HER-2/neu–transfected cell lines (14–16). On the other hand, down-regulation of HER-2/neu has been shown to confer enhanced sensitivity to the cytotoxicity of TNF in doxorubicin-resistant tumor cell lines (28). Studies have additionally shown that epidermal growth factor signaling in breast and cervical carcinoma cells can also modulate the cytotoxic effects of TNF (29).

In our study, we observed that scFv23/TNF, composed of the anti–HER-2/neu single-chain antibody fused to TNF, can overcome HER-2/neu–induced TNF resistance in HER-2/neu–overexpressing SKBR-3-LP cells. The results of our study suggest that TNF-R1 expression, caspase activation, and Akt phosphorylation are three critical factors that contribute to scFv23/TNF-induced cytotoxicity in TNF-resistant HER-2/neu–overexpressing SKBR-3-LP cells.

First, a critical factor in the mediation of scFv23/TNF cytotoxicity seems to be modulation of TNF-R1. Amplification of the HER-2/neu oncogene has been shown to lead to resistance of NIH 3T3 cells to TNF and this correlates with down-regulation of TNF receptor binding (16). The down-regulation of TNF-binding capacity by protein kinase C has also been shown to be associated with a decrease in TNF

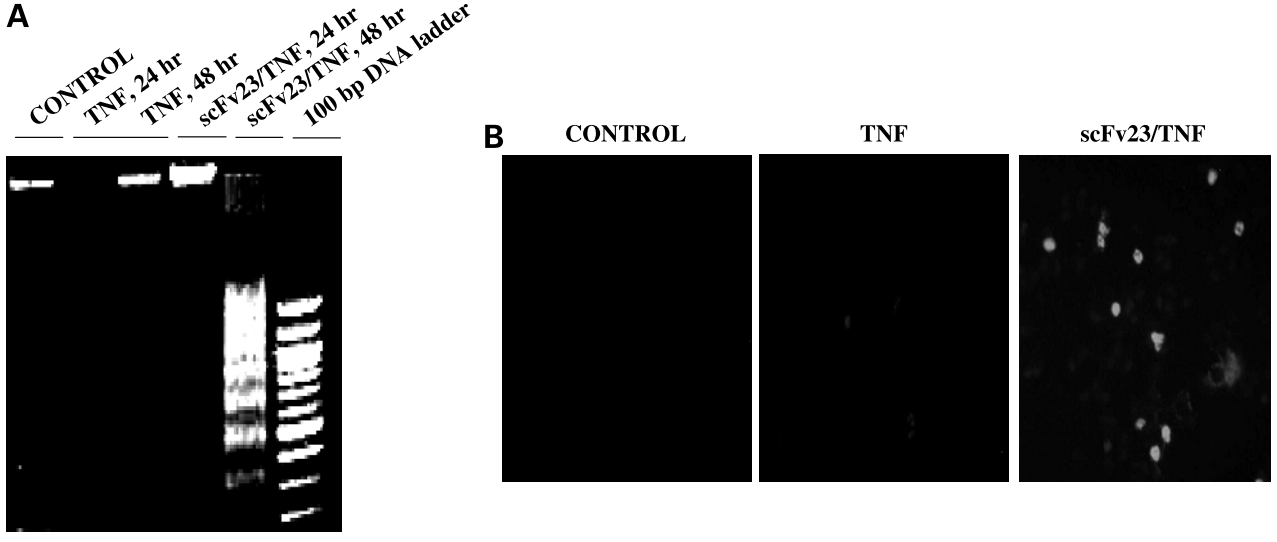

Figure 6. Effects of scFv23/TNF on apoptosis in HER-2/neu – overexpressing SKBR-3-LP cells. **A,** DNA fragmentation of apoptotic cells. SKBR-3-LP cells exposed to 200 nmol/L TNF or 200 nmol/L scFv23/TNF for 24 and 48 h were lysed. DNA was extracted, fractionated by electrophoresis, and stained with ethidium bromide. **B,** microscopic analysis of apoptotic cells. SKBR-3-LP cells were exposed to 200 nmol/L TNF or 200 nmol/L scFv23/TNF for 48 h. After treatment, the cells were washed with PBS, permeabilized in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate), and then fixed in 4% paraformaldehyde. Fixed cells were stained with *in situ* cell death detection kit (Roche). Cells undergoing apoptosis were determined by fluorescence microscopy (×200).

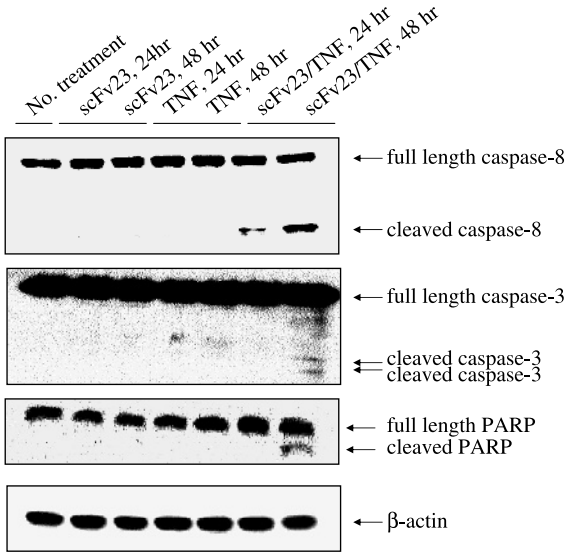

Figure 7. Effects of scFv23, TNF, and scFv23/TNF on the activation of caspase-8, caspase-3, and PARP cleavage. SKBR-3-LP cells were treated at the indicated times with 200 nmol/L scFv23, 200 nmol/L TNF, or 200 nmol/L scFv23/TNF for 24 and 48 h. After treatment, cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti–caspase-8, anti–caspase-3, and anti–PARP antibodies, followed by incubation with an anti-mouse horseradish peroxidase–labeled antibody and chemiluminescent detection. Actin was used as a loading control.

sensitivity (30). Therefore, we examined the effect of scFv23/TNF on the expression of TNF-R1. We observed that scFv23/TNF could induce up-regulation of TNF-R1 in a time-dependent fashion and blocking of the binding of scFv23/TNF to TNF-R1 was able to abrogate scFv23/TNF-induced cytotoxicity, suggesting that the immunocytokine scFv23/TNF sensitizes TNF-resistant HER-2/neu–overexpressing SKBR-3-LP cells to TNF via the modulation of TNF-R1. The TNF-mediated down-regulation of HER-2/neu in pancreatic tumor cells has been shown to be associated with an increase in TNF sensitivity (31). In the current study, we observed that treatment of SKBR-3-LP cells with scFv23/TNF resulted in the inhibition of HER-2/neu phosphorylation at 48 hours of exposure whereas treatment with TNF had no effect on the HER-2/neu phosphorylation (data not shown). We therefore suggest that the down-regulation of HER-2/neu phosphorylation by scFv23/TNF leads to the up-regulation of TNF-R1.

Second, a critical factor in the mediation of scFv23/TNF cytotoxicity seemed to be the involvement of various caspases. TNF-induced apoptosis is mainly mediated by TNF-R1 (32). Binding of TNF to TNF-R1 can induce the formation of signaling complexes, TNF-R1-TRADD-Fas-associated death domain-procaspase-8, resulting in the activation of caspase-8 (33). The activation of caspase-8 is thought to result in proteolytic activation of the other caspases (34). The activation of caspase-3 contributes to paclitaxel-induced apoptosis in HER-2/neu–overexpressing SKOV3.ip1 (35) and to immunotoxin-induced apoptosis (36). We observed that treatment with scFv23/TNF resulted in activation of caspase-8, caspase-3, and poly (ADP-ribose) polymerase cleavage in a time-dependent manner. Our data clearly suggest that the scFv23/TNF-induced cytotoxic mechanism was accompanied by induction of the apoptotic cascade through activation of caspase-8, caspase-3, and PARP cleavage via TNF-R1.

Finally, another important factor in the mediation of scFv23/TNF cytotoxicity is the modulation of Akt phosphorylation. The serine/threonine protein kinase Akt has been shown to have a pivotal role in cell cycle progression (37–39), angiogenesis (40), inhibition of apoptosis (41, 42), and cell growth (43). Overexpression of HER-2/neu is known to activate the Akt pathway and to confer resistance to apoptosis induced by many therapeutic drugs (44, 45). SKBR-3-LP cells which overexpress HER-2/neu had endogenous levels of phospho-Akt and Akt. Treatment with either scFv23 or TNF had no effect on the total cellular content or the phosphorylation Akt. On the other hand, treatment of cells with scFv23/TNF resulted in down-regulation of phosphorylated Akt. Our result suggests that Akt phosphorylation plays an important role in conferring TNF resistance on HER-2/neu–overexpressing SKBR-3-LP cells, and scFv23/TNF-induced cytotoxicity may be mediated, at least in part, by the inhibition of Akt survival signaling pathway.

Taken together, we observed that treatment of SKBR-3-LP cells with the immunocytokine scFv23/TNF resulted in up-regulation of TNF-R1 expression, down-regulation of Akt phosphorylation, and TNF-induced apoptosis through cleavage of caspase-8, caspase-3, and PARP. We summarized the observed differences in signaling events between TNF and scFv23/TNF in Fig. 9. Our *in vitro* mechanistic studies indicate that scFv23/TNF sensitizes TNF-resistant HER-2/neu–overexpressing SKBR-3-LP cells to TNF-induced apoptosis via the overexpression of TNF-R1, and we suggest that scFv23/TNF targeting the HER-2/neu may be an effective cytotoxic agent against HER-2/neu–overexpressing cancer cells, which are inherently resistant to TNF. In addition, the immunocytokine scFv23/TNF was more cytotoxic than TNF itself against MCF-7 breast tumor cell lines expressing intermediate levels of HER-2/neu

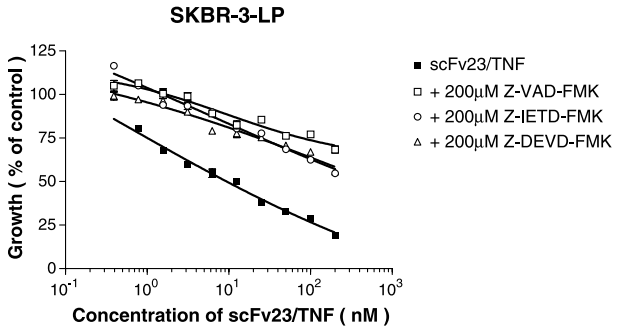

Figure 8. Influence of caspase inhibitors on the viability of scFv23/TNF-treated SKBR-3-LP cells. SKBR-3-LP cells were pretreated with or without 200 μmol/L general caspase inhibitor (z-VAD-fmk), 200 μmol/L caspase-8 inhibitor (z-IETD-fmk), or 200 μmol/L caspase-3 inhibitor (z-DEVD-fmk) for 2 h and then treated with various concentrations of scFv23/TNF. After 72 h of exposure, viability was determined using an XTT assay.

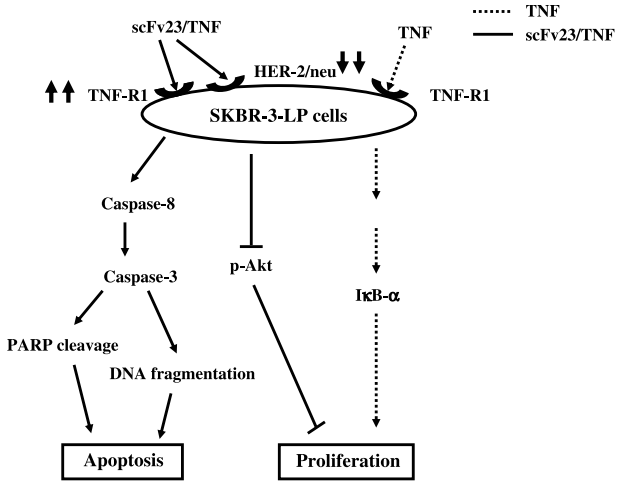

Figure 9. Summary of signal transduction effects of TNF and scFv23/TNF on HER-2/neu – overexpressing SKBR-3-LP cells.

(data not shown). We therefore suggest that the scFv23/TNF immunocytokine not only can overcome TNF resistance in HER-2/neu–overexpressing cells but may also be an excellent candidate for all breast cancer tumors, even those expressing modest amounts of HER-2/neu. *In vivo* pharmacokinetic, tissue disposition, and xenograft therapeutic studies are ongoing to examine whether this agent should be considered for clinical development.

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