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The immunocytokine scFv23/TNF targeting HER-2/neu induces synergistic cytotoxic effects with 5-fluorouracil in TNF-resistant pancreatic cancer cell lines☆

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ABSTRACT

Human pancreatic tumor cells are highly resistant to both tumor necrosis factor (TNF) and to chemotherapeutic agents. HER-2/neu expression has been proposed as a negative prognostic marker in pancreatic intraepithelial neoplasia. Our approach was to utilize HER-2/neu expression on the surface of tumor cells as a therapeutic target employing scFv23/TNF, immunocytokine composed of a single chain Fv antibody (scFv23) targeting the HER-2/neu and the cytokine TNF as the cytotoxic moiety, to deliver TNF directly to TNF-resistant pancreatic tumor cells. Using a panel of human pancreatic cell lines, which overexpress HER-2/neu, we evaluated the in vitro response of cells to TNF, scFv23/TNF, Herceptin, and a combination of scFv23/TNF with various chemotherapeutic agents. We found that all pancreatic cancer cell lines were highly resistant to the cytotoxic effects of TNF and that scFv23/TNF was highly cytotoxic to TNF-resistant HER-2/neu-expressing pancreatic cancer cell lines at levels rivaling that of conventional chemotherapeutic agents. Combination studies demonstrated a synergistic cytotoxic effect of scFv23/TNF with 5-fluorouracil (5-FU) in TNF-resistant pancreatic cancer cell lines. Mechanistic studies demonstrated that the 5-FU plus scFv23/TNF combination specifically resulted in a down-regulation of HER-2/neu, p-Akt and Bcl-2 and up-regulation of TNF-R1. In addition, the combination 5-FU plus scFv23/TNF induced apoptosis and this synergistic effect was dependent on activation of caspase-8 and caspase-3. Delivery of the cytokine TNF to HER-2/neu expressing pancreatic tumor cells, which are inherently resistant to TNF using scFv23/TNF may be an effective therapy for pancreatic cancer especially when utilized in combination with 5-FU.

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Abbreviations: scFv23/TNF, anti-HER-2/neu single chain Fv antibody fused to TNF; TNF, tumor necrosis factor; HER-2/neu, epidermal growth factor receptor-2; HER-1, epidermal growth factor receptor-1; TNF-R1, TNF receptor-1; TNF-R2, TNF receptor-2; p-Akt, phospho-Akt; 5-FU, 5-fluorouracil; PARP, poly ADP-ribose polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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1. Introduction

Pancreatic cancer remains one of the leading causes of cancer-related deaths in the United States and Europe [1–3]. This is a highly aggressive and metastatic tumor type virtually resistant to all chemotherapeutic and radiotherapeutic intervention [4,5]. Recent studies have demonstrated some clinical benefit to treatment with gemcitabine and gemcitabine-containing regimens [6–8].

There are numerous oncogenes such as HER-2/neu and HER-1 [9–13] which are over-expressed in pancreatic tumor biopsy specimens as well as mutations in various genes such as p53, Ki-ras, and p-21 which have been identified in pancreatic cancer [14–16]. Many of these genetic abnormalities play a major role in the development of the aggressive, metastatic, and therapy-resistant phenotype presented clinically. Experimental therapeutic approaches to use vaccines [17–20] or antibodies to target oncogene protein products [21–23] in pancreatic cancer are underway or have been completed to provide more focused control of tumor growth.

Immunocytokines are a novel class of recombinant agents composed of cytokines fused to antibodies. These fusion constructs are capable of re-directing their biological effects to target specific cells and to prevent non-target toxicity. We described a novel chemical conjugate of a tumor-targeting antibody and the cytokine tumor necrosis factor-alpha (TNF-α) [24,25]. Against antigen-positive cells, this chemical conjugate was more cytotoxic to target cells than TNF-α itself. More recently, we developed novel immunocytokine scFv23/TNF composed of a single-chain Fv antibody (scFv23) targeting the HER-2/neu antigen on the cell surface of tumor fused to the gene for TNF-α. This immunocytokine was also shown to be highly cytotoxic and specifically active against target cells resistant to TNF-α itself [26,27].

Because many human pancreatic tumor cell lines over-express HER-2/neu and additionally tend to be generally resistant to TNF and chemotherapeutic agents, the purpose of the current study was to examine the cytotoxic effects of the immunocytokine scFv23/TNF alone and in combination with various chemotherapeutic agents against a panel of four pancreatic lines (AsPc-1, Capan-1, Capan-2, and L3.6pl) which over-express HER-2/neu and the possible mechanisms which may account for the observed synergistic cytotoxic effects of scFv23/TNF in combination with 5-fluorouracil.

2. Materials and methods

2.1. Cell culture

L3.6pl and Capan-1 human pancreatic cancer cell lines were kindly provided by Dr. Jerald Killian and Dr. Paul Chiao (M.D. Anderson Cancer Center, Houston), respectively. AsPc-1 and Capan-2 human pancreatic cancer cell lines were kindly provided by Dr. Keping Xie (M.D. Anderson Cancer Center, Houston). All four human pancreatic cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.2. Chemotherapeutic agents and scFv23/TNF

5-Fluorouracil (5-FU) was from Roche Laboratories (Nutley, NJ). Cisplatin and etoposide (VP-16) were from Bristol Laboratories (Princeton, NJ). Doxorubicin was from Cetus Corporation (Emeryville, CA). Gemcitabine was from Eli Lilly Co. (Indianapolis, IN). Herceptin and bacteria-derived recombinant human tumor necrosis factor (rhTNF) that was purified to homogeneity with a specific activity of 5×10^7 units/mg were provided by Genentech (South San Francisco, CA). The immunocytokine scFv23/TNF was produced in a bacterial expression host, purified to homogeneity and assessed for biological activity as previously described [26].

2.3. Antibodies and chemicals

Monoclonal anti-HER-2/neu antibody (Ab) was purchased from Oncogene (San Diego, CA). Rabbit polyclonal anti-TNF-R1 Ab, rabbit polyclonal anti-TNF-R2 Ab, rabbit polyclonal anti-phospho Akt Ab, rabbit polyclonal anti-Akt Ab, mouse anti-Bcl-2 Ab, rabbit polyclonal anti-caspase-8 Ab, monoclonal anti-caspase-3 Ab, and monoclonal anti-PARP Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 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). Recombinant human TNF-R1:Fc fusion protein was purchased from Alexis (San Diego, CA).

2.4. In vitro cytotoxicity assay and combination studies of scFv23/TNF with conventional chemotherapeutic agents on pancreatic cancer cell lines

All human pancreatic cancer cells were seeded (1×10^4/well) in flat-bottom 96-well microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ) and 24 h later, scFv23/TNF, TNF, scFv23, Herceptin, or five chemotherapeutic agents (5-fluorouracil, cisplatin, etoposide, doxorubicin, and gemcitabine) were added in triplicate wells. For combination studies, scFV23/TNF and each of five chemotherapeutic agents were combined at their individual IC_{25} concentrations. To examine the effect of caspase inhibitor on the cytotoxicity of the combination, L3.6pl cells were pretreated with or without 200 μM general caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-FMK), or caspase-3 inhibitor (Z-DEVD-FMK) for 2 h and then treated with their individual IC_{25} concentrations. After incubation for an additional 72 h, remaining adherent cells were stained by adding 50 μl of crystal violet solution (0.5% (w/v) in 20% MeOH/H_2O). Dye-stained cells were solubilized by addition of 100 μl of Sorenson's buffer [100 mM sodium citrate (pH 4.2) in 50% ethanol], and absorbance was measured at 630 nm using an ELISA plate reader (Bio-Tek Instruments, Inc., Winooski, VT).

To analyze the cellular interaction between the two agents, for tested combination of the two agents combination index

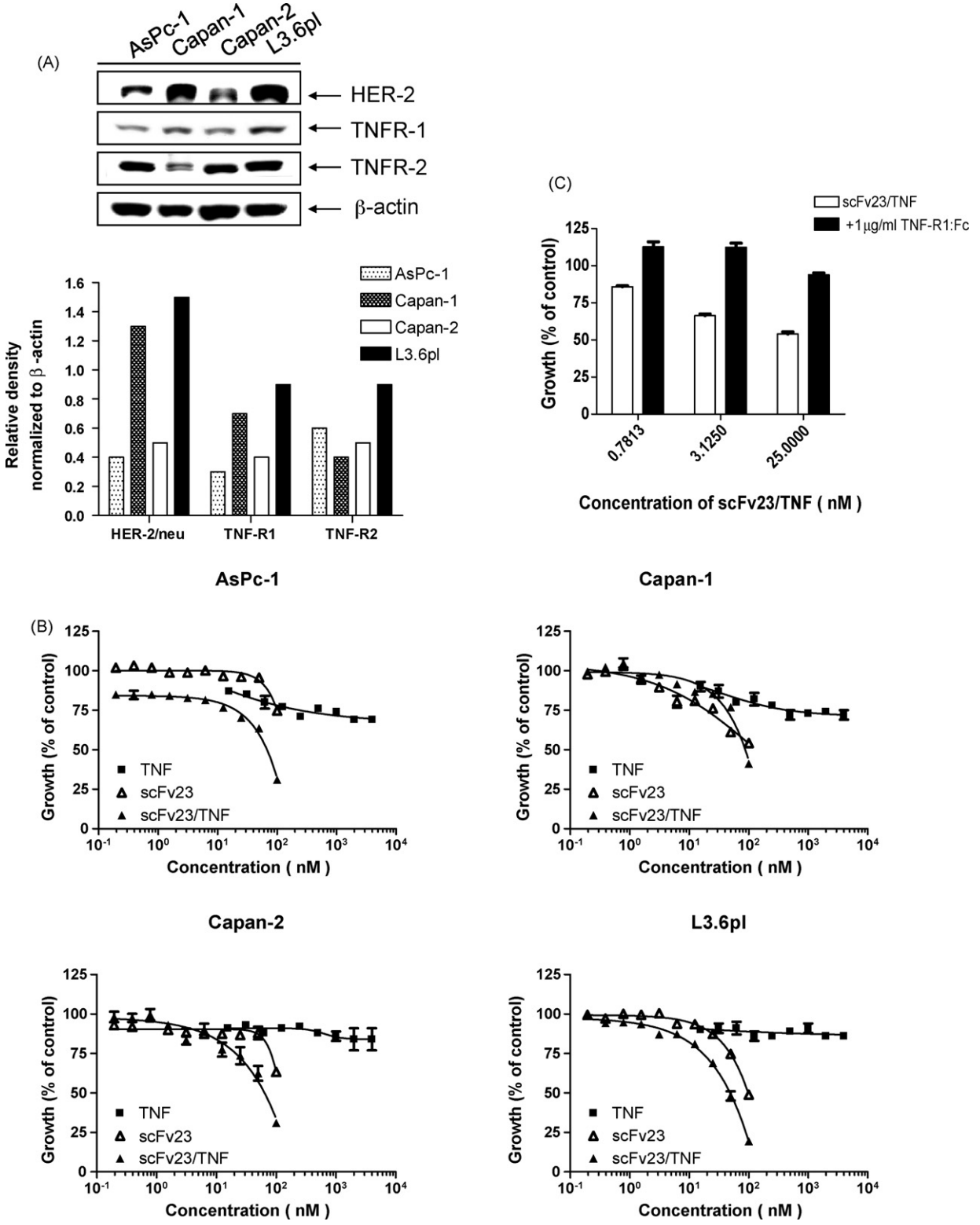

Fig. 1 – Expression pattern of HER-2/neu, TNF-R1, and TNF-R2 and sensitivity to scFv23/TNF. (A) Basal levels of HER-2/neu, TNF-R1, and TNF-R2 in four pancreatic cancer cell lines. AsPc-1, Capan-1, Capan-2, and L3.6pl cells were seeded at 5×10^5 cells/Ø 60 mm Petri-dish and incubated for 24 h after which cell lysates were collected. Whole cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti-HER-2/neu, anti-TNF receptor-1, anti-TNF receptor-2, and anti-β-actin antibodies, followed by incubation with an anti-mouse, anti-goat or anti-rabbit horseradish peroxidase-labeled antibody and chemiluminescent detection. Actin was used as a loading control for protein loading. Data are presented as the relative density of protein bands normalized to β-actin. Relative protein quantitation was performed using Flour Chem 8900. Representative of three independent experiments. Values are means ± S.D. (B) Growth inhibition of TNF, scFv23 or

(CI) values were calculated as proposed by Chou and Talalay [28]: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + \alpha D_1D_2/(Dx)_1(Dx)_2$, where $(D)_1$ and $(D)_2$ in combination kill X% of cells, and $(Dx)_1$ and $(Dx)_2$ are the estimated dose of the drug alone capable of producing the same effect of the combined drugs. $CI < 1$, $CI = 1$, $CI > 1$ indicate synergism, additive effect, and antagonism, respectively.

2.5. Assessment of the role of TNF-R1 in scFv23/TNF-induced cytotoxicity

L3.6pl cells were seeded (1×10^4 cells/well) in flat-bottomed 96-well microtiter plate (Becton Dickinson Labware) and 24 h later were pretreated with 1 μg/ml recombinant human TNF-R1:Fc fusion protein (Alexis) for 2 h, and then treated with scFv23/TNF. After incubation for 72 h, growth inhibition was determined by crystal violet staining.

2.6. Western blot analysis

To check the status of HER-2/neu, TNF receptor-1, and TNF receptor-2, four human pancreatic cancer cell lines (AsPc-1, Capan-1, Capn-2, and L3.6pl) 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). To examine the effect of combination treatment, AsPc-1, Capan-1, Capan-2, and L3.6pl cell lines were seeded at 5×10^5 cells/60 mm Petri-dish, allowed to grow overnight, and then treated with IC_{25} concentrations of 5-FU, scFv23/TNF, or combination for 48 h or different times. Cell lysates (50 μg) were separated by SDS-PAGE (8–15%), electrotransferred to PVDF membranes (Millipore Corporation, Bedford, MA), probed with different antibodies. After washing, the membrane was developed using ECL detection reagent (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Data are presented as the relative density of protein bands normalized to β-actin. Relative protein quantitation was performed using Flour Chem 8900 (Alpha Innotech Co, San Leandro, CA).

2.7. Detection of apoptosis

The development of apoptotic cell death was detected by TUNEL assay. To assess apoptosis, L3.6pl cells were plated on glass cover slips, allowed to adhere overnight and then treated with IC_{25} concentrations of 5-FU, scFv23/TNF, or combination for 48 h. 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, Indianapolis, IN). Cells undergoing apoptosis were identified by fluorescence microscopy (Nikon, Japan).

3. Results

3.1. HER-2/neu, TNF-R1, and TNF-R2 expression and cytotoxic activity of various agents against four pancreatic cancer cell lines

HER-2/neu has previously been found to be over-expressed in pancreatic tumor biopsy specimens and HER-2/neu expression has been proposed as a negative prognostic marker in pancreatic intraepithelial neoplasia [9]. We examined the basal level of the HER-2/neu, TNF-R1, and TNF-R2 expression by western blot analysis in four pancreatic cancer cell lines. All lines (AsPc-1, Capan-1, Capan-2, and L3.6pl) expressed HER-2/neu. L3.6pl cells expressed highest levels of HER-2/neu, TNF-R1, and TNF-R2 protein whereas AsPc-1 and Capan-2 cells expressed comparatively less HER-2/neu and TNF-R1 protein (Fig. 1A).

HER-2/neu plays a key role in modulation of cellular response to TNF [29,30]. We next evaluated the response of these pancreatic cancer cell lines to the cytotoxic effects of TNF, scFv23, or scFv23/TNF. All lines were resistant to TNF at doses of up to 4 μM whereas the cell lines showed specific sensitivity to scFv23 or scFv23/TNF. The ratio of IC_{50} values of TNF to scFv23/TNF was calculated for the cell lines. This ratio (targeting index) represents the ability of the scFv23 component of the scFv23/TNF to mediate the delivery of the TNF component to the target cell cytoplasm. scFv23/TNF showed IC_{50} values between 50 nM and 90 nM against TNF-resistant pancreatic cancer cell lines which express HER-2/neu (targeting index >44). Especially, L3.6pl cells expressing the highest levels of HER-2/neu, TNF-R1, and TNF-R2 were the most sensitive to scFv23/TNF (Targeting index >80) (Fig. 1B). Surprisingly, scFv23 showed a 2-fold gain in toxicity after conjugation with TNF. These data suggest that scFv23/TNF can overcome TNF cellular resistance associated with HER-2/neu expression.

The ability of various chemotherapeutic agents to inhibit cell proliferation *in vitro* was markedly different among the four lines tested. As noted above, all lines were highly resistant to the cytotoxic effects of TNF ($IC_{50} > 4$ μM). The chemotherapeutic agents 5-FU, cisplatin, etoposide, and Herceptin showed IC_{50} values between 1 μM and 300 μM whereas doxorubicin, gemcitabine, and scFv23/TNF were comparatively more active with IC_{50} values ranging between 6 nM and 500 nM. Surprisingly, scFv23/TNF was highly cytotoxic to HER-2/neu-expressing pancreatic cancer cell lines at levels rivaling that of conventional chemotherapeutic agents (Table 1).

3.2. The role of TNF-R1 in scFv23/TNF-induced cytotoxicity

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 to TNF-R1 using TNF-R1:Fc fusion protein.

scFv23/TNF in four pancreatic cancer cell lines. The cells were treated with various concentrations of TNF, scFv23 or scFv23/TNF for 72 h and then assessed growth inhibition by crystal violet staining. Values are means ± S.D. from at least four independent exposures. (C) Role of TNR receptor 1 in scFv23/TNF-induced cytotoxicity. To determine whether the cytotoxic effect of scFv23/NF was mediated entirely through interaction with cell surface TNF-R1, we blocked the binding of scFv23/TNF to TNF-R1 using 1 μg/ml TNF-R1:Fc protein. After 72 h of exposure, growth inhibition was determined by crystal violet staining. Values are means ± S.D. from at least four independent exposures.

Table 1 – IC_{50} of various agents against four human pancreatic cancer cell lines

Drug	IC_{50} (μM)			
	AsPc-1	Capan-1	Capan-2	L3.6pl
5-Fluorouracil (5-FU)	7.5	6	300	1
Cisplatin (CIS)	14	4.5	50	3.6
Etoposide (ETO)	28	2	40	2
Doxorubicin (DOX)	0.32	0.06	0.5	0.03
Gemcitabine (GEM)	0.2	0.02	0.15	0.006
scFv23/TNF	0.07	0.09	0.07	0.05
TNF	>4*	>4*	>4*	>4*
Herceptin	4.8	3.44	4.8	3.44

The IC_{50} values were determined after 72 h of exposure to the drugs and were defined as the concentration causing 50% growth inhibition in treated cells compared to control cells.
* Highest concentration achieved.

As shown in Fig. 1C, addition of TNF-R1:Fc was able to abrogate scFv23/TNF-induced cytotoxicity on L3.6pl cells. Our result clearly suggests that scFv23/TNF-induced cytotoxicity is principally mediated by interaction with the cell surface TNF-R1.

3.3. *Combination studies of scFv23/TNF with various chemotherapeutic agents*

We next performed studies combining scFv23/TNF in vitro with various chemotherapeutic agents. We found a synergistic cytotoxic effect of scFv23/TNF with 5-FU and an antagonistic effect of scFv23/TNF with doxorubicin in pancreatic cancer cell lines tested. However, the addition of cisplatin or gemcitabine to scFv23/TNF resulted in antagonistic cytotoxic effects in 3/4 cell lines tested whereas the addition of etoposide to scFv23/TNF resulted in a synergistic cytotoxic effect in 3/4 pancreatic cancer cell lines (Fig. 2A). These results suggest that targeting

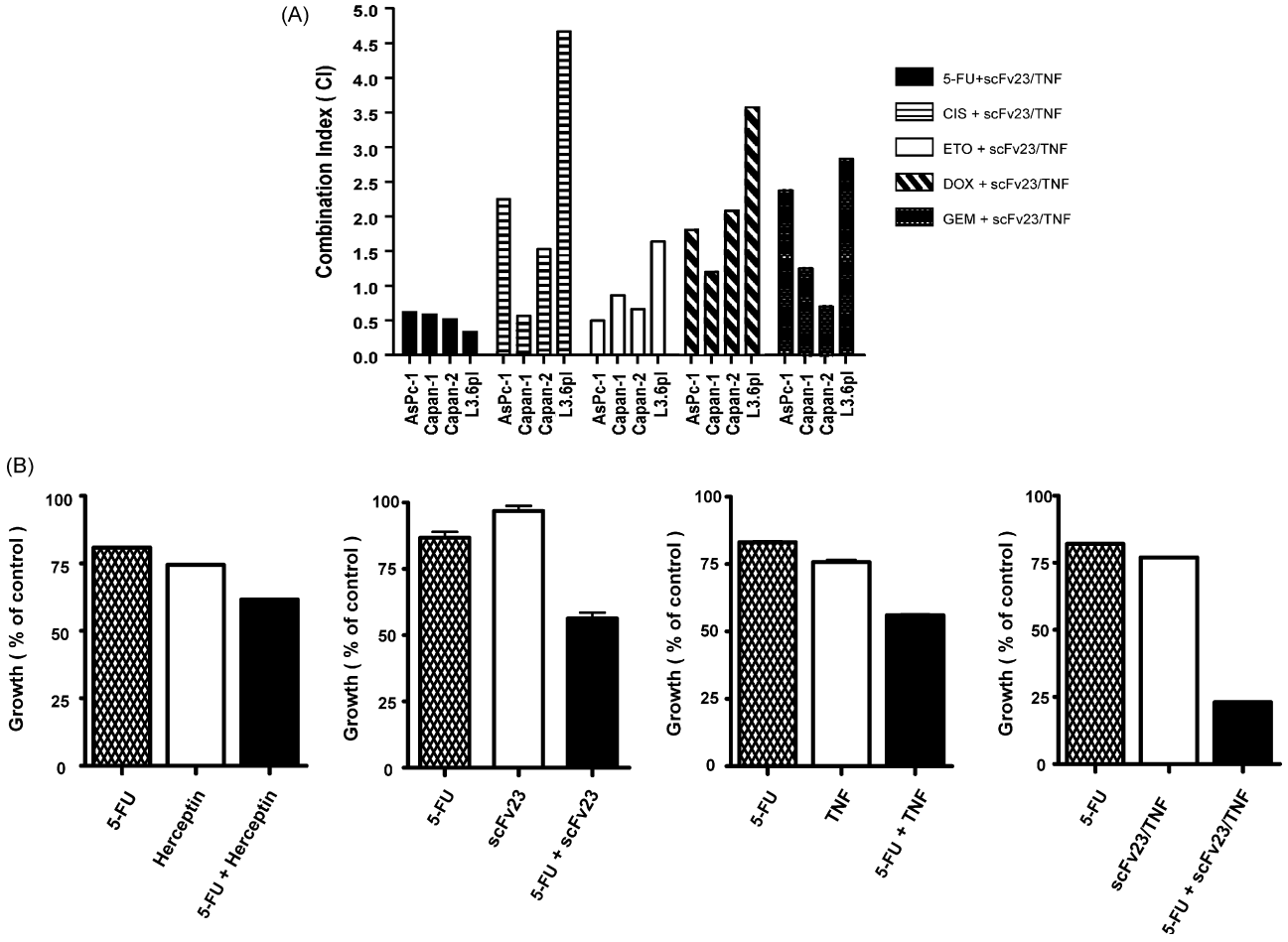

Fig. 2 – (A) Concomitant exposure to scFv23/TNF and five conventional chemotherapeutic agents against AsPc-1, Capan-1, Capan-2, and L3.6pl cells. After 72 h incubation, growth inhibition was determined by crystal violet staining. Values are means ± S.D. from at least four independent exposures. To analyze the cellular interaction between the 2 agents, for tested combination of the 2 agents combination index (CI) values were calculated as proposed by Chou and Talalay [28]. CI = 1 indicates an additive effect; CI > 1 indicated antagonism; and CI < 1 indicates synergism (see Section 2). (B) Effect of Herceptin, 5-fluorouracil, scFv23, TNF, scFv23/TNF or the combination on the growth of L3.6pl cells. The cells were treated with 4.5 μM Herceptin, 400 nM 5-fluorouracil, 50 nM scFv23, 625 nM TNF, 25 nM scFv23/TNF or the combination for 72 h and then assessed growth inhibition by crystal violet staining. Values are means ± S.D. from at least four independent exposures.

TNF-resistant HER-2/neu-expressing tumor cells using the immunocytokine scFv23/TNF may be an effective therapy for pancreatic cancer especially when utilized in combination with specific chemotherapeutic agents such as 5-FU, or etoposide.

For further study, we selected TNF-resistant L3.6pl cell line overexpressing HER-2/neu, which produce significantly higher incidence and number of lymph node and liver metastases than parent COLO 357 fast growing cells [31].

The immunocytokine scFv23/TNF is composed of a single chain Fv antibody (scFv23) targeting the HER-2/neu and TNF. To investigate the role of both TNF and scFv23 components in scFv23/TNF, we next examined the cooperative effect of combination treatment in L3.6pl cells. As shown in Fig. 2B, additive interaction was observed for 5-FU in combination with TNF or scFv23 whereas the addition of 5-FU to scFv23/TNF resulted in a synergistic cytotoxic effect. However, the cooperative effect of 5-FU plus Herceptin (anti-HER-2/neu antibody) is not great than that of 5-FU plus scFv23.

3.4. *Effect of combination treatment on HER-2/neu and TNF-R1 expression*

We next examined whether combination treatment can modulate the cellular expression of HER-2/neu and TNF-R1. Treatment of L3.6pl cells or Capan-2 cells with scFv23/TNF in combination with 5-FU induced down-regulation of HER-2/neu and up-regulation of TNF-R1 expression in a time-dependent fashion (Fig. 3). This result suggests that the cellular expression level of HER-2/neu and TNF-R1 may be directly correlated with the cytotoxic effect of combination treatment in TNF-resistant, HER-2/neu-overexpressing pancreatic cancer cell lines.

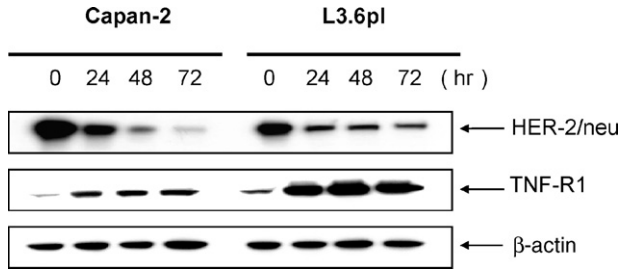

Fig. 3 – Effect of combination treatment on modulation of HER-2/neu and TNF-R1 expression. To determine whether combination treatment can modulate the expression of HER-2/neu and TNF-R1, we treated Capan-2 and L3.6pl cells with scFv23/TNF plus 5-FU. Whole cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti-HER-2/neu or anti-TNF-R1 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.

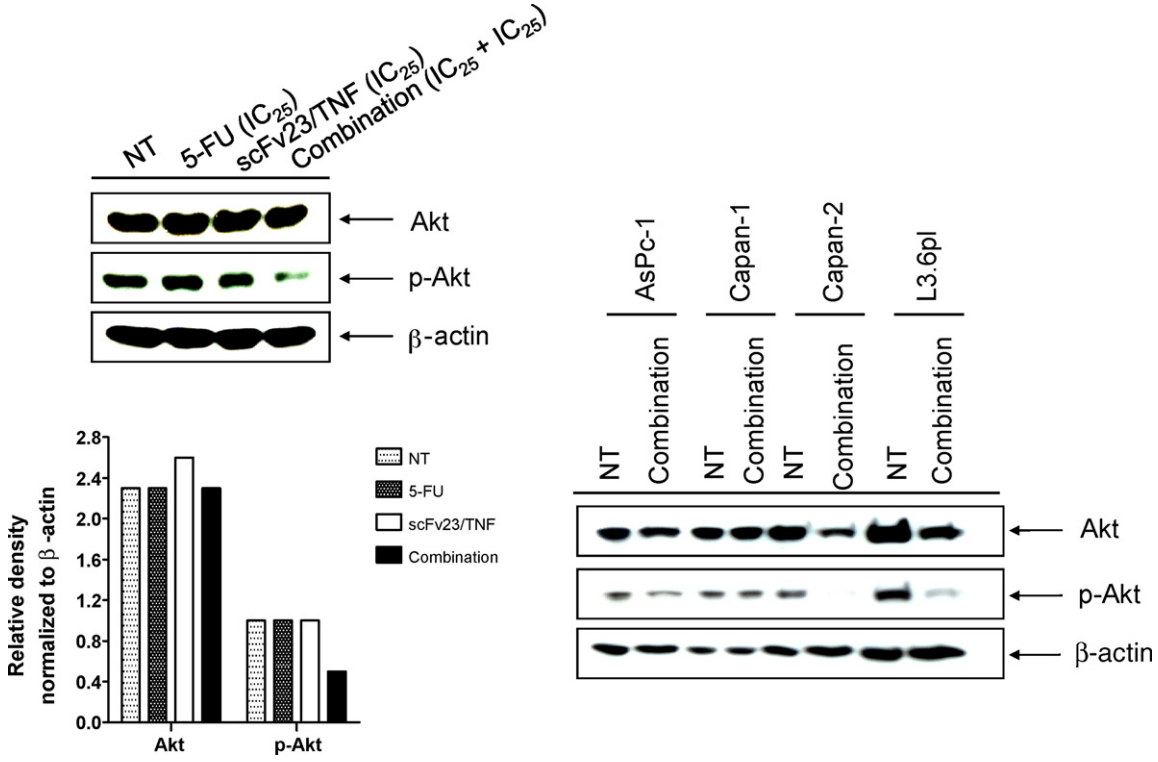

Fig. 4 – Inhibition of Akt expression by combination treatment. AsPc-1, Capan-1, Capan-2 or L3.6pl cells were treated with IC_{25} of 5-FU, scFv23/TNF or scFv23/TNF plus 5-FU combination for 48 h or 72 h. For combination studies, scFv23/TNF and 5-FU were combined at their individual IC_{25} concentrations. After treatment, cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti-Akt and anti-phospho-Akt antibodies, followed by incubation with an anti-rabbit horseradish peroxidase-labeled antibody and chemiluminescent detection. Actin was used as a loading control. Data are presented as the relative density of protein bands normalized to β-actin. Relative protein quantitation was performed using Flour Chem 8900. Representative of three independent experiments. Values are means ± S.D.

3.5. *Effect of 5-FU + scFv23/TNF combination treatment on Akt expression*

Overexpression of HER-2/neu has been shown to result in activation of different downstream pathways such as the Akt kinase pathway, which leads to cell proliferation and cell survival. To determine whether combination treatment affects this survival pathway, four pancreatic cancer cell lines were treated with IC_{25} doses of 5-FU, scFv23/TNF, or 5-FU + scFv23/TNF. The activation of Akt kinase was then assessed by Western blot analysis using antibodies to Akt and to phospho-Akt. As shown in Fig. 4, treatment of L3.6pl cells with 5-FU, scFv23/TNF as single agents or with the combination had no impact on the total levels of Akt while the combination treatment of scFv23/TNF plus 5-FU inhibited phosphorylation of the Akt protein by 50% after 48 h of treatment. In addition, combination treatment induced down-regulation of phospho Akt and Akt in AsPc-1, Capan-2, and L3.6pl but not in Capan-1 cells after 72 h of treatment. These results suggest that the cytotoxic effects of 5-FU plus scFv23/TNF may be mediated, at least in part, by an inhibitory effect on Akt phosphorylation events.

3.6. *Effect of 5-FU + scFv23/TNF combination treatment on Bcl-2 expression*

Increased levels of the anti-apoptotic protein Bcl-2 contribute to cellular resistance of tumor cells to a variety of chemotherapeutic agents including cyclophosphamide, methotrexate, anthracycline, cytarabine, paclitaxel, and corticosteroids [32]. To determine whether the cytotoxic effects of combination treatment are mediated through changes in cellular levels of Bcl-2, four pancreatic cancer cell lines were treated with IC_{25} doses of 5-FU, scFv23/TNF, or 5-FU + scFv23/TNF. As shown in Fig. 5, treatment of L3.6pl cells with 5-FU had no impact on cellular levels of Bcl-2 while scFv23/TNF and 5-FU + scFv23/TNF inhibited Bcl-2 expression levels by 43% and 71%, respectively. In addition, combination treatment induced down-regulation of Bcl-2 in AsPc-1, Capan-2, and L3.6pl but not in Capan-1 cells after 72 h of treatment. This result

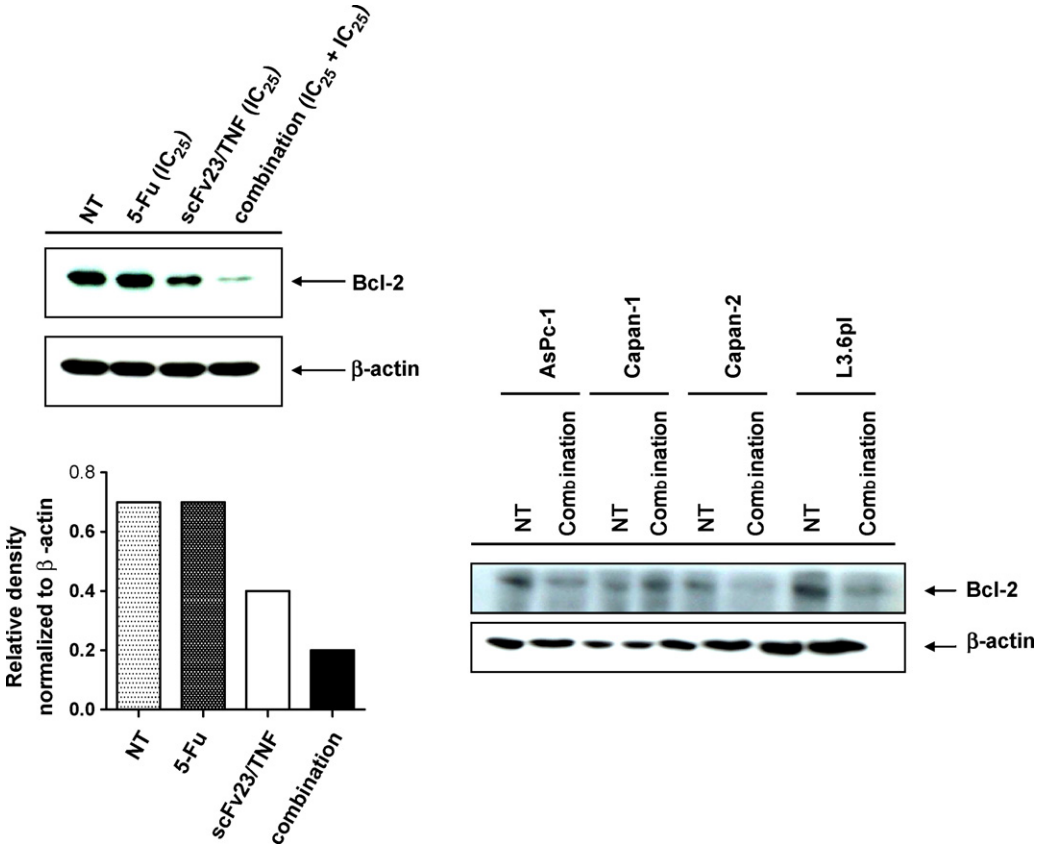

Fig. 5 – Inhibition of Bcl-2 expression by combination treatment. AsPc-1, Capan-1, Capan-2 or L3.6pl cells were treated with IC_{25} of 5-FU, scFv23/TNF or 5-FU plus scFv23/TNF combination for 48 h or 72 h. For combination studies, scFv23/TNF and 5-FU were combined at their individual IC_{25} concentrations. After treatment, cell lysates (50 μg) were analyzed by SDS-PAGE and immunoblotting with anti-Bcl-2 antibody, followed by incubation with an anti-mouse horseradish peroxidase-labeled antibody and chemiluminescent detection. Actin was used as a loading control. Data are presented as the relative density of protein bands normalized to β-actin. Relative protein quantitation was performed using Flour Chem 8900. Representative of three independent experiments. Values are means ± S.D.

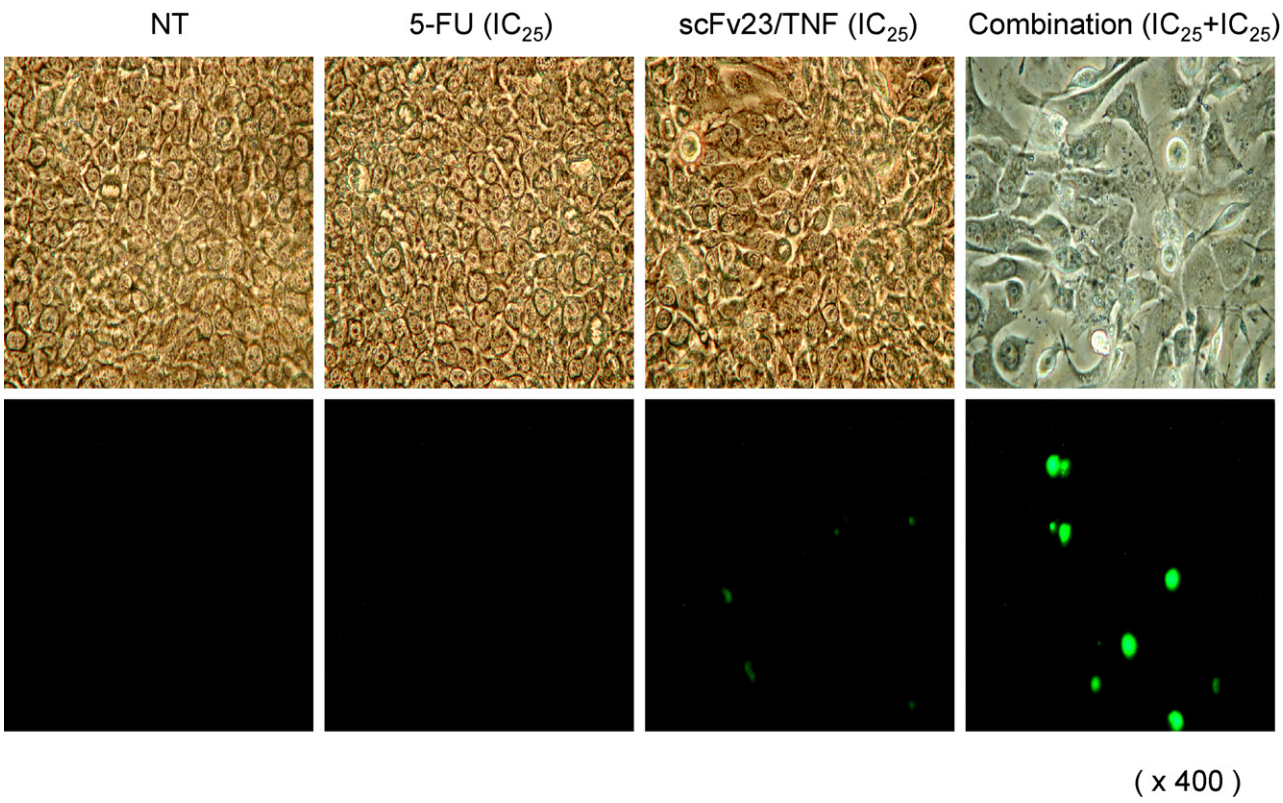

Fig. 6 – Induction of apoptosis by combination treatment. L3.6pl cells exposed to IC_{25} of 5-FU, scFv23/TNF, or combination for 48 h. For combination studies, scFv23/TNF and 5-FU were combined at their individual IC_{25} concentrations. After treatment, the cells were washed with PBS, permeabilized 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 microscope (400×).

suggests that the cytotoxic effects of 5-FU plus scFv23/TNF may be mediated, at least in part, by inhibition of Bcl-2 expression.

3.7. *Effect of 5-FU + scFv23/TNF combination treatment on apoptosis*

To determine whether the cytotoxic effect of combination treatment was associated with apoptosis, L3.6pl cells were assayed for apoptosis by TUNEL staining. L3.6pl cells were treated with IC_{25} doses of 5-FU, scFv23/TNF or 5-FU + scFv23/TNF. As shown in Fig. 6, 5-FU + scFv23/TNF-treated cells demonstrated a marked induction of apoptotic cell death within 48 h after treatment.

The caspase proteins are 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 L3.6pl cells during 5-FU + scFv23/TNF-induced cell death, we investigated the cleavage of caspase-8, caspase-3, and its substrate poly (ADP)-ribose polymerase (PARP). Treatment of L3.6pl cells with IC_{25} dose of 5-FU had no effect on caspase-8, caspase-3, and PARP cleavage whereas exposure of the cells to the scFv23/TNF or scFv23/TNF plus 5-FU combination resulted in cleavage of caspase-8, caspase-3, and PARP (Fig. 7A). Caspase-8 was activated after 24 h of combination treatment and then levels of cleaved caspase-8 were shown to decrease whereas levels of cleaved caspase-3 and PARP were increased 72 h after combination treatment (Fig. 7B).

To determine whether 5-FU + scFv23/TNF-induced apoptosis was dependent on activation of the caspase pathways, we examined the effect of caspase inhibitors on the cytotoxicity of 5-FU + scFv23/TNF against L3.6pl cells. As shown in Fig. 8, pre-treatment with the caspase inhibitors followed by combination treatment (5-FU + scFv23/TNF) was able to inhibit the synergistic cytotoxic effects observed. This result suggests that the synergistic cytotoxic effects of the combination may depend, at least in part, on a caspase-driven pathway.

4. Discussion

Human epidermal growth factor receptor-2 (HER-2/erbB-2) belongs to a family of four transmembrane receptors (HER-1, HER-3, and HER-4) and it plays a key role in HER family signaling events, cooperating with other HER receptors via a complex signaling network to regulate cell growth, differentiation, and survival [33–35]. Over-expression of HER-2/neu has been observed in several cancers where it is associated with multiple drug resistance, higher metastatic potential, and decreased patient survival [9,36–40].

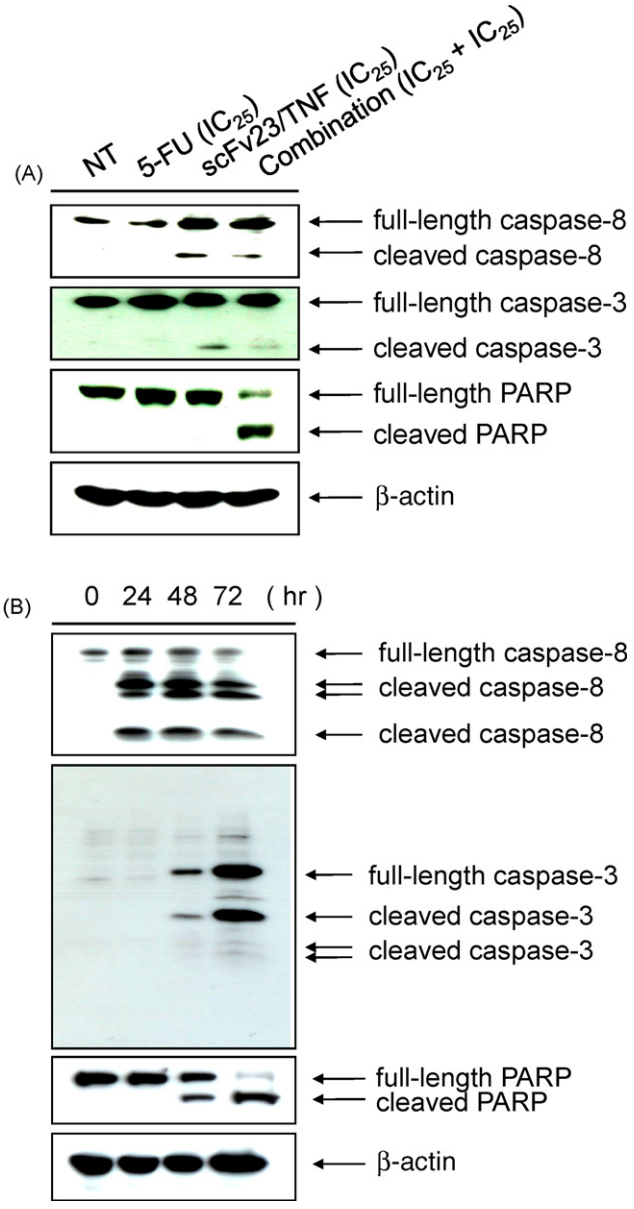

Fig. 7 – Effects of combination treatment on the activation of caspase-8, caspase-3, and PARP cleavage. L3.6pl cells were treated with IC_{25} of 5-FU, scFv23/TNF or combination for 48 h (A) or different times (B). For combination studies, scFv23/TNF and 5-FU were combined at their individual IC_{25} concentrations. 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.

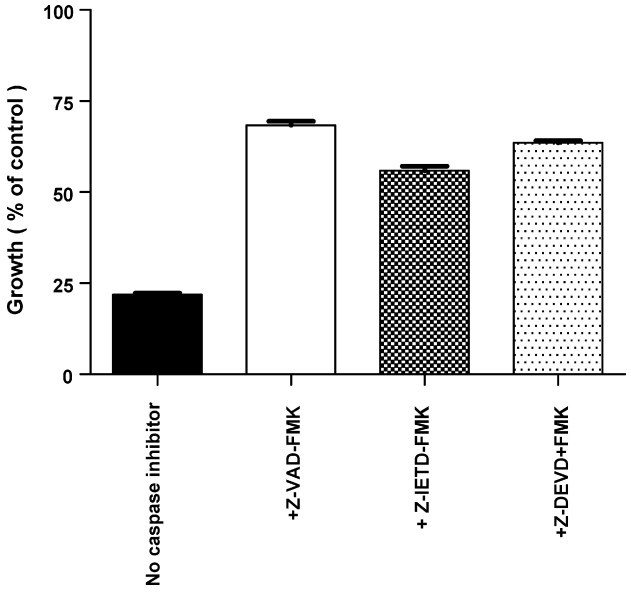

Fig. 8 – Influence of caspase inhibitors on the viability of 5-FU + scFv23/TNF-treated L3.6pl cells. L3.6pl cells pre-treated with or without 200 μM general caspase inhibitor (Z-VAD-FMK), caspase-8 inhibitor (Z-IETD-FMK), or caspase-3 inhibitor (Z-DEVD-FMK) (R&D) for 2 h and then treated with their individual IC_{25} concentrations. After 72 h of exposure, growth inhibition was determined by crystal violet staining. Values are means ± S.D. from at least four independent exposures.

To evaluate the influence of HER-2/neu expression in pancreatic cancer as it relates to clinical response to therapeutic agents, a variety of groups have used several HER-2/neu targeting strategies including using HER-2/neu targeted ribozymes [41–43], humanized anti-HER-2/neu antibody (Herceptin), and combination chemotherapeutic treatment regimens with Herceptin [44,21,45,46].

Our approach was to utilize HER-2/neu expression on the surface of tumor cells as a therapeutic target employing the immunocytokine scFv23/TNF, a fusion cytokine composed of TNF and a single chain Fv antibody (scFv23) targeting the HER-2/neu antigen, to deliver TNF directly to TNF-resistant pancreatic tumor cells. In this report, we observed that scFv23/TNF can overcome TNF resistance in HER-2/neu-expressing pancreatic cancer cell lines (Fig. 1B) and scFv23/TNF-induced cytotoxicity is mediated by interaction with the cell surface TNF-R1 (Fig. 1C). We previously observed that treatment with scFv23 or scFv23/TNF induced up-regulation of TNF-R1 in SKBR-3 breast cancer cell [27]. We also found that treatment of pancreatic cancer cell lines (Capan-2 and L3.6pl) with scFv23/TNF plus 5-FU induced down-regulation of HER-2/neu and up-regulation of TNF-R1 (Fig. 3). These results suggest that scFv23/TNF plus 5-FU can sensitize HER-2/neu-overexpressing cancer cells to TNF via down-regulation of HER-2/neu and up-regulation of TNF receptor-1 and the overexpression of TNF-R1 plays a crucial role in TNF sensitivity in HER-2/neu-overexpressing cancer cells.

The chemotherapeutic agents utilized in this study were selected to present a spectrum of different intracellular targets and are representative of the major classes of agents with therapeutic value. The potential combinations of tumor-targeted delivery of TNF in combination with chemotherapeutic agents have not been previously examined. Our studies combining scFv23/TNF and various chemotherapeutic agents clearly demonstrated a uniform synergistic effect of scFv23/TNF and 5-FU in all pancreatic tumor cell lines. Pegram

et al. reported that 5-FU has an antagonistic effect *in vitro* in combination with anti-HER-2/neu monoclonal antibodies whereas cisplatin, etoposide, and doxorubicin, previously showed a synergistic or an additive effect in combination with Herceptin [46]. However, we found a uniform synergistic effect of scFv23/TNF in combination with 5-FU and an antagonistic effect of scFv23/TNF in combination with doxorubicin against all four pancreatic cancer cell lines (Fig. 2A).

Over-expression of HER-2/neu is known to activate the Akt pathway and to confer resistance to apoptosis induced by many therapeutic drugs [47]. Han et al. [48] reported that the down-regulation of Akt1 with Akt1 siRNA could significantly enhance the sensitivity of AGS cells to 5-FU. Four pancreatic cancer cell lines (AsPc-1, Capan-1, Capan-2, and L3.6pl) which express HER-2/neu had endogenous levels of phospho Akt and Akt (Fig. 4). Treatment of three pancreatic cancer cell lines (AsPc-1, Capan-2, and L3.6pl) with combination 5-FU + scFv23/TNF resulted in significant reduction in phospho Akt and Akt. This result suggests that Akt phosphorylation plays an important role in 5-FU plus scFv23/TNF-induced cytotoxicity.

Over-expression of Bcl-2 has also been shown to contribute to the cellular resistance of a variety of chemotherapeutic drugs, including cyclophosphamide, methotrexate, anthracycline, cytarabine, paclitaxel, and corticosteroids [32]. Sasaki et al. reported that the level of Bcl-2 in cancer cells was an indicator of 5-FU efficacy [49]. We found that treatment of three pancreatic cancer cell lines with scFv23/TNF plus 5-FU resulted in significant reduction of Bcl-2 expression. However, treatment of L3.6pl cells with IC_{25} dose of 5-FU had no impact on the levels of Bcl-2. This result suggests that down-regulation of Bcl-2 by scFv23/TNF may be induced the sensitization of pancreatic cancer cell lines to be more sensitive to 5-FU.

We also found that another critical factor in the mediation of 5-FU + scFv23/TNF synergy is the caspase activation cascade. Binding of TNF to TNF-R1 can induce the formation of signaling complexes, TNF-R1-TRADD-FADD-pro-caspase-8, resulting in the activation of caspase-8 [50]. The activation of caspase-8 is though to result in proteolytic activation of the other caspases [51]. The activation of caspase-3 contributes to paclitaxel-induced apoptosis in HER-2/neu-overexpressing SKOV3.ip1 [52] and immunotoxin-induced apotosis [53]. We observed that treatment with 5-FU + scFv23/TNF resulted in activation of caspase-8, caspase-3, and PARP cleavage and the combination-induced cytotoxicity was inhibited by general caspase inhibitor, caspase-8 inhibitor, and caspase-3 inhibitor. Our data clearly suggest that 5-FU plus 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.

Taken together, we observed that the immunocytokine scFv23/TNF targeting HER-2/neu can overcome TNF resistance and induced a synergistic cytotoxic effect with 5-FU in TNF-resistant pancreatic cancer cell lines, which express HER-2/neu. Treatment of three pancreatic cancer cell lines (AsPc-1, Capan-2, and L3.6pl) with scFv23/TNF in combination with 5-FU resulted in down-regulation of HER-2/neu, p-Akt, Akt, and Bcl-2 and up-regulation of TNF-R1 and induction of apoptosis through cleavage of caspase-8, caspase-3, and PARP. Our results suggest that delivery of the cytokine TNF to HER-2/neu expressing TNF-resistant tumor cells using scFv23/TNF may be an effective therapy for pancreatic cancer especially when utilized in combination with specific chemotherapeutic agents. These observations provide a rationale for further developmental therapeutic studies of the immunocytokine scFv23/TNF against HER-2/neu expressing pancreatic xenograft models alone and in combination with selected chemotherapeutic agents.

REFERENCES

[1] Haycox A, Lombard M, Neoptolemos J, Walley T. Review article: current treatment and optimal patient management in pancreatic cancer. Aliment Pharmacol Ther 1998;12:949–64.

[2] Ward S, Morris E, Bansback N, et al. A rapid and systematic review of the clinical effectiveness and cost-effectiveness of gemcitabine for the treatment of pancreatic cancer. Health Technol Assess 2001;5:1–70.

[3] Kulke MH. Metastatic pancreatic cancer. Curr Treat Options Oncol 2002;3:449–57.

[4] Permert J, Hafstrom L, Nygren P, Glimelius B. A systematic overview of chemotherapy effects in pancreatic cancer. Acta Oncol 2001;40:361–70.

[5] Matsuno S, Egawa S, Arai K. Trends in treatment for pancreatic cancer. J Hepatobiliary Pancreat Surg 2001;8:544–8.

[6] van Moorsel CJ, Peters GJ, Pinedo HM. Gemcitabine: future prospects of single-agent and combination studies. Oncologist 1997;2:127–34.

[7] Carmichael J. Clinical response benefit in patients with advanced pancreatic cancer. Role of gemcitabine. Digestion 1997;58:503–7.

[8] Michael M, Moore M. Clinical experience with gemcitabine in pancreatic carcinoma. Oncology (Huntingt) 1997;11:1615–22.

[9] Tomaszewska R, Okon K, Nowak K, Stachura J. HER-2/Neu expression as a progression marker in pancreatic intraepithelial neoplasia. Pol J Pathol 1998;49:83–92.

[10] Sakorafas GH, Lazaris A, Tsiotou AG, Koullias G, Glinatsis MT, Golematis BC. Oncogenes in cancer of the pancreas. Eur J Surg Oncol 1995;21:251–3.

[11] Williams TM, Weiner DB, Greene MI, Maguire Jr HC. Expression of c-erbB-2 in human pancreatic adenocarcinomas. Pathobiology 1991;59:46–52.

[12] Lemoine NR, Hughes CM, Barton CM, et al. The epidermal growth factor receptor in human pancreatic cancer. J Pathol 1992;166:7–12.

[13] Ozawa S, Ueda M, Ando N, Abe O, Shimizu N. Epidermal growth factor receptors in cancer tissues of esophagus, lung, pancreas, colorectum, breast and stomach. Jpn J Cancer Res 1988;79:1201–7.

[14] Yokoyama M, Yamanaka Y, Friess H, Buchler M, Korc M. p53 expression in human pancreatic cancer correlates with enhanced biological aggressiveness. Anticancer Res 1994;14:2477–83.

[15] Hahn SA, Kern SE. Molecular genetics of exocrine pancreatic neoplasms. Surg Clin North Am 1995;75:857–69.

[16] Dergham ST, Dugan MC, Joshi US, et al. The clinical significance of p21 (WAF1/CIP-1) and p53 expression in pancreatic adenocarcinoma. Cancer 1997;80:372–81.

[17] Gjertsen MK, Gaudernack G. Mutated Ras peptides as vaccines in immunotherapy of cancer. Vox Sang 1998;74(2):489–95.

[18] Gunzburg WH, Salmons B. Novel clinical strategies for the treatment of pancreatic carcinoma. Trends Mol Med 2001;7:30–7.
[19] Jaffee EM, Hruban RH, Biedrzycki B, et al. Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. J Clin Oncol 2001;19:145–56.
[20] Kaufman HL, Di Jr VJ, Horig H. Immunotherapy for pancreatic cancer: current concepts. Hematol Oncol Clin North Am 2002;16:159–97.
[21] Buchler P, Reber HA, Buchler MC, et al. Therapy for pancreatic cancer with a recombinant humanized anti-HER2 antibody (herceptin). J Gastrointest Surg 2001;5:139–46.
[22] Xiong HQ, Abbruzzese JL. Epidermal growth factor receptor-targeted therapy for pancreatic cancer. Semin Oncol 2002;29:31–7.
[23] Buchsbaum DJ, Bonner JA, Grizzle WE, et al. Treatment of pancreatic cancer xenografts with Erbitux (IMC-C225) anti-EGFR antibody, gemcitabine, and radiation. Int J Radiat Oncol Biol Phys 2002;54:1180–93.
[24] Rosenblum MG, Cheung L, Mujoo K, Murray JL. An antimelanoma immunotoxin containing recombinant human tumor necrosis factor: tissue disposition, pharmacokinetic, and therapeutic studies in xenograft models. Cancer Immunol Immunother 1995;40:322–8.
[25] Rosenblum MG, Cheung L, Murray JL, Bartholomew R. Antibody-mediated delivery of tumor necrosis factor (TNF-alpha): improvement of cytotoxicity and reduction of cellular resistance. Cancer Commun 1991;3:21–7.
[26] Rosenblum MG, Horn SA, Cheung LH. A novel recombinant fusion toxin targeting HER-2/NEU-over-expressing cells and containing human tumor necrosis factor. Int J Cancer 2000;88:267–73.
[27] Lyu MA, Rosenblum MG. The immunocytokine scFv23/TNF sensitizes HER-2/neu-overexpressing SKBR-3 cells to tumor necrosis factor (TNF) via up-regulation of TNF receptor-α. Mol Cancer Ther 2005;4:1205–13.
[28] Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enyme Regul 1984;22:27–55.
[29] Lichtenstein A, Gera JF, Andrews J, Berenson J, Wara CF. Inhibitors of ADP-ribose polymerase decrease the resistance of HER2/neu-expressing cancer cells to the cytotoxic effects of tumor necrosis factor. J Immunol 1991;146:2052–8.
[30] Tang P, Hung MC, Klostergaard J. TNF cytotoxicity: effects of HER-2/neu expression and inhibitors of ADP-ribosylation. Lymphokine Cytokine Res 1994;13:117–23.
[31] Bruns CJ, Harbison MT, Kuniyasu H, Eue I, Fidler IJ. In vivo selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. Neoplasia 1999;150–62.
[32] Wuchter C, Karawajew L, Ruppert V, et al. Clinical significance of CD95, Bcl-2 and Bax expression and CD95 function in adult de novo acute myeloid leukemia in context of P-glycoprotein function, maturation stage, and cytogenetics. Leukemia 1999;13:1943–53.
[33] Lohrisch C, Piccart M. An overview of HER2. Semin Oncol 2001;28:3–11.
[34] Yarden Y. Biology of HER2 and its importance in breast cancer. Oncology 2001;61(2):1–13.
[35] Rubin I, Yarden Y. The basic biology of HER2. Ann Oncol 2001;12(l 1):S3–8.
[36] Hynes NE, Stern DF. The biology of erbB-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta 1994;1198:165–84.
[37] Singleton TP, Strickler JG. Clinical and pathologic significance of the c-erbB-2 (HER-2/neu) oncogene. Pathol Annu 1992;27:165–90.
[38] Stancovski I, Sela M, Yarden Y. Molecular and clinical aspects of the Neu/ErbB-2 receptor tyrosine kinase. Cancer Treat Res 1994;71:161–91.
[39] Torre EA, Salimbeni V, Fulco RA. The erbB 2 oncogene and chemotherapy: a mini-review. J Chemother 1997;9:51–5.
[40] Safran H, Steinhoff M, Mangray S, et al. Overexpression of the HER-2/neu oncogene in pancreatic adenocarcinoma. Am J Clin Oncol 2001;24:496–9.
[41] Thybusch-Bernhardt A, Aigner A, Beckmann S, Czubayko F, Juhl H. Ribozyme targeting of HER-2 inhibits pancreatic cancer cell growth in vivo. Eur J Cancer 2001;37:1688–94.
[42] Aigner A, Hsieh SS, Malerczyk C, Czubayko F. Reversal of HER-2 over-expression renders human ovarian cancer cells highly resistant to taxol. Toxicology 2000;144:221–8.
[43] Suzuki T, Anderegg B, Ohkawa T, et al. Adenovirus-mediated ribozyme targeting of HER-2/neu inhibits in vivo growth of breast cancer cells. Gene Ther 2000;7:241–8.
[44] Waldmann TA, Levy R, Coller BS. Emerging therapies: spectrum of applications of monoclonal antibody therapy. Hematology (Am Soc Hematol Educ Program) 2000;394–408.
[45] Butera J, Malachovsky M, Rathore R, Safran H. Novel approaches in development for the treatment of pancreatic cancer. Front Biosci 1998;3:E226–9.
[46] Pegram MD, Lopez A, Konecny G, Slamon DJ. Trastuzumab and chemotherapeutics: drug interactions and synergies. Semin Oncol 2000;27:21–5.
[47] Knuefermann C, Lu Y, Liu B, et al. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. Oncogene 2003;22:3205–12.
[48] Han Z, Hong L, Wu K, et al. Reversal of multidrug resistance of gastric cancer cells by downregulation of Akt1 with Akt1 siRNA. J Exp Clin Cancer Res 2006;25:601–6.
[49] Sasaki M, Kumazaki T, Tanimoto K, Nishiyama M. Bcl-2 in cancer and normal tissue cells as a predication marker of response to 5-fluorouracil. Int J Oncol 2003;22:181–6.
[50] Nagata S. Apoptosis by death factor. Cell 1997;88:355–65.
[51] Medema JP, Scaffidi C, Kischkel FC, et al. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 1997;16:2794–804.
[52] Ueno NT, Bartholomeusz C, Herrmann JL, et al. E1A-mediated paclitaxel sensitization in HER-2/neu-overexpressing ovarian cancer SKOV3.ip1 through apoptosis involving the caspase-3 pathway. Clin Cancer Res 2000;6:250–9.
[53] Keppler-Hafkemeyer A, Brinkmann U, Pastan I. Role of caspases in immunotoxin-induced apoptosis of cancer cells. Biochemistry 1998;37:16934–42.