Gastroenterology

Anti-cancer activity of anti-p185HER-2 ricin A chain immunotoxin on gastric cancer cells

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Abstract

Background and Aim: Overexpression of the human epidermal growth factor receptor 2 (HER-2) protein has been detected in gastric cancer and has been associated with an unfavorable prognosis. We investigated the anti-cancer effects of anti-p185HER-2 ricin A chain (RTA) immunotoxin, alone or in combination with 5-flurouracil on SGC7901-HER-2+ cells.

Methods: SGC7901-HER-2+ cells were obtained by transfecting SGC7901 cells with HER-2-pcDNA3.1. Anti-p185HER-2-RTA was prepared by chemical conjugation of anti-HER-2 monoclonal antibody (mAb) and RTA. The SGC7901-HER-2+ cells were incubated with RTA, anti-p185HER-2-RTA, and/or 5-flurouracil. The effects of drugs on cells were evaluated by MTT assay and Annexin V-fluorescein isothiocyanate and propidium iodide double staining flow cytometry. The expression of caspase-3, caspase-9, cyclooxygenase-2, and nuclear factor-κB/p65 were assayed by western blot. SGC7901-HER-2+ cells were transplanted into BALB/c nude mice to produce solid tumors in an attempt to study the immunotoxin activity in vivo.

Results: In vitro, anti-p185HER-2-RTA inhibited cell growth and induced apoptosis in SGC7901-HER-2+ cells. Anti-p185HER-2-RTA enhanced caspase-3 and caspase-9 activity, while downregulating the expression of cyclooxygenase-2 and nuclear factor-κB/p65. Its combination with 5-flurouracil further inhibited the growth of SGC7901-HER-2+ cells. In vivo, our data showed that anti-p185HER-2-RTA significantly inhibited the growth of SGC7901-HER-2+ cells-transplanted tumors.

Conclusions: Anti-p185HER-2-RTA inhibits the growth of SGC7901-HER-2+ cells. The effect may be related to the activation of caspase-3 and caspase-9 and inhibition of cyclooxygenase-2 and nuclear factor-κB/p65. Anti-p185HER-2-RTA plus 5-FU enhance anti-cancer activity, suggesting useful clues for further study for the treatment of HER-2 positive gastric cancers.

Key words

gastric cancer, human epidermal growth factor receptor 2, immunotoxin, ricin A chain, target therapy.

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Introduction

Gastric cancer is one of the most common cancers and is the second leading cause of cancer-related death in the world. A large proportion of patients are diagnosed when the tumor is at an unresectable stage. Although conventional chemotherapy, radiotherapy, and immunotherapy alone or in combination are active in metastatic disease, survival remains poor. Therefore, more effective therapeutic strategies are needed to improve clinical outcomes.

Human epidermal growth factor receptor 2 (HER-2) is a 185-kDa transmembrane tyrosine kinase receptor that belongs to the epidermal growth factor receptor family. Overexpression of HER-2 has been observed in various human cancers and has been linked to the malignant conversion of cancer cells. About 12–27% of gastric cancers exhibit overexpression of this receptor. Consequently, HER-2 could be a useful target for the treatment of gastric cancer. The use of monoclonal antibody (mAb) directed to HER-2 has been considered to be a useful approach for the specific targeting of cancer cells. HER-2-specific mAb trastuzumab, alone or in combination with chemotherapeutic drugs, has provided a survival benefit in patients with HER-2 positive breast cancer. Although the mAbs have shown promising results, treatment resistance remains a problem.

A method to improve the therapeutic activity of anti-HER-2 mAbs is to conjugate a toxin to create an immunotoxin; such compounds have displayed potent and selective killing of target cancer cells. Bacterial or plant toxins, such as Ricin A chain (RTA), gelonin, and Pseudomonas exotoxin A, have been used to prepare anti-HER-2 immunotoxins. Some previous studies showed that these immunotoxins displayed potent and specific anti-cancer activity in breast and ovarian cancers.
Moreover, immunotoxins have been shown to potentiate the cytoxic activity of chemotherapeutic agents\(^\text{13}\) and radionuclides.\(^\text{14}\) Therefore, the immunotoxins aroused great interest in HER-2 positive gastric cancers.

Immunotoxins can eliminate tumor cells not only by inhibiting protein synthesis but also by inducing apoptosis.\(^\text{13,15}\) Caspases have been recognized as critical mediators in the regulation of apoptosis. Caspase-9 is an important initiator caspase that is activated in the intrinsic pathway downstream of mitochondria, whereas caspase-3 performs a central role in both death-receptor and mitochondria-mediated apoptosis. It has been reported that the ricin or RTA immunotoxin’s effect is related to induction of apoptosis by activation of caspase-3.\(^\text{16,17}\) Several proteins, such as nuclear factor-κB (NF-κB) and cyclooxygenase-2 (COX-2), were associated with tumorigenesis in gastric cancers\(^\text{18,19}\) and might adversely affect the apoptotic process.\(^\text{20,21}\) However, the effect of HER-2 immunotoxins on them has not been established.

In the current study, we investigated the anti-cancer activity and possible mechanisms of the immunotoxin on HER-2 positive gastric cancer cells. We also explored the possibility of combining the immunotoxin and the chemotherapeutic agent 5-flourouracil (5-FU), which was commonly used in gastric cancer treatment, to augment the efficiency of killing target cells.

**Methods**

**Materials and drug preparation**

Samples of the human gastric cancer cell line SGC7901 were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The plasmid expressing HER-2 (HER-2-pcDNA3.1) was a generous gift from the National Key Laboratory of Infectious Diseases (Hangzhou, China). RTA was purchased from Sigma (St Louis, MO, USA). Mouse anti-human HER-2 monoclonal antibody was purchased from AbD Serotec (Oxford, UK). An Annexin V-fluorescein isothiocyanate (V-FITC) Kit was purchased from Bender Medical Systems GmbH (Vienna, Austria). Antibodies used in the western blot analysis were rabbit anti-active caspase-3 and caspase-9 (Chemicon, Temecula, CA, USA), which recognizes only the cleaved large subunit (17 kDa of caspase-3 and 37 kDa of caspase-9); rabbit anti-COX-2, rabbit anti-NF-κB/p65; and rabbit anti-β-actin polyclonal primary antibodies (Santa Cruz, CA, USA). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody. Six-week-old athymic female nude mice (BALB/c) were obtained from the Shanghai Cancer Institute and kept in laminar airflow benches. The animal study protocol complied with the guidelines of the Chinese Council for Animal Care and was approved by the Institutional Animal Care and Use Committee.

**Cell transfection**

The HER-2-pcDNA3.1 was transfected into SGC7901 cells using Lipofectamine 2000 (Gibco BRL, Paisley, UK) according to the manufacturer’s instructions. After transfection, individual colonies were picked and expanded in the presence of 300 μg/mL of G418 (geneticin) (Gibco BRL). Stable HER-2 expression in SGC7901 cells was confirmed by western blot analysis. Blots were analyzed for measurement of the band intensities.

**Preparation of immunotoxin**

To prepare the immunotoxin, RTA was conjugated to the anti-HER-2 mAb using the heterobifunctional reagent N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) (Pierce, Rockford, IL, USA)\(^\text{22}\) at a 1:1 molar ratio. Gel filtration chromatography on Blue-Sepharose 4B (GE Healthcare, Piscataway, NJ, USA) with 0.1 M phosphate-buffered saline (PBS) (pH 7.5) was used to absorb the components that contained RTA and removed the excessive SPDP and mAb fragments. Next, the conjugate (anti-p185\(^\text{HER-2}\)-RTA) was separated from the free toxin by gel filtration on Sephadex G-100 (GE Healthcare) eluted with 0.1 M PBS (pH 7.5).

**Cell culture and treatments**

The SGC7901 cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA) in an air incubator containing 5% CO\(_2\) at 37°C. When the adherent cells became confluent, cells were treated with the different drugs at the indicated concentrations for 48 h. The drugs included anti-HER-2 mAb (1 μg/mL), RTA (0.01, 0.1, or 1 μg/mL), anti-p185\(^\text{HER-2}\)-RTA (0.01, 0.1, or 1 μg/mL), and 5-FU (20 μg/mL) alone, as well as anti-p185\(^\text{HER-2}\)-RTA (0.01, 0.1, or 1 μg/mL) combined with 5-FU (20 μg/mL). With respect to anti-p185\(^\text{HER-2}\)-RTA, the 0.01, 0.1, or 1 μg/mL concentrations referred to the mass concentrations of RTA.

**Growth proliferation assay**

*In vitro*, the growth inhibition effect of drugs on SGC7901 cells was determined by MTT (Sigma) dye absorbance of living cells. Cells (1 × 10\(^6\) cells per well) were seeded in 96-well plates. After exposure to the different drugs for 48 h, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and the plates were incubated for an additional 4 h at 37°C. The MTT solution in the medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 150 μL of dimethylsulfoxide (DMSO) was added to each well before absorbances (A) at 570 nm were measured. The cell survival was calculated as the ratio of A\(_{570\text{nm}}\) in wells containing a drug to that in control wells with no drug. The percent growth inhibition (GI) was calculated according to the formula: GI = (1 - A\(_{\text{drug}}\)/A\(_{\text{control}}\)).

**Flow cytometry analysis of apoptosis**

Flow cytometry was used to analyze the effect of the drugs on cell apoptosis. The Annexin V-FITC/propidium iodide (PI) apoptosis detection kit was used to measure typical apoptosis and necrosis according to the manufacturer’s instructions. Briefly, suspensions were washed twice and adjusted to a concentration of 1 × 10\(^6\) cells/mL with ice-cold PBS. Next, they were double stained with Annexin V-FITC and propidium iodide for at least 20 min at room temperature in the dark, after which PBS binding buffer was added without washing. Flow cytometry was carried out with a 488-nm laser coupled to a cell sorter (FacsCalibur;
BD Biosciences, San Jose, CA, USA). Cells stained only with Annexin V-FITC were considered early apoptotic, whereas cells stained with both propidium iodide and Annexin V-FITC were considered late apoptotic.

**Western blot assay**

Cells were harvested and washed three times with ice-cold PBS after treatments. Cell lysates were prepared for western blot analysis of caspase-3, caspase-9, COX-2, NF-κB/p65, and β-actin using whole cellular protein extraction kits (Active Motif, Carlsbad, CA, USA). A DC protein assay kit (Bio-Rad, Richmond, CA, USA) was used to determine the protein concentration in each cell lysate. Protein was mixed with 2× sodium dodecyl sulfate (SDS) sample buffer. Forty micrograms of protein were separated in a 10% polyacrylamide gel and blotted on a nitrocellulose membrane (Bio-Rad). Nitrocellulose membranes were blocked with 5% BSA (Sigma) in TBS (25 mM tris-HCl, 150 mM sodium chloride, pH 7.2) for 2 h at room temperature. Blots were incubated with anti-caspase-3, anti-caspase-9, anti-COX-2, anti-NF-κB/p65, or anti-β-actin specific rabbit polyclonal IgG primary antibody at a 1:500 dilution overnight at 4°C. Blots were washed in washing buffer (PBS with 0.1% Tween-20) three times and then incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:2000 dilutions) for 2 h at room temperature. All blots were developed using the enhanced chemiluminescence (ECL) detection system (Supersignal Dura kit, Pierce) following the manufacturer’s instructions. Blots were scanned and analyzed for measurement of the band intensities. The expression of β-actin was used as a loading control.

**In vivo study**

Human cancer xenograft models were established using methods reported previously. Aliquots of 1×10⁷/mL SGC7901-HER-2+ cells were injected subcutaneously into the right hypochondrium of BALB/c nude mice in a volume of 0.2 mL. Animals were randomly divided into eight groups as follows: (i) normal control; (ii) 1 μg anti-p185HER-2-RTA; (iii) 10 μg anti-p185HER-2-RTA; (iv) 100 μg anti-p185HER-2-RTA; (v) 50 mg/kg 5-FU; (vi) 1 μg anti-p185HER-2-RTA + 50 mg/kg 5-FU; (vii) 10 μg anti-p185HER-2-RTA + 50 mg/kg 5-FU; and (viii) 100 μg anti-p185HER-2-RTA + 50 mg/kg 5-FU. The above 0.01, 0.1, or 1 μg referred to the mass of RTA in anti-p185HER-2-RTA. All drugs and isometric normal saline were given intraperitoneally twice per week. The length (L) and the width (W) of the transplanted tumor mass were measured on the 1st, 7th, 14th, 21st, 28th, and 35th day following the initial injection. The mice were killed on the 36th day and the tumors were dissected and weighed individually. Tumor volume (TV) was estimated using the formula TV (mm³) = L (mm) × W² (mm²)/2 as previously reported.

**Statistics**

All results are represented as mean ± SD. Statistical differences were calculated using Student’s t-tests. SPSS 13.0 was used to analyze the data; P-values < 0.05 were considered to be significant.

**Results**

**Transfection of HER-2-pcDNA3.1 into SGC7901 cells**

The SGC7901-HER-2+ cells were obtained by transfecting SGC7901 cells with HER2-pcDNA3.1. Western blot analysis (Fig. 1) demonstrated the HER-2-transfected clones (Lane 1 and 2) expressed 20 times more HER-2 than untransfected cells (Lane 3 and 4).

**Effect of anti-HER-2 mAb on cells**

The effect of anti-HER-2 mAb was tested in our study. Two cells were incubated with anti-HER-2 mAb (1 μg/mL). The inhibition of cell growth caused by the anti-HER-2 mAb was measured by MTT assay (Fig. 2a) and the percentage of apoptotic cells was detected by Annexin V-FITC and PI double staining methods (Fig. 2b). The absorbance at 570 nm in the anti-HER-2 mAb group was similar to that of the control group in both the SGC7901-HER-2+ and the untransfected SGC7901 cell lines, and so was the percentage of apoptotic cells. Anti-HER-2 mAb alone had little effect on the proliferation and apoptosis of cells.
Growth inhibition of SGC7901-HER-2+ cells

The SGC7901-HER-2+ cells were incubated with RTA (0.01, 0.1, and 1 μg/mL), anti-p185HER-2-RTA (0.01, 0.1, and 1 μg/mL), and/or 5-FU (20 μg/mL), and the inhibition of cell growth caused by the drugs was measured by MTT assay (Fig. 3). The SGC7901-HER-2+ cells treated with anti-p185HER-2-RTA exhibited concentration-dependent inhibition of cell growth. The GIs (48 h) induced by anti-p185HER-2-RTA (0.01, 0.1, or 1 μg/mL) alone or in combination with 5-FU for SGC7901-HER-2+ cells were significantly higher than those for untransfected SGC7901 cells. For treatment with RTA, there was no significant difference at any concentration between the GIs for SGC7901-HER-2+ cells and untransfected SGC7901 cells (P > 0.05). The GIs of anti-p185HER-2-RTA in combination with 5-FU were significantly higher than when either drug was used alone for SGC7901-HER-2+ cells.

Induction of apoptosis in SGC7901-HER-2+ cells

We next investigated the effect of different concentrations of anti-p185HER-2-RTA and/or 5-FU on SGC7901-HER-2+ cell apoptosis. The percentage of apoptotic cells was detected by Annexin V-FITC and PI double staining methods (Fig. 4a,b). Anti-p185HER-2-RTA induced SGC7901-HER-2+ cell apoptosis in a
Effects of anti-p185HER-2-RTA alone or in combination with 5-FU on SGC7901-HER-2+ cells in vivo

We observed the tumor volumes of the transplanted tumor mass on the 1st, 7th, 14th, 21st, 28th, and 35th day following the initial injection. The mice were killed on the 36th day and the tumors were dissected and weighed individually. The tumor growth situations of different groups on the 36th day and the tumor masses dissected from the nude mice are shown in Figure 6a,b. We found that the tumor volume and weight increased much less in the anti-p185HER-2-RTA treatment groups than in the control group. Moreover, the effect was progressive and in a concentration-dependent manner (Fig. 6c,d). A greater inhibition of tumor growth was obtained in the combination groups than in groups of either single agent.

Discussion

Overexpression of HER-2 has been reported in several human cancers, including gastric cancer, and has been associated with an unfavorable prognosis. Recently, HER-2 has been shown to be an attractive target for the treatment of gastric cancer. In experimental models, preclinical data show significant anti-cancer efficacy of anti-HER-2 therapies (e.g. immunotoxins that target HER-2) against the growth of human gastric cancer with HER-2 overexpression in vitro and in vivo.29 To improve clinical responses, however, combination therapies with standard chemotherapeutic drugs are required.26,27

In the present study, we investigated the effect of an immunotoxin composed of anti-HER-2 mAb and RTA on gastric cancer cells. The mAb targeting HER-2 had shown little effect on the proliferation and apoptosis of cells. Our data demonstrated that anti-p185HER-2-RTA targeted SGC7901-HER-2+ cells and inhibited the growth of SGC7901-HER-2+ cells. The anti-cancer effect of anti-p185HER-2-RTA might be correlated with the activation of caspase-9 and caspase-3 and downregulation of COX-2 and NF-kB/p65. In addition, anti-p185HER-2-RTA in combination with 5-FU enhanced anti-cancer activity.

The present data showed that anti-p185HER-2-RTA decreased cell proliferation in a concentration-dependent manner. The GIs (48 h) of anti-p185HER-2-RTA for SGC7901-HER-2+ cells were significantly higher than those for untransfected SGC7901 cells, whereas RTA had similar effects on the two types of cells. Our results indicated that anti-p185HER-2-RTA targeted SGC7901-HER-2+ cells and displayed greater anti-cancer activity than RTA. This finding is consistent with Shinohara et al.’s28 report that anti-HER-2 immunotoxin has specific anti-cancer activities against gastric cancer cells overexpressing HER-2.

Several previous studies reported that immunotoxins’ effect related to apoptosis.14,15 Abnormal apoptosis may break the balance between cell death and cell proliferation, which may lead to tumorigenesis, progression, and treatment failure.29 Consequently, modulating apoptotic susceptibility should be considered.
a critical goal in cancer treatment. In our study, induction of apoptosis by anti-p185HER-2-RTA in a concentration-dependent manner was confirmed by flow cytometry analysis, suggesting that induction of SGC7901-HER-2+ cells apoptosis is an important mechanism for the anti-cancer effect of anti-p185HER-2-RTA. By detecting the essential proteins in the caspase cascade by western blot analysis, we found that anti-p185HER-2-RTA could activate caspase-9 and caspase-3. This finding indicates that caspase-9/caspase-3-mediated apoptosis may be involved in the anti-cancer activity of anti-p185HER-2-RTA against SGC7901-HER-2+ cells.

Nuclear factor-κB is a transcription factor for the inducible expression of genes, including COX-2 that may be related to cell proliferation. NF-κB might also protect cancer cells from apoptosis by activating anti-apoptotic genes.\textsuperscript{20} Other reports have shown that NF-κB inhibition can induce apoptosis and increase chemotherapeutic sensitivity.\textsuperscript{31,32} The activated form of NF-κB is a heterodimer, which usually consists of p65 and p50 subunits. Yamanaka et al.\textsuperscript{33} used anti-p65 immunostaining to show that NF-κB is constitutively activated in gastric carcinoma. In the current study, western blot analysis illustrated that anti-p185HER-2-RTA could downregulate NF-κB/p65 expression, which implies that inhibiting NF-κB/p65 expression might be correlated to the anti-cancer effect of anti-p185HER-2-RTA.

COX-2, an induced form of COX, is frequently overexpressed in gastric cancer\textsuperscript{34} and has been associated with tumor angiogenesis, tumor progression, and patient survival.\textsuperscript{35} COX-2 can upregulate Bcl-2, which may contribute to decreased cytochrome c release and further reduced caspase-9 and caspase-3 activation, thus resulting in the inhibition of apoptosis.\textsuperscript{21} Therefore, anti-p185HER-2-RTA-induced activation of caspase-9 and caspase-3 may
be linked to the downregulation of COX-2 expression. Previous studies have shown that HER-2 can regulate COX-2 expression through direct transcriptional mechanisms, while other studies have shown that activation of the HER-2 signaling pathway is associated with expression of COX-2. Thus, downregulation of COX-2 may be related to the inhibition of HER-2 in anti-p185HER-2-RTA treated cells.

A recent study from Kim and colleagues has shown that trastuzumab in combination with chemotherapeutic agents clinically used for gastric cancers, such as cisplatin, 5-FU, and oxaliplatin, can exert synergistic or additive effects. Nevertheless, reports about the effects of anti-HER-2 immunotoxin plus chemotherapeutic agents used for gastric cancers are rare. In the present study, we first confirmed that anti-p185HER-2-RTA in combination with 5-FU enhanced the anti-cancer effect in SGC7901-HER-2+ cells and induced significantly more apoptotic cells than each drug did alone. Moreover, the combination therapy could further enhance caspase-3 and caspase-9 activity. Detailed mechanisms of the combination therapy should be investigated further, and efforts to understand them are currently under way.

In the present study, we also tried to investigate the anti-cancer effects of anti-p185HER-2-RTA in vivo on tumors that developed from SGC7901-HER-2+ transplanted cells. In the nude mice model, anti-p185HER-2-RTA could inhibit the growth of tumors. The interactions between the immunotoxin and 5-FU may permit effective treatment with lower doses of each agent. However, our data are preliminary and require studies with a larger number of samples to clarify the effect of anti-p185HER-2-RTA.

Kim et al. have also reported several natural HER-2-amplified gastric cancer cell lines, such as SNU-216 and NCI-N87, in which
Figure 6  (a) The tumor growth situations of different groups in SGC7901-HER-2+ cells-transplanted nude mice on the 36th day after initial injection as described in the Materials and methods section. (b) The tumor masses of different groups dissected from the nude mice. (c) The tumor volumes of different groups after treatment with anti-p185HER-2-RTA and/or 5-FU. Tumor volumes were measured on the 1st, 7th, 14th, 21st, 28th, and 35th day following the initial injection as described in the Materials and methods section. (d) The tumor weights of different groups after treatment with anti-p185HER-2-RTA and/or 5-FU. The mice were killed by cervical dislocation on the 36th day after tumor administration, and the tumors were dissected and weighed individually.
the anti-cancer effect of anti-HER-2 mAbs has been proved. Our study mainly compared the effect of anti-p185HER-2-RTA in HER-2 expressing and non-expressing cell lines. Different consequences may occur in tumors with native HER-2 expression. It may be affected by the number of HER-2 binding sites, the sensitivity of different cells to drugs and some other factors, and further studies are focused on them.

In conclusion, the present study shows that anti-p185HER-2-RTA targets SGC7901-HER-2+ cells and inhibits the growth of SGC7901-HER-2+ cells. Anti-p185HER-2-RTA in combination with 5-FU can enhance anti-cancer activity. The anti-cancer effects of anti-p185HER-2-RTA may be correlated with activation of caspase-9 and caspase-3 and downregulation of COX-2 and NF-xB/p65. Our study provides useful clues for further research on anti-p185HER-2-RTA alone or combination chemotherapy for the treatment of HER-2 positive gastric cancers.

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