Research Article

Construction and Characterization of a Novel Fusion Protein MG7-scFv/SEB against Gastric Cancer

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Antibody-targeted superantigen has been developed into a new strategy to treat many malignant tumors. In this study, for specific targeting to gastric cancer cell, superantigen SEB (Staphylococcal Enterotoxin B) was genetically fused to the single-chain variable fragment of gastric carcinoma-associated antibody MG7(MG7-scFv) that recognizes the MG7 antigen frequently expressed in gastric cancer cell. The recombinant MG7-scFv/SEB fusion proteins are expressed in E. coli as inclusion bodies, and the purified MG7-scFv/SEB retains high binding affinity with gastric cancer cell SGC-7901 (positive MG7 antigen expression). When incubated with effector cell—peripheral blood mononuclear cells (PBMCs), MG7-scFv/SEB could effectively inhibit the proliferation and induce apoptosis of SGC-7901. After being treated with MG7-scFv/SEB, PBMCs remarkably increased the production of Th1 cytokines (IFN-gamma, IL-2), and slightly increased the production of Th2 cytokines (IL-4, IL-10) in vitro. It was observed that gastric-tumor-bearing rats administrated with MG7-scFv/SEB showed more inflammatory cell infiltration, more significant tumor inhibition, and longer survival time than those of rats treated with SEB or NS (Normal Saline). The data indicated that MG7-scFv/SEB fusion protein could specifically target gastric cancer cell, enhance the activity of T cells and induce tumor cell apoptosis to exert the antitumor effect on gastric cancer.

1. Introduction

In recent decades, oncologists have made strenuous attempts to improve the prognosis of gastric cancer patient; however, due to unresponsiveness to majority of the available therapies, gastric cancer still ranks as one of the most frequent and lethal cancers [1–3].

Therefore, alternative treatment approaches have been made to treat malignant tumor, such as monoclonal antibody that has become a new potential immunotoxin for anticancer therapy [4–7]. Nevertheless, monoclonal antibody has some deficiencies, such as high molecular weight, low penetration ability, slow clearance in the blood [5, 8–10], and so forth. These deficiencies result in the low tumor-to-blood biodistribution ratio [11–15]. In addition, patients may produce human anti-mouse antibody (HAMA) against most antibodies used for clinical therapy as they are from murine hybridoma [15, 16]. How can we circumvent these problems and enhance the antitumor effect of the immunotoxin?

Single-chain variable fragment (scFv), denoted as the fusion of the immunoglobulin heavy and light chain variable regions, can partially get rid of the shortcomings of the whole antibody [17]. The scFv that consists of variable heavy-chain antibody connected to the variable light chain by a flexible linker is the smallest antibody fragment that retains the entire antigen-binding region for a particular antibody [18, 19], and the molecular weight of scFv is only one-sixth of the whole antibody [20]. Recently, scFv has had possible clinical utility [18, 21, 22].

The other part of this immunotoxin is the superantigen. Superantigen is a family of bacterial or viral protein, named from its ability to polyclonally activate large fractions (2%–20%) of the T-cell population cells at picomolar concentrations [23, 24]. Its unique feature is that it bypasses antigen-processing mechanism, specifically binds to T-cell receptor variable region beta-chain (TCR-Vβ) segment, and forms a trimolecular complex along with major histocompatibility complex class II (MHC-II) [23, 25–27]. This trimolecular
complex can trigger T cells proliferation and affect cytokines production (e.g., IFN-γ, TNF-alpha, IL-2, and IL-12) and strong cytolytic activity [28–30]. This discovery confirms the effective application of superantigen in tumor immunotherapy. SEB, a well-characterized 28-kDa exotoxin produced by some strains of staphylococcus aureus, is one of the most commonly used exogenous superantigens for tumor therapy [30–32].

Here, we constructed a fusion protein consisting with chemically synthesized MG7-scFv against human gastric-cancer-associated antigen MG7 and superantigen SEB. The fusion protein was expressed in E. coli [33]. Then the antigen-binding activity of the fusion protein was determined by cell-ELISA and the antitumor effect was examined both in vitro and in vivo. So far as we know, it is the first time to report on a preliminary investigation into the target antitumor effect of MG7-scFv/SEB fusion protein on gastric cancer.

2. Materials and Methods

2.1. Reagents. The expression vector pET-32a (+) was purchased from Novagen (Germany). All enzymes were purchased from TaKaRa (Japan). Anti-SEB rabbit polyclonal antibody and HRP-labeled goat anti-rabbit were purchased from Novagen (Germany). All enzymes were purchased from Shanghai No. 2 Chemical Reagent Factory (China).

2.2. Cell Lines and Animals. Human gastric adenocarcinoma SGC-7901 cell line and human normal gastric mucosal cell lineGES-1 were preserved by our Laboratory. PBMCs were separated from healthy volunteers. Cells were maintained in RPMI-1640 and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO2. The steps for sonication and centrifugation were repeated five times. The inclusion bodies were dissolved in freshly prepared denaturation buffer (8 M guanidine hydrochloride, 0.1 M Tris-HCl, 2 mM EDTA, 10 mg/mL DTE, pH 8.0), incubated at room temperature for 4 hours, and then centrifugated at 18,000 g for 20 minutes at 4°C. The supernatant was purified by Ni2+-His Bind Resin affinity chromatography, and the purified fusion protein was eluted with 300 mM of imidazole. The solution was slowly dialyzed against renaturation solution I (2 M urea, 50 mM Tris-HCl, 2 mM GSH, and 1 mM GSSG) for 24 hours at 4°C, then continued dialyzed against renaturation solution II (50 mM Tris–HCl, 5 mM EDTA, pH 8.0) for 10 hours at 4°C. Then, the upstream 158-residue N-terminal fusion sequence was cleaved from enterokinase recognition sequence DDDDK by enterokinase. Digested proteins were dialyzed against 10 mM Tris-HCl pH 7.5 and then proteins were applied to a Superdex-75 column (2.6 × 100 cm) and eluted in 10 mM PBS (pH 7.6) containing 0.15 M NaCl. Fractions containing proteins of interest were collected and dialyzed against 10 mM Tris–HCl pH 7.5. The fusion protein was confirmed by Western blot, and stored at −70°C for further analysis.

2.4. Antigen-Binding Activity. Briefly, SGC-7901 or GES-1 cells were suspended at a concentration of 2 × 105/mL. 100 μL of the cell suspension was added to 96-well microplate, and incubated at 37°C for 48 hours. After discarding the medium, the plate was washed three times by filling the wells with 200 μL PBS, and then fixed with cold methanol/aceton solution (1:1) for 30 minutes, blocked with 0.3% H2O2/methanol at 37°C for 30 minutes. Serial diluted concentrations of MG7-scFv/SEB and SEB were added to the wells, respectively, (NS was added as negative control) and incubated at 37°C for 60 minutes. Washed twice with PBS, the well was then added with rabbit anti-SEB antibodies, incubated at 37°C for another 60 minutes. Washed twice again, the well was added with sheep anti-rabbit secondary antibodies, incubated at 37°C for 30 minutes. Each well was added with TMB (3,3′, 5,5′-tetramethylbenzidine) solution, incubated for 30 minutes, and then added with equal volume of 2 M H2SO4, and the optical density was read at 450 nm.

2.5. Effect of MG7-scFv/SEB on Gastric Cancer Cell Proliferation In Vitro. MG7 antigen positive-expression SGC-7901 cells were used as target cells. PBMCs were used as effector cells. SGC-7901 cells were suspended at a concentration of 5 × 104/mL, and 100 μL of the cell suspensions were added to 96-well microplate. After an overnight incubation, the cells were co-cultured with PBMCs at effector: target (E: T) ratios of 10: 1, and treated with various concentrations (0.01 μg/mL, 0.1 μg/mL, 1.0 μg/mL, and 10 μg/mL) of MG7-scFv/SEB or SEB for 48 hours, respectively. Moreover,
cells were incubated with 1.0 μg/mL of MG7-scFv/SEB for different periods of time (0, 6, 12, 24, 48, and 72 hours). The cell viability after treating with MG7-scFv/SEB or SEB was measured by cell counting kit-8 (CCK-8) assay. In brief, CCK-8 (10 μL) was added to each well of a 96-well microplate containing 100 μL of culture medium and incubated at 37°C for 2 hours. The absorbance was then measured at 470 nm using a Bio-Rad 550 spectrophotometric microplate reader. Six wells were repeated per group and all experiments were performed in triplicate.

2.6. Effect of MG7-scFv/SEB on Gastric Cancer Cell Apoptosis. SGC-7901 cells were incubated in 60 mm culture dish with PBMCs, allowed to adhere overnight, and then treated with different concentrations (0.1 μg/mL, 1.0 μg/mL, and 10 μg/mL) of MG7-scFv/SEB or NS (normal sodium). Each group of cells was harvested after 24 hours. The cell suspension was pelleted by centrifugation at 800 g for 5 minutes and resuspended in 400 μL lysis buffer, mixed by gentle vortex. The sample was incubated with proteinase K (10 μg/mL) at 65°C for 120 minutes, and centrifuged for 10 minutes to precipitate DNA. After supernatant removal, DNA pellet was washed with 750 μL 70% ethanol, centrifuged again to remove trace ethanol, and air dried for 10 minutes at room temperature. Each test sample was resuspended in the gel-loading buffer, and then 15 μL of each sample solution was loaded to a 1.5% agarose gel and electrophoresed at 100 V for 45 minutes. Ethidium bromide-stained DNA could be visualized by transillumination with UV light and photographed.

2.7. Cytokines Release In Vitro. PBMCs were adjusted to 1 × 10^6/mL. 100 μL of the cell suspension was seeded in 96-well microplate, and incubated at 37°C for 1 hour to adhere. The cells were cocultured with 1.0 μg/mL concentrations of SEB and MG7-scFv/SEB, respectively, (NS used as negative control). The supernatant was harvested at 12, 24, 36, 48,
and 72 hours after incubation. Then, the cytokines (IL-2, IFN-gamma, IL-4, and IL-10) secreted by PBMCs were measured using a standard sandwich ELISA technique with corresponding kits.

2.8. Inhibition Effect of MG7-scFv/SEB on Gastric Cancer In Vivo. Rats models of gastric cancer were established by inoculating $1 \times 10^7$ SGC-7901 cells into left hind limbs of rats. After 12 days, the mean diameter of tumor nodule was nearly 1.0 cm. Sixty tumor-bearing rats were randomly divided into 3 groups: NS control group, SEB-treated group, and MG7-scFv/SEB-treated group. Then, rats in MG7-scFv/SEB group were administrated with $100 \mu$g/mL MG7-scFv/SEB via the vena caudalis, once every other day for 6 times. The equivalent quality of SEB or NS was administrated in SEB group or control group, respectively. One-half rats in each group were sacrificed on the 15th day, and the subcutaneous tumors were isolated and weighed. The other half of rats in each group were observed for their survival period.

2.9. Inflammatory Cells Infiltration and Necrosis of Tumor Nodule. The tumor nodules were taken from tumor-bearing rats on the 15th day after the first injection. The subcutaneous tumor nodules were fixed in 10% formalin, and embedded in paraffin. Sections were observed by hematoxylin and eosin (H&E) dyeing. Grading evaluation criteria were referred to Q Wang (35). Briefly, the evaluation of tumor necrosis was $\sim$, no necrosis; $+$, less than 1/3 of the tumor size; $++$, 1/3-2/3 of the tumor size; $+++$, more than 2/3 of the tumor size. The evaluation of the infiltration of inflammatory cell both in and around the tumor was $\sim$, no inflammatory cell infiltration; $+$, minimal inflammatory cell infiltration; $++$, medium inflammatory cell infiltration; $+++$, intense inflammatory cell infiltration.

2.10. Statistical Analysis. All the results are expressed as mean $\pm$ standard error of the mean (SEM). Student’s $t$-test or ANOVA was used to analyze the results. $P < .05$ was considered statistically significant.

3. Results

3.1. Construction and Expression of MG7-scFv/SEB. The expression vector pET32a(+)MG7-scFv/SEB was constructed for the production of fusion protein MG7-scFv/SEB, in which the superantigen SEB was linked to the C-terminal of the anti-MG7 scFv via a (Gly4Ser)$_3$ flexible linker facilitating both correct folding of the MG7-scFv and SEB (Figure 1). The expression of the fusion protein MG7-scFv/SEB was confirmed by Western blot analysis.

Table 1: The infiltration of inflammatory cell and necrosis of tumor nodule in gastric-cancer-bearing rats after combined therapy with MG7-scFv/SEB, SEB, or NS.

<table>
<thead>
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<th>Groups</th>
<th>Tumor necrosis$^1$</th>
<th>Infiltration of inflammatory cells inside the tumors$^2$</th>
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<td>NS</td>
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<td>MG7-scFv/SEB</td>
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$^1$Tumor necrosis: $\sim$, no necrosis; $+$, less than 1/3 of the tumor size; $++$, 1/3-2/3 of the tumor size; $+++$, more than 2/3 of the tumor size.

$^2$Infiltration of inflammatory cells: $\sim$, no infiltration; $+$, minimal infiltration; $++$, medium infiltration; $+++$, intense infiltration.
Figure 6: Production of cytokines by stimulated PBMCs: (a) IFN-γ production, (b) IL-2 production, (c) IL-4 production, (d) IL-10 production. The productions of IFN-γ and IL-2 are much more than those of IL-4 and IL-10 (P < .05). It indicated that SEB mainly activates Th1 cell to upregulate cellular immunity; meanwhile, it slightly activates Th2 cell.

using the anti-SEB antibody. As shown in Figure 2, Western blotting analyses revealed that a protein of about 76 kDa was strongly expressed before digestion with enterokinase, and a protein of about 56 kDa was expressed after digestion with enterokinase.

3.2. Binding Potential of MG7-scFv/SEB to SGC-7901. The binding potential of MG7-scFv/SEB to SGC-7901 gastric cancer cells was examined by using cell-ELISA. As shown in Figure 3(a), the MG7-scFv/SEB fusion protein demonstrated a high binding capacity to the SGC-7901 cells, whereas recombinant SEB exhibited very low binding affinity. As shown in Figure 3(b), both MG7-scFv/SEB fusion protein and SEB demonstrated a poor binding capacity with control cell GES-1 (human normal gastric mucosal cell line, negative expression of MG7 antigen). These findings indicated that MG7-scFv/SEB fusion protein can specifically target MG7-positive cell with high affinity.

3.3. Antitumor Effect of MG7-scFv/SEB on Gastric Cancer In Vitro. As shown in Figure 4, both SEB and MG7-scFv/SEB could effectively inhibit the proliferation of SGC-7901 cell which was cocultured with PBMCs, and the inhibitory effect exhibited a time- and dose-dependent manner (part of data not shown). In the absence of effective cell, the fusion protein MG7-scFv/SEB had no obvious inhibition effect of SGC-7901 cell. The data indicated that the antitumor effect of MG7-scFv/SEB fusion protein is dependent on the function of PBMC.

The characteristics of DNA ladders indicated inter-nucleosomal cleavage of DNA during apoptosis. The results of agarose gel electrophoresis of DNA for the SGC7901 cells showed an overt ladder shaped band in 1.0 μg/mL and 10 μg/mL MG7-scFv/SEB-treates group; the ladder-shaped band became weaker in 0.1 μg/mL treated group, and no DNA ladders were detected in NS treating group (Figure 5).
For this reason, monoclonal antibody against MG7 has been recognized as an effective strategy for targeting therapy of gastric cancer.

In this study, we synthesized scFv against MG7, fused it with SEB to construct MG7-scFv/SEB fusion protein, and detected its biological characteristics. Cell-ELISA results showed that MG7-scFv/SEB fusion protein could specifically target MG7-positive gastric cancer cell SGC-7901 with high affinity, suggesting the correct folding of fusion protein during the process of renaturation. CCK-8 assay indicated that the fusion protein MG7-scFv/SEB had significant inhibitory effect on SGC7901; nevertheless, there was no obvious influence on the cell negative expression of MG7 antigen. Some further experiments showed that MG7-scFv/SEB could mainly increase the production of Th1 cytokines (IL-2, IFN-gamma) and activate cellular immunity to mediate tumor cell apoptosis and necrosis. These results indicated that MG7-scFv/SEB was not only endowed with the specificity target to the MG7 antigen, but also maintained the immunogenicity of superantigen SEB. Given the results we had, SEB was apparently more effective to produce cytokines and inhibit the proliferation of tumor cell than MG7-scFv/SEB at the same concentration. We considered that it was probably due to smaller molecular weight of SEB than that of MG7-scFv/SEB. But taking the antigen-specific antitumor effect into account, MG7-scFv/SEB is more potential for clinical application than SEB as the less cytotoxic effect on normal cells.

We used rat as the recipient in tumor xenograft model because rat has intact immune system which is necessary for specificity of SEB. Although the immune system of rat somehow can reject human tumor cells, the baseline of especially the latter. The results in Figure 8 showed that the survival times of the rats in MG7-scFv/SEB and SEB groups were longer than that of NS group within 60-day observation ($P < .05$). These results indicated that MG7-scFv/SEB fusion protein effectively inhibited gastric cancer growth in vivo.

As shown in Table 1, extensive inflammatory cells were observed in tumor nodules of gastric-cancer-bearing rats treated with MG7-scFv/SEB; relatively less inflammatory cells were present in the tumors of SEB-treated rats; little inflammatory cells were found inside and around tumors of NS control group.

4. Discussion

SEB, an exotoxin produced by some strains of staphylococcus aureus, is a bacterial superantigen that has been deeply studied in tumor therapy [30, 31, 35]. As a superantigen, SEB can polyclonally activate CTL (cytotoxic T lymphocytes), and massively produce cytokines in vivo [31]. It is capable of killing tumor cells with similar injuring effect on normal cells, to some extent, limiting its clinical application. The application of hybridoma technology and genetic engineering technology to fuse superantigen with a tumor-specific vector to generate a fusion protein that can selectively target tumor cells can effectively limit these adverse effects. The antigen of MG7 had high specificity to gastric cancer, whose positive rate in gastric cancer was 82.8%–91.2% [36, 37]. For this reason, monoclonal antibody against MG7 has been recognized as an effective strategy for targeting therapy of gastric cancer.
rejection is same for all groups. So this model still can
tell the difference between different groups even if there is
xenograft rejection. Tumor-bearing rats model experiment
showed that the average weight of tumor nodules in MG7-
scFv/SEB group was significantly lighter than those in SEB
or NS group ($P < .05$) at the same injection volume. The
survival time of tumor-bearing rats in MG7-scFv/SEB group
was prolonged compared with those in other two groups
($P < .05$). The immunohistochemistry results of tumor
nodule showed that large number of inflammatory cells
(neutrophils, lymphocytes, and monocytes) existed inside
and around the tumor nodules in MG7-scFv/SEB-treated
group ($P < .05$). The data suggested that MG7-scFv/SEB
could effectively activate T cell to inhibit the growth of gastric
cancer cell in vivo. Interestingly, the antitumor effect of
MG7-scFv/SEB was significantly stronger than that of SEB in
the same dosage. Maybe it was due to the specifically target
ability to tumor cell of MG7-scFv/SEB.

Taken together, we finished the construction, expression,
and purification of fusion protein MG7-scFv/SEB. The
fusion protein retains high binding affinity to MG7-positive
gastric cancer cell SGC-7901 and the immunogenicity of
SEB. These characteristics ensure that MG7-scFv/SEB can
specifically kill tumor cell without obvious side effect on
normal cell. Our data provided a basis for further investigation
of tumor immunotherapy.

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