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Original article

The tetravalent anti-DR5 antibody without cross-linking direct induces apoptosis of cancer cells

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand can induce apoptosis in many tumor cell lines. This apoptotic effect is mediated by interaction of TRAIL and its receptors, which include Death Receptor 4 (DR4) and Death Receptor 5 (DR5). Some antibodies to DR4 or DR5 do not have anti-tumor ability without cross-linking but exhibit anti-tumor ability in the presence of a cross-linking reagent. Here, we suggest that the tetravalent anti-DR5 antibody can induce apoptosis of cancer cells independent of cross-linking reagent. The single-chain variable fragment of the anti-DR5 antibody, HSA (human serum albumin) – p53 gene, comprising residues 490–513 of HSA and the tetramerization domain of human p53 were assembled into the tetravalent antibody by an overlapping PCR. Results of size exclusion HPLC indicated that the purified protein exhibited a major peak (tetramer) and a minor peak (dimer). MTT assay demonstrated the tetravalent antibody without cross-linking could inhibit survival of Jurkat and EC9706 cells in a dose-dependent manner while the monovalent antibody could not inhibit survival of Jurkat and EC9706 cells. IC50 of Jurkat cell was 3.2 mg/L and IC50 of EC9706 cell was 3.9 mg/L. Furthermore, the Annexin V/PI assay and the Hoechst 33258 staining showed that the tetravalent antibody could efficiently induce apoptosis of Jurkat and EC9706 cells. Therefore, the tetravalent anti-DR5 antibody can act as a direct agonistic antibody, and initiate efficient apoptotic independent of cross-linking reagent. Thus, the tetravalent anti-DR5 antibody will be a new kind of candidate for potential cancer therapeutics.

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily [1]. Many studies have indicated that TRAIL can induce apoptosis in cancer cells originating from multiple tissues and organs while with minimum cytotoxicity toward normal tissues both *in vitro* and *in vivo* [2,3]. This apoptotic effect is mediated by interaction of TRAIL and its receptors, which include Death Receptor 4 (DR4) [4] and Death Receptor 5 (DR5) [5].

After binding the TRAIL, the ligand receptor complexes oligomerize and transmit the apoptotic signal through intracellular domains of DR4 or DR5, which render a scaffold for specific adaptor proteins (such as Fas-associated death domain and initiator caspases), and form the death inducing signaling complex (DISC) [6]. DISC actives autocleavage of the initiator caspases resulting in activation of the death-executing caspase cascade and ultimately cell apoptosis [7].

Although many tumors express DR4 or DR5, they also typically express TRAIL decoy receptors that have the potential to limit the therapeutic efficacy of the recombinant TRAIL [3]. Agonistic Abs that specifically bind DR4 or DR5 bypass this problem, and offer the advantage of having a longer half-life *in vivo* [8].

Some antibodies to DR4 or DR5 do not have anti-tumor ability without cross-linking but exhibit anti-tumor ability in the presence of a cross-linking reagent [9,10]. Indeed, it has been shown that strategies that increase oligomerization of DR4 or DR5 can amplify the TRAIL-induced apoptosis [11,12]. Therefore, we speculated that the tetravalent anti-DR5 antibody, which could induce oligomerization of DR5 may also activate the TRAIL death pathway and exert tumoricidal activity even if the monovalent anti-DR5 antibody could not activate the TRAIL death pathway. In this study, we found that the tetravalent anti-DR5 antibody could direct induces apoptosis of cancer cells *in vitro*.

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Table 1
Oligonucleotides used for the construction of the pSecTag2A-3T.

Primer	Sequence (from the 5′-end to the 3′-end)
L1	GGCAAGCTTAGATATTGTGATGACACAGT Hind III
L2	GGAGCCGCCGCCGCCAGAACCACCACCACCAGAACCACC
H1	GGCGGCGGCGGCTCCGGTGGTGGTGGATCCGAGGTCAGCTBCAGCAGTC
H2	GTTGTGTCACCAAGTGGGGTTGAGGAGACAGTGAGAGTGGA
P53a	ACCCCACTTGGTGACACAACTCACACATCCGGAAAACCACTGGATGGAGAATATTTCACCCTTCAGATCCGTGGGCGTGAGCGCTTCG
P53b	TGGCTCCTTCCCAGCCTGGGCATCCTTGAGTTCCAAGGCCTCATTCAGCTCTCGGAACATCTCGAAGCGCTCACGCCCACGGATCT
P53	CGCCTCGAGTGGCTCCTTCCCAGCCT Xho I

The underlined nucleotides in primers L1 and p53 indicate the sites of action of the endonucleases mentioned.

2. Materials and methods

2.1. Cell lines and culture conditions

The human esophageal cancer cell lines (EC9706 cell, NEC cell) and human lymphocyte leukemia cell lines (Jurkat cell, K562 cell) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cancer cells were cultured with RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco) and maintained at 37 °C in 5% CO_2.

2.2. The production of the tetravalent anti-DR5 antibody

The VL and VH of the anti-DR5 antibody were amplified from the hybridoma of YM366EC [13] and subsequently were linked in tandem by (GGT GGA GGC GGT TCA)3, ultimately were cloned into the eukaryotic expression vector pSecTag2A. This vector expressing single-chain variable fragment (scFv) was named pSecTag2A-3M. The DNA sequence of scFv has been deposited in the GenBank database (accession numbers KM052656). The expression product of pSecTag2A-3M was a monovalent antibody.

The scFv, HSA (human serum albumin) – p53 gene, comprising residues 490–513 of HSA [14] and residues 319–360 of the human p53 [15,16] was assembled by an overlapping PCR with seven synthetic oligonucleotides (Table 1). The PCR product was cloned into the eukaryotic expression vector pSecTag2A. The vector expressing the tetravalent antibody was named pSecTag2A-3T. The expression product of pSecTag2A-3T was a tetravalent antibody.

Plasmids of pSecTag2A-3M and pSecTag2A-3T were respectively transfected into CHO cells. CHO cells were grown and induced. Expression products were purified with Ni^{2+}-NTA superflow affinity chromatography. Finally, expression proteins were analyzed by Size exclusion HPLC (Beckman).

2.3. DR5 expression assays

Culture cells (1×10^6) were collected by centrifugation at 4 °C for 5 min at 1,000 rpm. After washing, cells were re-suspended in FACS buffer (PBS containing 2% FBS and 0.01% sodiumazide) and incubated with 10 μg/ml of the purified antibody YM366EC for 1 h at 4 °C. The mouse IgG1 was set as a negative control. After washing two times with FACS buffer, cells were incubated with 50 μl of 1:100 diluted FITC-conjugated goat anti-mouse IgG1 for 30 min at 4 °C with gentle shaking, then washed two times with FACS buffer and fixed in 300 μl of 1% paraformaldehyde. Samples were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson).

2.4. Cell survival assays

The tumor cell survival was indirect measured by a modified MTT (Sigma) assay [17]. Cells at 4×10^5 per ml were treated with indicated graded doses of the monovalent antibody and the tetravalent antibody respectively. After 16 hours plating, 0.1 volumes of 5 mg/ml MTT in PBS were added to each well. Then, cells were incubated for 4 h at 37 °C before DMSO was added to dissolve the formazan products. Finally, the absorbance was measured at 570 nm with an ELISA reader (Bio-Tek). Cell survival rate was calculated by the following formula: cell survival rate (%) = (absorbance of the tetravalent antibody/absorbance of the monovalent antibody) × 100%.

2.5. Cell apoptosis assays by Hoechst 33258 staining

Cells were treated with 4 μg/ml of the monovalent antibody and the tetravalent antibody respectively. Cells were harvested after 16 h incubation period and centrifuged at 400 g for 5 min. After washing with cold PBS, cells were fixed in 50 μL of 3% polyformaldehyde solution for 10 min, and then exposed to 15 μL of Hoechst 33258 (Sigma) in a final concentration of 16 mg/L for 15 min. After washing, 10 μL of cell pellet was dropped on a glass slide and the image was observed under a fluorescence microscope (Zeiss). The apoptosis of cancer cells was determined by the alteration of nuclear morphology and fluorescent density.

2.6. Cell apoptosis assays by flow cytometry

Cancer cells (1×10^6) were treated with 4 μg/ml of the monomeric antibody and the tetrameric antibody respectively. After incubated for 16 hours, cancer cells were collected for analysis by using the Annexin V-FITC apoptosis detection kit according to the protocol (Becton-Dickinson). Cancer cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson) using the software program CellQuest.

2.7. Statistical analysis

All data were presented as the mean ± SD of three independent experiments. Data were analysed by Student's-test with SPSS 15.0 to determine their significant differences. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The production of the tetravalent anti-DR5 antibody

The genetically engineered constructs – the monovalent and tetravalent anti-DR5 antibodies – were schematically shown in Fig. 1. The tetravalent antibody was a homotetramer formed association of four scFv fusion products with residues 319–360 of the human p53 [15]. Results of size exclusion HPLC indicated that the purified protein exhibited a major peak (65.5%) with a retention time appropriate for the tetramer and a minor (34.5%) peak with a retention time appropriate for the dimer (Fig. 2).