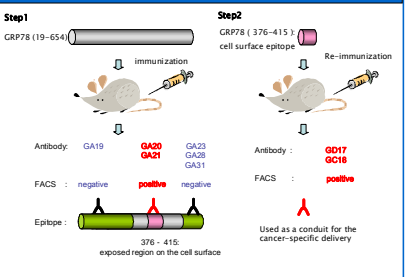


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ABSTRACT

Clinical trials using monoclonal antibodies have been conducted for several cancer types. Generally, the molecular target of a therapeutic anticancer antibody should be the cancer-specific membrane proteins. GRP78, a member of the heat-shock protein 70 (HSP70) family, plays critical roles in protecting the cells against endoplasmic reticulum (ER) stress-induced apoptosis. GRP78 is expressed in all tissues and normally is localized in the ER. In malignant cells, the expression of GRP78 is highly elevated, correlating with tumor proliferation, survival, metastasis, and drug resistance. Furthermore, cancer-specific surface localization of GRP78 is also reported, suggesting that surface-located GRP78 may serve as a potent target for cancer therapies including antibody-based immunotherapy. In this study, we isolated the monoclonal antibody against GRP78, termed GD17 mAb, which recognizes the membrane-located GRP78 of cancer cells. Using a single-chain antibody fragment (scFv) derived from GD17 mAb and a truncated form of Pseudomonas exotoxin A (PE40) lacking its natural binding domain Ia, a fully recombinant immunotoxin (GD17-PE40) was constructed. The bacterially expressed and purified immunotoxin GD17-PE40 induced cell death *in vitro* against several cancer cell lines with IC50's ranging from 1 to 10 nM, while normal cell lines remained unaffected. Furthermore, GD17-PE40 demonstrated potent antitumor activity *in vivo* with reduction of the growth of the tumor xenograft derived from 22Rv1 prostate cancer cell. These data indicate that immunotoxin targeting of membrane-located GRP78 on cancer cells is a new therapeutic approach for patients with several types of cancer.

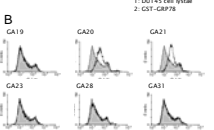
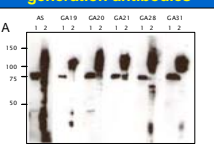
Two-step strategy for generating surface located-GRP78 antibody



Step 1: Antibodies against GRP78 (binders) were obtained from mice immunized with whole GRP78 protein. Evaluation of the FACS binding of the binders showed that only two (GA20, GA21) out of 54 binders were identified to be antibodies bound to surface-located GRP78. The region of GRP78 exposed on the cell surface was identified by epitope mapping of GA20 and GA21.

Step 2: To generate an antibody to surface-located GRP78 with a higher affinity, GRP78 (376-415) was used for re-immunization. Finally, the two best binders, GD17 and GC18, were identified and showed an EC50 of 6.4e-11 and 9.1e-11, respectively.

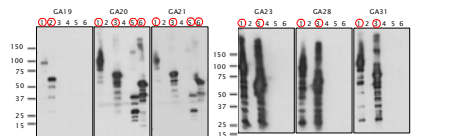
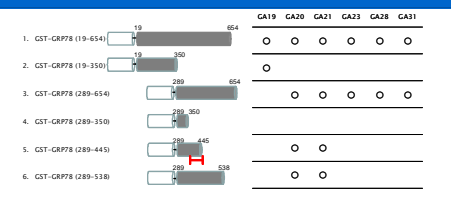
Binding properties of first generation antibodies



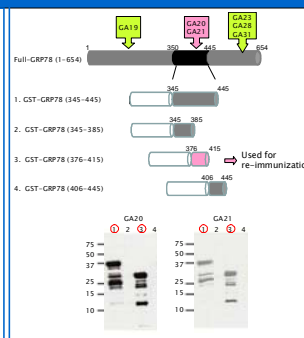
A: DU145 whole cell lysate (lane 1) and purified GST-GRP78 (lane 2) were subjected to SDS-PAGE and blotted. The blot was incubated with anti-serum (AS, x3000) derived from mice immunized with GRP78, and anti-GRP78 antibodies as indicated.

B: DU145 cells were stained with mAbs and analyzed by flow cytometry. GA20 and GA21 bound to surface-located GRP78.

Identification of the GRP78 epitope exposed on the cell surface

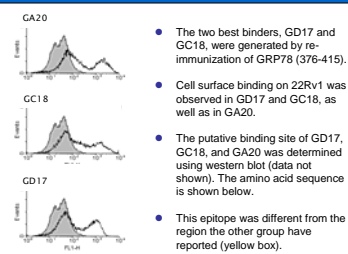


Schematic illustration of full-length and truncated GRP78 fused to the GST protein. Expression of the constructs were induced by IPTG in E. coli. The binding region for each antibody was determined by western blotting. Results are summarized in the right panel. The epitope of GA20 and GA21 is in the region corresponding to at least 95 amino acids (350-445).



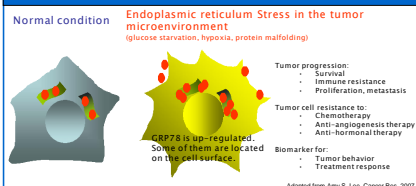
GRP78 at 345-445 was further divided into three regions (345-385, 376-415, 406-445) and used for western blot. Data shows the epitope of GA20 and GA21 to be within the amino acid region 376-415 of GRP78 and was used for re-immunization.

Generation of antibody to surface-located GRP78 by re-immunization



- The two best binders, GD17 and GC18, were generated by re-immunization of GRP78 (376-415).
- Cell surface binding on 22Rv1 was observed in GD17 and GC18, as well as in GA20.
- The putative binding site of GD17, GC18, and GA20 was determined using western blot (data not shown). The amino acid sequence is shown below.
- This epitope was different from the region the other group have reported (yellow box).

Introduction



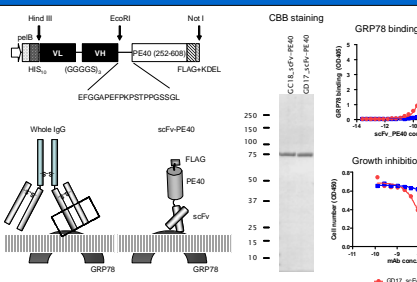
GRP78, a member of the heat-shock protein 70 (HSP70) family, is expressed in all tissues and normally localized in the endoplasmic reticulum (ER). Because cancer-specific surface localization of GRP78 has been identified in malignant cells, it can serve as a conduit for cancer-specific delivery of cytotoxic agents, e.g. toxins, via an antibody to surface-located GRP78.

First, we identified the region of GRP78 exposed on the cell membrane which is recognized by antibodies against surface-located GRP78.

Second, after immunizing this region, we identified a monoclonal antibody, GD17, which binds to the cell-surface located GRP78 with high affinity.

Finally, we developed a recombinant surface-located GRP78 specific single-chain immunotoxin based on the GD17 mAb and investigated the potency of antitumor activity *in vitro* and *in vivo*.

Generation of scFv-PE40 based on whole IgG



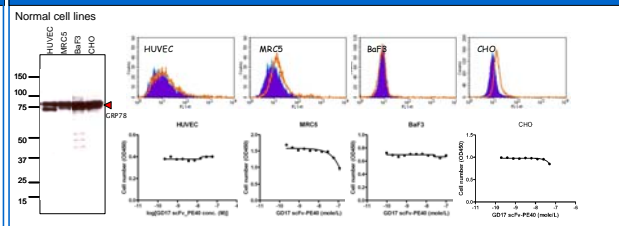
A schematic representation of the scFv-toxin fusion protein
The scFv antibody fragment is fused to the PE40 (252-408) by the 20-amino acid linker, and a COOH-terminal flag tag followed by the ER retention signal with KDEL.

Expression and purification of scFv-PE40
Recombinant immunotoxins, GD17_scFv-PE40 and GC18_scFv-PE40, were purified from a soluble periplasmic extract. The quality of purified scFv-PE40 was assessed by SDS PAGE and CBS staining.

GRP78 binding activity
Immunotoxin binding was assayed by an ELISA technique. Binding affinity of GD17_scFv-PE40 was higher than GC18_scFv-PE40.

Growth inhibition activity
Immunotoxin binding was assayed by an ELISA technique. Binding affinity of GD17_scFv-PE40 was higher than GC18_scFv-PE40.

GRP78 expression and sensitivity to GD17_scFv-PE40 cytotoxicity in normal and tumor cells

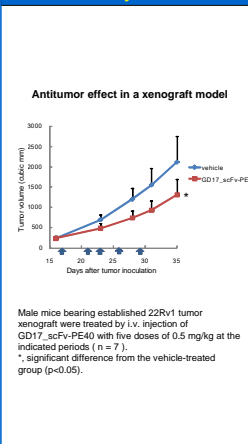


The expression of target protein GRP78 was confirmed by western blotting on whole cell lysate and FACS analysis using GD17 mAb.

Surface localization of GRP78 was detected in cancer cell lines, but was slightly low or undetectable on normal human and mouse cell lines.

Three cancer cell lines were found to be sensitive to the GD17_scFv-PE40. Despite the high level of GRP78 expression, normal cell lines were found to be less sensitive.

GD17_scFv-PE40 anti-tumor efficacy in vivo



Antitumor effect in a xenograft model

Male mice bearing established 22Rv1 tumor xenograft were treated by i.v. injection of GD17_scFv-PE40 with five doses of 0.5 mg/kg at the indicated periods (n = 7). *, significant difference from the vehicle-treated group (p < 0.05).

CONCLUSIONS

- The region of GRP78 corresponding to region 376-415 was identified as a novel epitope of surface-located GRP78.
- Monoclonal antibody GD17 binds to surface-located GRP78 with high affinity.
- Surface-located GRP78 targeting the immunotoxin GD17_scFv-PE40 was generated based on the GD17 monoclonal antibody.
- Cytotoxic activity is likely to be associated with the GRP78-binding affinity of the immunotoxin.
- GD17_scFv-PE40 showed toxicity toward tumor cell lines but less or no toxicity to normal cell lines *in vitro*.
- GD17_scFv-PE40 demonstrated significant antitumor efficacy in a xenograft mouse model.
- Surface-located GRP78 was identified as a target molecule for antibody-based therapy.