INTRODUCTION

In the 1980s, we developed several monoclonal antibodies (McAbs) against gastric cancer and colorectal cancer, which were named Mg (against gastric cancer) antibodies and Mc (against colorectal cancer) antibodies.1-3 The Mg series monoclonal antibodies include Mg7, Mgr-1, Mgb-1, and Mgd-1. The Mc series monoclonal antibodies include Mc5, Mc3, Mc7, and Mc10. Gastric cancer and colorectal cancer were positively stained by these antibodies. However, among antigens recognized by these antibodies, only Mg7-ag, Mgr1-ag, and Mc3-ag have been identified. Mg7-ag was found to be heterogeneous nuclear ribonucleoprotein a2/b1.4 Mimicry epitope (epitopes that mimic the original antigens, mimotope) vaccines of Mg7 showed strong antitumor activities.5,6 Human 37-kDa laminin receptor precursor was the antigen recognized by Mggr1 McAb and was involved in multidrug resistance of gastric cancer.9 The Mc3 antigen was identified recently. Thioredoxin-like 2 was suggested to be the Mc3 antigen, and overexpression of Mc3-Ag was found in colorectal cancer cells compared with para-cancerous tissues and adjacent normal tissues.10 Antigens recognized by other monoclonal antibodies still remain to be identified.

In this article, we report the selection and identification of anti-idiotype (anti-id) antibodies of Mgb1 and Mc5 McAb using phage-displayed single-chain variable fragment (ScFv) libraries. After purification, the anti-id antibodies were approximately 30 kd and could be recognized by Mgb1/Mc5 McAb. Anti-id antibodies significantly blocked the binding of Mgb1 and Mc5 to gastric cancer/colorectal cancer cells, respectively, suggesting that the antibodies were specific to Mgb1 and Mc5. Antibodies against gastric and colorectal cancer could be detected in mice at 6 weeks after immunization with the anti-id antibodies. At week 8, antibody titers reached 1:400. The anti-id antibodies may be useful as novel reagents for developing vaccines against gastric cancer and colorectal cancer. (Journal of Biomolecular Screening 2010:308-313)

Key words: gastric cancer, colorectal cancer, anti-idiotype antibody, phage display library, monoclonal antibody
and assembled into ScFv DNA by PCR (7 cycles: 94 °C × 1 min, 63 °C × 4 min). Afterward, the ScFv DNA was amplified, with primers incorporating the SfiI site at the 5′ end and a NotI site at the 3′ end. The ScFv DNA was then ligated into phagemid vector pCANTAB5E (Amersham Biosciences, Piscataway, NJ) after digestion with SfiI and NotI. The recombinants were electrotransformed into Escherichia coli TG1 and incubated for 1 h at 37 °C in 2× YT medium containing ampicillin and glucose (2× YT-AG medium). Bacteria were infected with M13K07 helper phage for 1 h at 37 °C with shaking. After centrifugation, the bacteria were gently resuspended in 2× YT medium containing ampicillin and kanamycin (2× YT-AK medium) and incubated overnight at 37 °C with shaking. The supernatant containing the recombinant phage was harvested by centrifugation, and the phages were precipitated with PEG8000 and NaCl and resuspended in 2× YT medium, filtered through a 0.45-μm filter and stored at 4 °C.

Biopanning of the phage ScFv libraries with MGb1 and MC5 antibodies

The filtered phages were incubated with blocking buffer for 15 min and then added to culture flasks coated with MGb1/ MC5 McAb and incubated for 2 h at 37 °C. The supernatant was removed and the flasks were washed with phosphate-buffered saline (PBS) 3 times. Then E. coli TG1 at the log phase was added to the flasks and incubated at 37 °C for 1 h. Ampicillin, glucose, and M13K07 helper phage were added and incubated for 1 h. The bacteria were harvested and resuspended with 2× YT2AK medium and incubated overnight. Phages in the supernatant were then harvested and filtered as described above and underwent the next round of panning. After 4 rounds of panning, the infected bacteria were plated onto SOBAG agar plates and grown overnight.

Selection of the phages expressing the anti-MGb1/MC5 ScFv by enzyme-linked immunosorbent assay

Colonies from the plates were picked and inculated into individual tubes. Supernatant from each tube was screened with 96-well plates coated with either MGb1 or MC5. Murine IgG was used as negative control. Horseradish peroxidase (HRP)–conjugated anti-M13K07 phage monoclonal antibody was used as the secondary antibody. Tetramethylbenzidine (TMB) substrate was used for visualization.

Soluble expression, purification, and characterization of the anti-MGb1/MC5 ScFv

Phages from positive colonies identified by enzyme-linked immunosorbent assay (ELISA) were used to infect E. coli HB2151. Periplasmic proteins were prepared and examined by dot blotting. The ScFv fragments were then purified with the RPAS purification kit (Amersham Biosciences) and characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

Inhibition of MGb1/MC5 McAb binding to gastric/colorectal cancer cells by anti-idiotype antibodies

We added 1 × 10⁶ KATO III cells (gastric cancer cell line) or SW480 cells (colorectal cancer cell line) to a 96-well plate after washing with PBS and then centrifuged them at 1200 rpm for 15 min. Then, 0.25% glutaraldehyde diluted with PBS was used to fix the cells for 12 min. Cells were then washed 3 times by PBS and then blocked with 3% bovine serum albumin (BSA). MGb1 McAb/MC5 McAb (final concentration of 40 μg/mL) were mixed with purified corresponding ScFvs at different concentrations and then added to each well. After incubation at room temperature for 1 h, the cells were washed with PBS buffer containing 0.1% Tween-20. HRP-conjugated goat antimouse antibody was used as the secondary antibody. TMB substrate was used for visualization. Purified ScFv proteins from nonscreened phages were used as negative controls.

Immunization of mice with purified ScFv and detection of antibody titer by cell ELISA

Bab/c mice were immunized with purified ScFv proteins of MGb1 or MG7 conjugated with complete Freund’s adjuvant by intraperitoneal injection, and mice immunized with only complete Freund’s adjuvant were set as the negative control group (10 mice in each group). Mice were boosted at day 14. Murine sera were prepared at weeks 5, 6, 7, and 8 and diluted 1:100. KATO III and SW480 cell plates were prepared and blocked as described above in the inhibition assay. The diluted sera were added to the wells and incubated at room temperature for 2 h. After washing 3 times with PBS, HRP-labeled goat antimouse antibody was then added and incubated at room temperature for 1 h. To detect peroxidase activity, 200 μL of ortho-phenylenediamine (OPD) substrate solution was added to each well and incubated for 10 min at room temperature followed by addition of 50 μL 3 M HCl to stop the reaction. The plates were read at 450 nm on a multwell plate reader. To determine the titer of the antibodies, murine sera at week 8 were diluted 1:100, 1:200, 1:400, and 1:800 and underwent cell ELISA assay as described above. To further verify the specificity of the antibodies induced by anti-id proteins, another cell ELISA assay was performed using the GES-1 cell, an immortalized human gastric epithelial cell line that does not express the MC5 or MGb1 antigen. For all ELISA assays, triplicate wells were used for each serum sample from the mice.

RESULTS

Selection of anti-MGb1/MC5 McAb antibodies

After 4 rounds of panning, 132 colonies positive for MGb1 and 134 colonies positive for MC5 were identified by ELISA.
二十个MGb1阳性克隆和21个MC5阳性克隆，这些克隆显示了与对应抗体的最高特异性，并且没有对正常小鼠IgG（如ELISA所示，数据未示出）的回应，进行了限制酶SfiI和NotI的插入。ScFv片段的插入被证实于所有克隆中，这进一步被序列分析所确认。

**Protein purification and characterization**

二十个MGb1阳性克隆和21个MC5阳性克隆被诱导表达可溶ScFv。来自克隆的外周蛋白质被准备并用于检测其与MGb1或MC5抗体的亲和力（图1）。显示了最佳亲和力的克隆被选择并表达ScFv。外周蛋白质被进一步纯化。通过SDS-PAGE分析，ScFv蛋白质的大小约为30 kd（图2）。Western blot检测表明，纯化的ScFv蛋白质可以被MGb1或MC5单克隆抗体识别，但不能被正常小鼠IgG识别（图3）。

**Inhibition of MGb1/MC5 McAb binding to gastric/colorectal cancer cells by anti-idiotype antibodies**

为了进一步测试筛选的ScFv蛋白质的特异性，进行了细胞ELISA竞争实验。结果如图4所示，抗-抗体显著抑制了MGb1/MC5抗体对胃癌/结直肠癌细胞的结合，但对其他抗体没有影响。这些发现表明，抗-抗体特异性针对MGb1和MC5。

**Immunization of mice and detection of the antibody titer**

为了检测抗-抗体的免疫原性，将这些纯化的抗-抗体与小鼠免疫。在不同时间点，制备小鼠血清，进行了细胞ELISA检测以检测抗体的滴度。如图5所示，随着免疫时间的延长，胃癌/结直肠癌细胞的抗体滴度增加。在第8周，胃癌/结直肠癌细胞的抗体滴度达到1:400（图6）。使用GES-1细胞的细胞ELISA结果在图7中呈现。没有检测到血清对GES-1细胞的反应，无论是免疫的小鼠还是非免疫的小鼠。

**DISCUSSION**

胃癌和结直肠癌是消化系统的主要恶性肿瘤。几种胃癌和结直肠癌
cancer vaccines have been developed and are in clinical use. Major targets of gastric cancer vaccines are patient-specific cytotoxic T lymphocyte (CTL) peptides isolated from the draining lymph nodes of the patients, gastrin, tumor markers such as carcinoembryonic antigen (CEA), and other cancer antigens such as MAGE. And for colorectal cancer, CEA, CD55, GA733 antigen, CTL peptide, ras peptides, and p53 were the major molecules used to develop vaccines. Among these targets, cancer CTL peptides were mostly patient specific and difficult to isolate and identify. Tumor markers such as p53, CEA, and gastrin had moderate or poor specificity and sensitivity for gastric cancer and colorectal cancer. MC5 and MGb1 were monoclonal antibodies developed in our institute by immunizing mice with colorectal/gastric cancer cells. By immunohistochemical staining, it was found that 99.2% of the colorectal cancer specimens were MC5 positive and 78% of gastric cancer specimens were MGb1 positive, suggesting that antigens recognized by these antibodies were abundant in either cancer type. However, these antigens have not been identified. MGb-1 and MG7 are other MG series antibodies against
gastric cancer developed in our institute. Human 37-kDa lamin receptor precursor was identified as an MGR1 antigen, which was found be associated with multidrug resistance of gastric cancer. Inhibiting MGR1-Ag expression significantly sensitized gastric cancer cells to chemotherapeutic drugs. For MG7, approximately 90% of gastric cancer cells expressed MG7-Ag. Vaccines based on MG7 mimicry peptide induced significant anticancer immune response.

To improve the efficacy of gastric cancer vaccines and provide novel candidate molecules for colorectal cancer vaccine, we constructed the phage-displayed murine ScFv libraries from mice immunized with MGB1 McAb or MC5 McAb. By screening the library, anti-id antibodies against MGB1 and MC5 were identified. These anti-id antibodies could inhibit the binding of the McAb to gastric or colorectal cancer cells, suggesting that the anti-id antibodies were specific for the original McAbs. Immunization of mice with anti-id antibodies induced antibodies against gastric cancer or colorectal cancer but not against cells that do not express MGB1 or MC5 antigen, which indicated that these ScFv proteins were both immunogenic and specific. Given that almost all colorectal cancer cells express MC5 and that 78% of gastric cancer cells express MGB1-Ag, the MC5 and MGB1 anti-id antibodies would serve as good candidates for a colorectal and gastric cancer vaccine. Although MGR1 antigen was present in only 18% of gastric cancer cells, its role in multidrug resistance of gastric cancer cells indicated that MGR1 antigen-based vaccines may provide additional benefit by sensitizing cancer cells to chemotherapeutic drugs. Including multiple epitopes such as MG7-Ag, MGR1-Ag, and MGB1 anti-id antibody in gastric cancer vaccine development seems to be a reasonable strategy, and it would be very interesting to test if the MGR1 anti-id antibody vaccines can affect the drug sensitivity of gastric cancer. More work is needed to clarify whether vaccines based on MC5 anti-id antibodies would provide a protective effect on colorectal cancer and whether multiepitope gastric cancer vaccines, including MG7-Ag, MGB1 anti-id antibodies, and MGR1-Ag, would be more effective than single-epitope vaccines. Research on this aspect is under way in our institute.

REFERENCES


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