Development of 36 Clones Producing Human Monoclonal Antibodies Specific to Cancer Cells and their Ability to Inhibit Cancer Cell Growth

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Abstract. The development of a specific antibody for cancer therapy could enable a potent strategy for overcoming cancer. As ideal immunotherapy, a human monoclonal antibody (HuMoAb) might have a useful antitumor effect without any lethal toxicities. Thirty-six unique clones producing HuMoAbs were successfully developed using tumor infiltrating lymphocytes collected from 28 patients with several malignant solid tumors. The 36 tumor-specific immunoglobins were found among 9,450 clones after 43 fusions by the conventional hybridoma method. Among these 36 HuMoAbs, 9 had a remarkable tumor-specific reaction and no reaction with normal tissues, as determined with quantum dots-streptavidin and a fluorescence microscope. The inhibition of cell proliferation by the HuMoAbs was evaluated with the MTT assay. Over 40% cell growth inhibition was confirmed with 4 of the 36 HuMoAbs. Two of the antibodies had highly-specific reactivity to carcinomatous lesions with strong growth inhibition and up to 94.3% inhibition of the control growth. In conclusion, 36 clones with HuMoAbs that have specific reactions with cancer cells were successfully established. These HuMoAbs might be utilized as either anticancer or drug delivery agents.

Recently, new drugs targeting particular molecules responsible for cancer progression or metastasis, such as angiogenesis inhibitors, targeting antibodies, or kinase inhibitors, have been developed, and the clinical application of such drugs has progressed. Nineteen drugs targeting particular molecules have been approved as anticancer agents by the government health systems in several countries, based on the results of clinical trials (1). Since the laboratory technique for the production of monoclonal antibodies (MoAbs) was introduced by Milstein and Köhler in 1975, it has enabled the massive production of MoAbs responding to cancer cells (2). Cancer therapy using a specific antibody reacting to a targeted molecule has been attempted, and various kinds of antibodies have been developed demonstrating the potential for cancer therapy. FDA-approved MoAbs in the USA are Trastuzumab (Herceptin, humanized anti-HER-2 antibody), Rituximab (Rituxan), Gemtuzumab ozogamicin (Mylotarg), Alemtuzumab (Campath), Cetuximab (Erbitax, chimeric mouse-human monoclonal antibody for EGFR-positive colorectal cancer), Ibritumomab Tiuxetan (Zevalin), Tositumomab (Bexxar), Erlotinib (Tarceva), and Bevacizumab (Avastin, recombinant humanized version of the murine antihuman VEGF monoclonal antibody). Among these approved antibodies, Trastuzumab (Herceptin), Cetuximab (Erbitax), and Bevacizumab (Avastin) have been emphasized for solid tumor treatment. Trastuzumab, the first clinically applied monoclonal antibody for HER-2 overexpressing metastatic breast cancer, has been approved in the USA since 1998 (3) and is now used worldwide with good efficacy for breast cancer. Cetuximab and Bevacizumab have been approved for metastatic colorectal cancer in the USA since 2004 and have high efficacy for advanced colorectal cancer (4, 5). Thus, at present, several monoclonal antibodies have been approved as therapeutic agents for human malignancy and some of them are clinically applied. In addition, some targeting antibodies had synergistic antitumor effects with other anticancer agents. The use of humanized or murine/human chimeric monoclonal antibodies is now well established for the treatment of human malignancies (6, 7). Such antibodies are effective as mono-therapy and in combination with chemotherapy. For example, HER-2-specific trastuzumab (Herceptin) for breast cancer and CD20-specific rituximab (Mabthera, Rituxan) for follicular non-Hodgkin’s B-cell
lymphoma have been reported to improve patients’ overall survival (8-11). However humanized or murine/human chimeric monoclonal antibodies that might produce a human anti-mouse antibody (HAMA) response that causes severe toxicities in several human organs (12-14). The ideal strategy to prevent such immunological reactions is to use fully human MoAbs (HuMoAbs), which would never elicit immune responses, thereby realizing the clinical application of MoAbs with ultimate safety.

Tumor infiltrating lymphocytes (TILs) are known to be composed of mononuclear lymphocytes and macrophages that infiltrate around malignant tumors as part of the immunological reaction (15-18). TILs have been used in clinical trials against several types of human malignancies because these cells have been reported to generate highly specific immunological activity against human tumors (19-21). Although the clinical efficacy of TILs in immunotherapy, such as a direct effect on malignant tumors, still remains unclear, the role of TILs in the host immune response has significant potential as a useful therapy in clinical application against malignant tumor cells.

We propose that an ideal immunotherapy using MoAbs against human tumor cells would be possible with the production of HuMoAbs utilizing TILs, which can be clinically collected from the resected human specimen. This strategy could result in antitumor effects on human tumor cells without any lethal toxicities from the immune response. Conventional human hybridoma technology with TILs offers a unique possibility to investigate novel humoral immunity against human cancer and to overcome cancer using HuMoAbs (23-24).

**Materials and Methods**

*Hybridoma technique. Lymph node lymphocytes and TILs from lung-, gastric-, pancreas-, or colon-carcinoma patients were obtained during surgery and prepared by mechanical disruption of the tissue. All the patients gave informed consent. The study was approved by the ethical committee of Keio University (Approval No. 13-57). The resulting single cell suspension was fused at 1:1 with mouse myeloma P3U1 with polyethylene glycol 1500 (PEG 1500, Roche Diagnostics Co., Manheim, Germany), and 100-μL aliquots were distributed at a density of 5×10⁵/mL/well in 96-well plates. The hybridomas were cultured in RPMI 1640 containing 10% fetal calf serum plus hypoxanthin aminopterin tyrmidin (HAT) supplement (Sigma Chemical Co., St. Louis, MO, USA). After about 2 weeks in culture, the antibodies produced by the hybridomas were screened by ELISAs. The supernatant from each well was incubated with cancer cells that had been fixed with 2% paraformaldehyde. Horse radish peroxidase (HRP) H (Vector Laboratories, Burlingame, CA, USA)-conjugated goat anti-human immunoglobulins (Cappel, Cooper Biomedical, Inc., PA, USA) were used as the second antibody and O-phenylenediamine dihydrochloride was used as the substrate. Positive clones were tested for the ability of the antibodies produced to bind the cancer cells. In the wells where reactivity was detected, limiting dilution and subsequent re-cloning were performed repeatedly.*

*Antibody purification. The MoAbs produced by the cloned hybridoma cells were isolated on a Protein-A affinity column. Purity was determined by SDS-PAGE.*

**Cell lines.** The following human cell lines were obtained from Immuno-Biological Laboratories Co. Ltd. (Gunma, Japan): gastric cancer (MKN-45, MKN-74, HSC-3), lung cancer (PC-3, PC-9), and colon cancer (HT-29, LoVo). The following human cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA): lung cancer (A549), colon cancer (Colo205, DLD-1), pancreatic cancer (PANC-1, HPAC), and human umbilical vein endothelial cells (HUVECs). A human lung cancer cell line, HLC-1, and a human pancreatic cancer cell line, SUIT2, were provided by Dr. Yamada (Nihon University, Tokyo, Japan) and Dr. Inokuchi (National Cancer Center Hospital of Kyushu, Japan), respectively. A human pancreatic cancer cell line, PK-8, was obtained from the Cell Research Center of the Biomedical Research Institute of Development, Aging and Cancer, Tokoh University. SKS, a human gastric cancer cell line that is transplantable to nude mice, was established from fresh cancer tissues in our laboratory. Each of the above cancer cell lines was transplanted into BALB/cA/cnu nude mice (Nihon Clea Co., Tokyo, Japan) in order to form tumors. Tumors were resected aseptically. Necrotic tumor tissues were removed and the remaining non-necrotic tumor tissues were minced or sliced according to each use.

*Detection of antibody on cancer cell surface. In view of antibody on cancer cell surface, inorganic fluorophores, quantum dot which has a high photobleaching threshold was used. Each fresh tumor tissue was minced and filtrated. Single cells were separated and biotinylated antibodies which were previously prepared were conjugated to the tumor for 60 min in human serum. Quantum dots-streptavidin (2 μg/mL) was added sequentially to antibody-tumor cell complex. The reaction proceeded for 30 min, and then the complex was washed with phosphate buffered saline. All the steps were carried out at 0°C. Trypan blue was added to the labeled cells and an aliquot of each cell suspension was placed in a 96-well plate. Each sample was imaged immediately with a fluorescence microscope.*

*Immunohistochemical staining. For the staining procedure, the DAKO staining kit; Code K 1500 (DAKO CORP., CA, USA), Catalysed Signal Amplification (CSA) system with HRP was used following the manufacturer’s instruction. CSA is a very sensitive immunohistochemical staining procedure incorporating a signal amplification method based on the peroxidase-catalysed deposition of a biotinylated phenolic compound followed by a secondary reaction with streptavidin-peroxidase. Sections from normal tissues and from the carcinomas in the transplanted nude mice were deparaffinized and rehydrated according to standard protocols. After blocking endogenous biotin and suppressing non-specific background staining as prescribed by the manufacturer, biotinylated link antibody was applied to the tissues and an incubation of 30 min followed. The slides were then gently rinsed with phosphate buffered saline with Tween 20 and placed in a fresh buffer 3 times for 3 to 5 min each. The staining was completed with a 3 to 5 min incubation with 3,3’-diaminobenzidine tetrahydrochloride, which is oxidized to a brown-colored precipitate by the peroxidase, and stained with hematoxylin and eosin finally.*
Proliferation assay. The MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.] assay was used to screen the human lung adenocarcinoma cell line HLC-1, the human stomach adenocarcinoma cell line MKN-45, the human pancreas adenocarcinoma cell line PANC-1 or PK-8, and HUVEC as the normal control. The trypsinized cells were diluted to 3×10^4 cells/mL in 5% FCS with 5% human serum and 50 μL of cell suspension was added to each well of a 96-well plate. The antibodies were added to each plate at concentrations of 100, 50, 25, 12.5, and 6.25 μg/mL and medium alone as controls were included. Normal human IgM (Chemicon International, Inc., CA, USA) was used as a control antibody. The plates were incubated at 37°C for 6 days. The medium was exchanged with fresh antibody solution every 2 days. Upon completion of the antibody incubation, 50 μL of MTT solution was added to each well, and after incubation for 5 hours, 10% SDS buffer was added to each well. Absorption was measured at a wavelength of 550 nm after overnight incubation. In this assay, primarily human carcinoma cells that had the same origin as each antibody were used. The antibodies that gave a positive response were tested with another cell line.

Results

HuMoAbs. Lymphocytes were obtained from 28 patients and a total of 43 fusions were performed with the conventional hybridoma method. The total number of growing clones was 9450, in which there were 36 tumor-specific HuMoAbs. The origins of these antibodies were 23 from lung, 8 from stomach, 4 from pancreas and 1 from colon. Twenty-seven of these antibodies were IgM and 9 were IgG (Table I). Forty percent of the growing hybridomas were counted, 40% of them were IgMs and 60% were IgGs. Seventy-five percent (27/36) of the clones producing tumor-specific antibodies produced IgMs. Among the 36 antibodies, 2 (HuMoAbs A and B) had both specific and intense reactivity to carcinomatous lesions with strong growth inhibition. HuMoAb A was derived from the TILs of a lung cancer patient and HuMoAb B was produced from those of a gastric cancer patient.
Binding activity of each antibody. Twenty-three out of the 36 HuMoAbs had a tumor-specific reaction without reaction to normal tissues, and 9 of them (2 gastric cancer, 4 lung cancer and 3 pancreatic cancer) had remarkable tumor-specific reactions (Table II). For example, Figure 1 shows the definite specific reactivity of HuMoAb-A to the surface of lung and pancreatic cancer cells but not to normal cells in vitro.

Reactivity pattern of antibodies. The reactivity patterns of 23 out of 36 the HuMoAbs were determined by immunohistochemistry Table III shows that 5 HuMoAbs attached to the cancer cell surface in over 75% of the cells, 2 attached to more than 50% of the cancer cells and 3 attached to less than 50% of the cancer cells. HuMoAb-B reacted specifically to tumor tissue from all 4 lung, all 4 pancreatic, all 4 gastric and all 4 colon cancer cell lines, but not to normal cells (Figure 2).

Functional activity of antibodies. Twelve of the HuMoAbs had the ability to inhibit cell proliferation, and more than 40% up to 94.3% inhibition was confirmed in 4 out of the 36 HuMoAbs (Table IV). Direct incubation with HuMoAb-B in vitro resulted in significant inhibition of cell proliferation in the cancer cell lines (MKN-45, HLC-1, PANC-1) in a dose dependent manner (Figure 3) but not in the normal HUVEC cells.

Discussion

The MoAbs obtained in the present study using the conventional human hybridoma technology were fully human MoAbs. No prior information about the antigens targeted by the HuMoAbs or the individual HuMoAb was reactivity with a particular antigen expressed specifically on a cancer cell was determined. After the production of many clones, the significant clones that had specific reactions with cancer cells or cancer tissues were selected. Thus, these HuMoAbs might help in the discovery of important new antigen targets on cancer cells. Although it has been reported that only IgMs are obtained using this hybridoma method (25), our HuMoAbs included IgGs that showed tumor-specific binding ability or the ability to inhibit cell proliferation. The detailed mechanisms of these HuMoAbs and the identification of the specific antigens on the cancer cells are not known. Further investigations are needed to clarify the mechanisms and targets of the new molecules.

The development and clinical application of HuMoAbs is still being studied. A12, SC-1, and GAH have recently been reported to be established HuMoAbs. A12, a HuMoAb to the insulin-like growth factor 1 receptor, was reported to inhibit in vivo growth and increase the number of apoptotic cells in breast, renal, and pancreatic tumors (26). SC-1, a HuMoAb isolated from a patient with gastric signet ring cell carcinoma, was shown to induce apoptosis in gastric cancer...
and showed little toxicity in Phase II clinical trials (18, 27-32). Previously, we reported the development of GAH, cancer-specific human monoclonal antibody, which was obtained by using the same techniques described in the present study (33). GAH demonstrated high binding specificity for cancer cells of the stomach and colon, suggesting that a drug delivery system using GAH may be a potent and useful means of targeting cancer cells (34). We have also developed Hoawks-1, another novel HuMoAb, that was derived from TILs of lung cancer and inhibits cancer cell growth by morphological alteration (35).

The HuMoAbs in the presented study are still under investigation for identification of their specific antigens and confirmation of functional analysis on cancer and normal cells in vitro and in vivo.
cells. Some of the tumor-specific HuMoAbs could bind to tumor cell surfaces but not to normal cells. Interestingly, this feature was specific not only to the original cancer cells, but also to other cancer cells, suggesting that these tumor-specific antibodies can be used effectively as delivery systems for cytotoxic agents to tumors without specific toxicities to the other organs (36, 37). Tumor-specific HuMoAbs-drug conjugates are ideal for drug delivery to a tumor to overcome cancer without toxic effects or immunological reactions. Tumor-specific reactions with HuMoAbs and delivery systems with HuMoAbs-drug conjugates might be future tools for conquering cancer.

Conclusion

Thirty-six clones stably producing HuMoAbs that react specifically to cancer cells were successfully established. Several of these antibodies demonstrate strong binding capacity specific to cancer cells or tissues and some of these antibodies show direct inhibitory effects on cancer cell proliferation.

References


