A Novel Human Monoclonal Antibody Derived from Tumor-infiltrating Lymphocytes in Lung Cancer Inhibits Cancer Cell Growth with Morphological Changes

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Abstract. Tumor-infiltrating lymphocytes (TIL) were isolated from surgically resected human samples using hybridoma techniques, and human monoclonal antibodies (HuMoAbs) were produced. Stable antibody-producing hybridoma cell lines were established and, on the basis of reactivity to human cancer cell lines, a clone of HuMoAb, named HoAKs-1, was selected. By confocal microscopy, we confirmed that HoAKs-1 showed specific and intense reactivity to the cell membrane of HLC-1 and PANC-1, whereas the antibody did not show reactivity to human umbilical vein endothelial cells (HUVECs). Using xenografts formed from epithelial cancer cell lines in nude mice, we also demonstrated a broad spectrum of reactivity of the antibody in 6 out of 14 xenografts. In addition, using surgically resected clinical specimens from two patients with lung cancer, we showed that HoAKs-1 had specific reactivity to cancerous lesions but not to normal sites. In an in vitro experiment, HoAKs-1 induced morphological changes with neurite-like cytoplasmic processes in MKN-45, HLC-1 and PANC-1, whereas no morphological alterations were observed in HUVECs. Growth inhibition rates by HoAKs-1 were 63% in HLC-1 and 47% in MKN-45, while no growth inhibition occurred in normal HUVECs. We confirmed that HoAKs-1 recognized a 55 kDa protein by determining the molecular weight of the HoAKs-1 reacting protein. In conclusion, we successfully produced a novel HuMoAb, HoAKs-1, which reacted specifically to cancer cells and inhibited cell growth with morphological changes. HoAKs-1 may have the potential to be utilized as an anticancer agent, without causing any immunological reaction in humans.

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Key Words: Tumor-infiltrating B lymphocyte, monoclonal antibody, lung cancer, hybridoma.
foreign proteins, chimeric humanized Abs have been developed by combining the variable regions of a murine antibody with the constant region (Fc) of a human antibody. However, chimeric Abs still show a toxic immune response against the foreign variable regions. Fully human MoAbs (HuMoAbs) are therefore the ideal Abs for clinical application, as these Abs will have a specific effect on malignant tumors, but not on normal human tissue.

In this study, we elucidated the specificity of reactions against cancer cells and the resulting biological functions of these cells of a newly established HuMoAb, HoAKs-1, produced successfully by hybridoma techniques on TIL obtained from human lung cancer tissue.

**Materials and Methods**

**Hybridoma preparation and screening.** Lymph node lymphocytes and TIL from patients with lung, pancreatic, or gastric carcinomas were collected during surgery with the patients’ informed consent. The study was approved by the internal review board of Keio University’s School of Medicine, Japan (Approval No. 13-57). Cell suspensions of single cancer and lymphocyte cells were prepared from the samples. The suspensions were exposed to polyethylene glycol 1500 (PEG 1500, Roche Diagnostics Co., Manheim, Germany) and then were fused at a 1:1 ratio with mouse myeloma P3U1 and placed in 96-well plates. The hybridomas were cultured in RPMI 1640 containing 10% FCS and hypoxanthin aminopterin thymidine (HAT) supplement (Sigma Chemical Co., MO, USA) and then screened for human antibody production using ELISAs.

The Cell ELISA method was used for the initial screening of antibody activity. The supernatant from each well was incubated with cancer cells that were fixed moderately with 2% paraformaldehyde. Horseradish peroxidase H (Vecter Laboratories, Burlingame, CA, USA)-conjugated goat anti-human immunoglobulins (Cappel, Cooper Biomedical, Inc., PA, USA) were used as the second antibody, while o-phenylenediamine dihydrochloride was used as the substrate. In the wells where reactivity was detected, corresponding hybridomas were cloned using limiting dilution.

**Antibody purification.** Monoclonal antibodies produced by the cloned hybridoma cells were purified from the culture supernatant on a Protein-A affinity column. Purity was determined by SDS-PAGE (sodium dodecyl sulfate / polyacrylamide gel electrophoresis).

**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-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) 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 for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku 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/cAic-nu (Nihon Clea Co., Tokyo, Japan) in order to form tumors.

**Reactivity of HoAKs-1 to cancer cells detected by confocal microscopy.** HLC-1, PANC-1 and HUVEC cells were cultured in their respective complete growth media at 37°C in a humidified incubator. The cells were plated on 96-well plates and incubated at 37°C for 24 hours, followed by the removal of supernatant from each well. Purified HoAKs-1 and control human IgM were biotinylated using a biotinylination reagent (Amersham Life Sciences, Buckinghamshire, UK). After incubation for 60 min on ice with biotinylated HoAKs-1 (50 µg/ml) or human IgM (50 µg/ml), the supernatant was then removed from each well followed by the addition of 20 nM Q-dot streptavidin (Quantum Dot Corporation, Hayward, CA, USA) to each of the wells. Immunoreactivity on the cell surface was visualized with a confocal microscope (CSU10-R, Yokogawa Electronic Corporation, Tokyo, Japan).

**Selection of HoAKs-1.** After evaluation for reactivity to cancer cells, a hybridoma clone, HoAKs-1 was selected. This clone produced human IgM monoclonal Abs that showed reactivity to MKN-45, HLC-1 and PANC-1, but not to normal cells or HUVECs in the initial screening or secondary evaluation. HoAKs-1 was purified from the supernatant of the respective hybridoma cells and was stored at −4°C until use.

**Immunohistochemical staining.** Purified HoAKs-1 and the control human IgM were biotinylated using a biotinylation reagent (Amersham Life Sciences). The formalin-fixed, paraffin-embedded sections were deparaffinized and then blocked for 30 min at room temperature with blocking reagent (Blocking One, Nacalai Tesque, Inc., Kyoto, Japan). After incubation with biotinylated HoAKs-1 (50 µg/ml) or human IgM (50 µg/ml) for 30 min at room temperature, each section was washed three times with PBS/20%-Tween and then stained with the Dako staining kit (DAKO Corp., CA, USA). Streptavidin-peroxidase (Vecter Laboratories) was used to detect the biotinylated antibodies that reacted with the tissue sections, while 3, 3′-diaminobenzidine tetrahydrochloride was used as the substrate. After counterstaining with hematoxylin, the sections were examined by light microscopy.

**Morphological alteration of human cancer cell lines by the antibody.** MKN-45, HLC-1, PANC-1 and HUVEC cells were cultured in their respective complete growth media at 37°C in a humidified incubator. The cells were plated on 96-well plates and incubated with each antibody (70 µg/ml) or human IgM (50 µg/ml) and then screened for human antibody production using ELISAs.

**MTT proliferation assay.** The MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5 diphenyl tetrazolium bromide: Sigma Chemical Co.) assay was performed in order to detect any growth inhibition after incubation with each antibody. MKN-45, HLC-1 and HUVEC cells were plated on 96-well plates at 1.5x10³ cells / well and incubated at 37°C for 6 days with each antibody at a final concentration of 70 µg/ml. Normal human IgM (Chemicon International, Inc., CA, USA) was used as a control antibody. Growth inhibition was evaluated by the MTT assay.

**Analysis of antigen recognized by HoAKs-1.** Cultured PANC-1 cells were collected. Using sonication, a whole-cell lysate was prepared and infused with anti-PANC-1 antibodies (anti-PANC-1 IgM). The antibody was then removed by centrifugation followed by elution with 0.1 M HCl. After elution, the antibody was precipitated with 50% PEG 6000 and dialyzed against dilution buffer containing 10% FCS. The precipitated antibody was then used for the MTT proliferation assay.
in ice-cold RIPA buffer (0.1% sodium dodecyl sulfate, 1% Nonidet-P40, 0.5% deoxycholate, 50 mM Tris-HCl, 150 mM/L NaCl, pH 8.0) with 1/1000 volume of proteinase inhibitor mix stock solution (5 mg/ml of leupeptin, 5 mg/ml of pepstatin A and 5mg/ml of Chymostatin, all purchased from Peptide Institute, Inc., Osaka, Japan). Quantification of the protein in these samples was performed using Lowry’s method. The soluble and insoluble fractions of PANC-1 cells were prepared by first homogenizing the cells in TNE-buffer (10 mM Tris-HCL, pH 7.6, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors. The cells were then centrifuged at 10,000 rpm for 20 min, followed by separation of the supernatant as the soluble fraction and the precipitate as the insoluble fraction. The insoluble fraction was also lysed with RIPA-buffer containing protease inhibitors. After approximately 30 μg of protein from the whole-cell lysate were obtained, the soluble and insoluble fractions were electrophoresed on 2~15% gradient polyacrylamide gels (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). The proteins were transferred to PVDF membranes (Immobilon-P, Millipore, MA, USA) under non-reducing conditions using a semi-dry electroblotter (Bio-Rad Laboratories, CA, USA). The membranes were then incubated with a blocking reagent, followed by overnight incubation at 4°C with HoAKs-1. Horseradish peroxidase-conjugated goat anti-human immunoglobulins (anti human Igs-HRP) (Cappel) were used as the second antibody, with immunoreactivity being detected using a Western blot luminal reagent (Santa Cruz Biotechnology Inc., CA, USA).

Results

Reactivity to cancer cell lines detected by confocal microscopy. Confocal microscopy showed intense reactivity to the cell surface (cell membrane) in HLC-1 and PANC-1 cells, but not in HUVEC cells (Figure 1).

Table I. The reactivity of HoAKs-1 to various kinds of epithelial cancer cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Positive /Negative for reactivity</th>
<th>Rate of positive reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLC-1</td>
<td>Positive</td>
<td>2/3</td>
</tr>
<tr>
<td>A549</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>PC-9</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PANC-1</td>
<td>Positive</td>
<td>3/3</td>
</tr>
<tr>
<td>PK-8</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>SUIT2</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MKN-45</td>
<td>Positive</td>
<td>1/4</td>
</tr>
<tr>
<td>MKN-74</td>
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<td></td>
</tr>
<tr>
<td>HSC-3</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>SKS</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Colon cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-29</td>
<td>Negative</td>
<td>0/4</td>
</tr>
<tr>
<td>LoVo</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Colo205</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>DLD-1</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>6/14 (43%)</td>
</tr>
</tbody>
</table>

Reactivity in xenografts. Fourteen xenografts, established in nude mice derived from 14 human cancer cells as described in the Materials and Methods section, were examined in order to evaluate the specificity of HoAKs-1. This demonstrated a 6 out of 14 positive rate for reactivity of the Ab. HoAKs-1 had reactivity to all 3 pancreatic cancers, 3 out of 4 lung cancers, 1 out of 3 gastric cancers, and none of the 3 colon cancer cell lines (Table I, Figures 2, 3).

Specific reaction to cancer lesions in clinical specimens. Immunohistochemical staining showed HoAKs-1 reactivity to the primary cancer lesions, but not to normal lung tissue of the patient who provided TIL-producing HoAKs-1 (Figure 4; Case 1). HoAKs-1 also showed specific staining in cancer lesions, but not in normal lung tissue of another patient (Figure 4; Case 2).

HoAKs-1-induced morphological alterations in cancer cells. While non-malignant HUVECs did not show any morphological changes after incubation with 70 μg/ml of HoAKs-1, incubation of the MKN45, HLC-1 and PANC-1 cancer cell lines with 70 μg/ml of HoAKs-1 for 48 h resulted in altered morphology seen as neurite like cytoplasmic processes (Figure 5).
Figure 2. Purified HoAKS-1 was biotinylated using a biotinylation reagent. Formalin-fixed, paraffin-embedded sections were deparaffinized and blocked with blocking reagent (Blocking One, Nacalai Tesque, Inc., Kyoto, Japan). After incubation with biotinylated HoAKS-1 (50 ng/ml), each section was washed, and then stained using a Dako staining kit. Streptavidin-peroxidase was used to detect the biotinylated antibodies that reacted with the tissue section, while 3,3'-diaminobenzidine tetrahydrochloride was used as the substrate. After counterstaining with hematoxylin, the sections were examined by light microscopy. HoAKS-1 showed reactivity to all of the three pancreatic cancer cell lines, PANC-1, PK8 and SUIT2.

Figure 3. Formalin-fixed, paraffin-embedded sections were deparaffinized and blocked with blocking reagent. After incubation with biotinylated HoAKS-1 (50 ng/ml), each section was stained using a Dako staining kit. Streptavidin-peroxidase and 3,3'-diaminobenzidine tetrahydrochloride was added to each section. After counterstaining with hematoxylin, the sections were examined by light microscopy. HoAKS-1 showed reactivity to two of the three lung cancer cell lines, HLC-1 and A549.
HoAKs-1 inhibited cancer cell growth. After 6 days of incubation with HoAKs-1 at a concentration of 70 Ìg/ml, the growth inhibition rate for MKN-45, HLC-1 and HUVEC cells was 63%, 47% and 2%, respectively, compared with similar incubation of each control cell with non-specific human IgM (Figure 6).

Antigen recognized by HoAKs-1. Using Western blot techniques with the PANC-1 cell lysate, it was confirmed that the HoAKs-1 reacting protein was contained in the insoluble fraction and had a molecular weight of approximately 55 kDa (Figure 7).

Discussion

It has been reported that TIL are critical markers of immunological activity and prognosis in patients with primary or metastatic malignant melanoma and also correlate with the degree of T-cell infiltration in the clinical response (22, 23). A high CD4+ T-cell to TIL ratio has been associated with better prognosis in primary and metastatic melanoma (24, 25). Nonetheless, the broader applicability of TIL in the treatment of cancer patients remains unclear and further study is therefore required. TIL have the potential to be a powerful method for predicting prognosis in cancer patients and also as a means of establishing novel treatment approaches. In the present study, we found that the success rate for establishing HuMoAbs by means of a hybridoma technique was 6 times higher with TIL than with lymphocytes from lymph nodes (data not shown). In addition, we have already obtained 36 clones to produce HuMoAbs, which showed specific reactivity to malignant cancer cells associated with some biological reaction, such as the growth inhibition caused by HoAKs-1. This finding suggested the system we utilized in the present study will be an optimal procedure for developing novel HuMoAbs.

Approximately 75 MoAbs have been approved for either human use or for clinical trials, with approximately 20% of these Abs being currently recognized as antitumor agents. Four of these agents have already been approved for clinical use in cancer patients (26-31). These agents include Campath-1H (Alemtuzumab, humanized anti-CD52 for chronic lymphocytic leukemia), Herceptin (Trastuzumab, humanized anti-Her-2/neu protein for breast cancer),
Rituximab (humanized anti-CD20 for chronic lymphocytic leukemia and non-Hodgkin’s lymphoma) and Gemtuzumab ozogamicin (humanized anti-CD33, CMA-676 for acute myeloid leukemia). In addition, other humanized Abs have also been developed for use against the anti-epidermal growth factor receptors. For example, h-R3 and C225 (Cetuximab) have been used clinically for the treatment of head and neck, lung and colon cancers.

At present, almost all clinically applied Abs are chimeric or humanized MoAbs that contain part of a murine antibody in the Fab regions or in the complementary determining regions (CDR), respectively. The foreign protein of the murine antibody has the potential to stimulate immune responses, with recognition of the murine protein at the initial treatment inducing human anti-mouse antibodies (HAMA) that may result in anaphylaxis and allergy at the second administration of the Abs. In addition, HAMA formation is also known to decrease serum half-life, resulting in reduced effectiveness of the Abs.

Thus, HuMoAbs appear to be ideal for treatment in humans, as these antibodies will be utilized without any accompanying immunological toxicity. 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 (32). Similarly, SC-1, a HuMoAb isolated from a patient with gastric signet ring cell carcinoma, was shown to induce apoptosis in gastric cancer cells in vitro and in vivo, and showed little toxicity in Phase II clinical trials (33-39). We previously reported the development of a HuMoAb, GAH, using the same techniques described in the present study (40). GAH demonstrated high binding specificity for cancer cells of the stomach and colon, suggesting that a GAH drug delivery system may be a potent and useful means of targeting cancer cells (41).

In conclusion, we successfully developed a HuMoAb, HoAKs-1, that showed specific reactivity to several cancer cell lines, observed by confocal microscopy as binding of the antibody to the cell membrane of the cancer cells. At the same time, we confirmed that HoAKs-1 did not react with normal cells or HUVECs. The specific reactivity of HoAKs-1 to cancerous lesions was also confirmed by immunohistochemical staining of clinical specimens. Interestingly, in immunohistochemical staining of xenografts, HoAKs-1 showed intense reactivity to several kinds of cancer characterized by extremely poor prognosis, such as pancreatic or lung cancer cell lines. HoAKs-1 also induced morphological changes in cancer cells, seen as elongated cytoplasmic processes-like neurites, in addition to causing growth inhibition in vitro. Although further research is necessary before HoAKs-1 can be applied clinically with both safety and effectiveness, the successful production of this HuMoAb may result in a beneficial outcome in clinics for treating cancer cells.

References


Received June 9, 2005
Accepted July 8, 2005