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HUMAN-HUMAN HYBRIDOMAS SECRETING ANTIBODIES SPECIFIC TO HUMAN LUNG CARCINOMA

H. MURAKAMI, S. HASHIZUME, H. OHASHI, K. SHINOHARA, K. YASUMOTO, K. NOMOTO, AND H. OMURA

Food Science and Technology Institute (H. M., H. O., K. S., H. O.), Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812, Japan, Morinaga Institute of Biological Science (S. H.), Shimosueyoshi 2-1-1, Tsurumi-ku, Yokohama 230, Japan, and Second Surgery (K. Y.), Medical Institute of Bioregulation (K. N.), Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812, Japan

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SUMMARY

Human Namalwa cells were screened in serum-free medium and in 6-thioguanine, then fused with human lymphocytes from lymph nodes of lung adenocarcinoma cancer patients. Extensive testing using 14 lung cancer cell lines, 11 other cancer cell lines and 4 normal fibroblast lines identified monoclonal antibodies produced by 4 hybridoma clones that reacted specifically with lung adenocarcinoma cells. These monoclonal antibodies also reacted with lung adenocarcinoma tissues and not normal tissues or erythrocytes of any blood type. These hybridoma clones grew and stably secreted the antibodies in serum-free medium as well as in serum-containing medium.

Key words: human hybridoma; serum-free medium; lung adenocarcinoma.

INTRODUCTION

The technology developed by Kohler and Milstein (10) for preparing antibody-secreting hybridomas has led to attempts to obtain monoclonal antibodies (MoAb) specific to human cancer for diagnostic and therapeutic uses. Some of the MoAb originating from mouse-mouse hybridomas are reported to be applicable to this end (1,4,7-9,12,16,17,19,20). However, human MoAb so far reported against lung cancer are few (6,18). For example Glassy et al. (6) have reported that the WLNA6 MoAb reacted strongly with T293, an oat cell carcinoma of the lung, had weak reactivity with the HeLa and CaSki cervical carcinoma cell lines, and had no reactivity with lung carcinoma Calu-1, prostate carcinoma Ln-Cap, or WI-38 and 350Q normal fibroblasts. Sikora et al. (18) have also shown that specificity studies on 12 MoAb clearly show a wide range of weak binding to G/CCM glioma, MOR lung adenocarcinoma, Calu-1 squamous cell lung carcinoma and HT29 colorectal carcinoma. However, human MoAb highly specific for lung adenocarcinoma have not yet been reported. It is important to make such MoAb since almost half of lung cancer patients suffer from adenocarcinoma. To make specific MoAb-secreting hybridomas, we first used a HAT-sensitive human lymphoma cell line (NAT-30) from Namalwa cells as a fusion partner with B-lymphocytes. The NAT-30 not only gave fusion efficiency comparable to the LICR-LON-HMy2 lymphoblastoid line (14), SKO-007 myeloma line (15), GM4672 lymphoblastoid cell line (3) and LTR228 lymphoblastoid cell line (11), but also proliferated indefinitely in a growth factor-supplemented serum-free medium. We report here that we made human-human hybridomas from which MoAb specifically reactive to human lung adenocarcinoma were obtained. The hybridomas can proliferate in serum-free medium, facilitating purification of the MoAb.

MATERIALS AND METHODS

Establishment of hypoxanthine guanine phosphoribosyl transferase (HGPRT)-deficient human lymphoma lines for fusion. Namalwa, a human B lymphoma cell line, was first cultured in a 2:1:1 mixture of RPMI 1640, Ham's F12 and Dulbecco's modified Eagle's media (RDF), supplemented with 10 μg/ml of insulin, 35 μg/ml of transferrin, 10 μM of ethanolamine and 2.5 nM of selenium (ITES)(13). About 50% of the cells died from this treatment, but after subculturing for 2 weeks, the remaining cells were almost 100% viable. These viable cells were cloned in soft agar (0.24%) in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (FCS). The resultant clones were screened in DME/10% FCS medium containing dead Namalwa (5×10^5 cells/ml), prepared by freezing and thawing of Namalwa cells, to obtain clones which could start growing even in a number of dead cells during selection in HAT medium. HGPRT-deficient mutants were selected in DME/10% FCS medium containing 30 μg/ml of 6-thioguanine, and the resultant mutants were tested for aminopterin sensitivity with a dose range of 4×10^{-7}-10^{-5} M. The mutant clone, NAT-30, was selected on the basis of level of aminopterin sensitivity, fusing efficiencies and growth rates.

Preparation of lymphocytes from lymph nodes. Lymphocytes were prepared from lymph nodes of lung cancer patients as follows. The lymph nodes were cut into small pieces and pressed between two slide glasses. The turbid suspension squeezed out was washed twice with DME by

centrifugation and used as a lymphocyte cell suspension in cell fusion experiments.

Fusion procedure. Fusion of the lymphocytes with NAT-30 was carried out essentially as described by Köhler and Milstein (10). Usually, 8×10^7 lymphocytes and 3×10^7 NAT-30 cells were suspended in 1 ml of 42.5% (w/v) polyethylene glycol (M.W., 1500; BDH Chemicals, England) in DME containing 15% dimethyl sulfoxide for 1 min and incubated at 37° C for another 1 min, followed by the addition of 9 ml of DME. The cells were then pelleted at 200 ×g. Hundred μl cell suspensions containing 10^4 lymphoma cells in a well were cultured for one day, and then 100 μl of DME/15% FCS medium containing 200 μM hypoxanthine, 0.8 μM aminopterin and 32 μM thymidine (2 × concentration of HAT medium) was further added to the wells. The cultures were maintained at 37° C in humidified 7% O_2/5% in HAT medium for 14–28 days. The medium was then changed to 100 μM hypoxanthine/16 μM thymidine and further maintained for about 7 days, and then changed to DME/10% FCS or RDF/ITES medium. In all the cases, the medium was changed every 3 days. Control cultures of lymphoma cell lines contained no surviving cells after 1 week in HAT medium. The first putative hybridoma cells could be detected at about 2 weeks after fusion.

Indirect immunofluorescent assay. This was essentially carried out by the method of Dorreen et al. (5). For testing cell lines, human cancer and normal cell lines were cultured to confluency. These lines were fixed with 0.05% glutaraldehyde and washed three times with phosphate-buffered saline (PBS). Fixed cells were then washed in 3% bovine serum albumin (BSA) in PBS and incubated in the BSA solution for 1 hr at room temperature to prevent non-specific adsorption of immunoglobulin onto the test cells in the next step. After the incubation, the cells were washed five times with PBS and incubated with the spent media of hybridoma clones for 3 hr. After incubation at room temperature, the cells were washed in PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human Ig (1:50, Tago). After a further 3 hr at room temperature, the cells were washed three times and observed in a fluorescent microscope. To check reactivity with cancer tissues, frozen sections of human tissues were fixed for 30 min in 10% formaldehyde and washed three times with PBS. Fixed sections were washed with 10% goat serum in PBS and incubated in the serum solution for 30 min at room temperature to prevent non-specific adsorption. The following procedures were the same as described above. Erythrocyte suspensions from which lymphocytes and platelets had been removed by centrifugation were transferred to a 96-well plate and fixed with 0.05% glutaraldehyde. Other procedures were the same as described above.

Results

HGPRT-deficient human lymphoma cells to be used as a fusion partner with human B lymphocytes were selected by a series of applications of exposures to serum-free ITES medium and media containing dead Namalwa and 6-thioguanine. Among HGPRT-deficient clones obtained, NAT-30 clone was used for hybridization, since the growth rate of these cells in the serum-free medium was the best.

Fusion of NAT-30 was carried out with human lymphocytes from lymph nodes of lung cancer patients bearing lung adenocarcinoma. Through repeated fusions more than 2000 hybridoma clones were obtained. The proportion of hybridoma clones secreting IgM, IgA and IgG were 4, 2 and 1, respectively. These MoAb were tested for reactivity to lung

TABLE 1

REACTIVITIES OF MONOCLONAL ANTIBODIES TO VARIOUS CELL LINES BY INDIRECT IMMUNOFLUORESCENT ASSAY[a]

Cell line	HF10B4	HF10F7	HB4G7	HB4C5	H-7	H-9	Human plasma
Human cancer lines							
Lung, adenocarcinoma							
PC-8	+++	++	+++	+	−	−	−
PC-3	++	+	+	+	−	−	−
PC-9	−	−	−	−	−	−	−
PC-14	+	−	−	−	−	−	−
HLC-1-109	+	+	+	−	−	−	−
ABC-1	+	−	+	−	−	−	−
Lung, squamous carcinoma							
QG-56	−	−	−	−	−	−	−
PC-1	+	−	−	−	−	−	−
PC-10	−	−	−	−	−	−	−
EBC-1	+	−	+	−	−	−	−
Lung, small cell anaplastic carcinoma							
QG-90	−	−	−	−	−	−	−
SBC-1	++	+	++	+	−	−	−
Lung, large cell anaplastic carcinoma							
PC-13	+	+	+	−	−	−	−
Lung, oat cell carcinoma							
PC-6	+	−	+	−	−	−	−
Bladder							
KU-1	−	−	−	−	−	−	−
NBT-2	−	−	−	−	−	−	−
Breast							
ZR-75-1	−	−	−	−	−	−	−
ZR-75-30	−	−	−	−	−	−	−
Epidermoid							
A431	−	−	−	−	−	−	−
Renal							
KPK-1	−	−	−	−	−	−	−
KPP-1	−	−	−	−	−	−	−
Melanoma							
G361	−	−	−	−	−	−	−
Bowes	−	−	−	−	−	−	−
Stomach, adenocarcinoma							
MKN-28	−	−	−	−	−	−	−
MKN-45	+	+	+	−	−	−	−
Normal human cell lines							
Lung, fibroblast							
WI-38	−	−	−	−	−	−	−
Flow 2000	−	−	−	−	−	−	−
Foreskin, fibroblast							
Flow 7000	−	−	−	−	−	−	−
Intestine, fibroblast							
Flow 11 000	−	−	−	−	−	−	−

[a]The cells were cultured to confluency, fixed with 0.05% glutaraldehyde and used for the assay. +++; more than 70%, ++; 50-70%, +; 20-50% of the total cells were stained with fluorescent dye-conjugated anti-human IgM after the treatment with each MoAb. −; no anti-human IgM bound to the cells was microscopically detectable. Positiveness was easily discriminated as every positive cell lines, when stained with the dye-conjugated anti-human IgM, exhibited more than 20% of the population reactive with the antibody.

TABLE 2

REACTIVITIES OF MONOCLONAL ANTIBODY FROM HYBRIDOMA CLONE, HF10B4, TO VARIOUS TISSUES AND ERYTHROCYTES BY INDIRECT IMMUNOFLUORESCENT ASSAY[a]

Sample		Reactivity
Lung adenocarcinoma	Patient 1	+
	2	+
	3	+
	4	+
	5	+
	6	+
	7	+
Normal lung[b]	patient 1	−
	2	−
	3	−
	4	−
	5	+
	6	−
	7	−
Normal kidney[c]	patient 8	−
Normal liver[c]	8	−
Erythrocyte blood type		
A	normal adult 1	−
B	2	−
AB	3	−
O	4	−

[a]Tissues were sliced with a thickness of 10 μm on slide glass, fixed with 10% formaldehyde and used for the assay (5). Erythrocytes were sedimented by centrifugation, fixed with 0.05% glutaraldehyde and used for the assay. +; more than 20% of cells in the tissue was stained with the dye-conjugated anti-human IgM after the treatment with MoAb from HF10B4 cells. −; no IgM was detectable on the tissue or cells.

[b]Histologically normal tissues adjacent to the lung cancer of the same patient whose cancer tissues were assayed in a.

[c]From patients bearing stomach cancer.

adenocarcinoma cell line PC-8 by the indirect immunofluorescent assay. Four clones (HF10B4, HF10F7, HB4G7 and HB4C5) of the tested hybridomas were detected to secrete MoAb reacting against the lung adenocarcinoma. Those MoAb were all IgM class with lambda light chains. The chromosome number of the HB4C5 clone after 6 months' culture was 56.5 ± 5.3, compared with 46.3 ± 0.5 for NAT-30 cells, and the chromosome numbers of other clones were also apparently more than that of NAT-30.

IgM in the spent media of these 4 positive hybridoma clones were then examined for specificities on a panel of 29 established human cell lines: 14 lung, 2 bladder, 2 breast, epidermoid, 2 renal, 2 melanoma, and 2 stomach cancers, and 4 normal fibroblasts. These results are summarized in Table 1.

MoAb from 4 clones strongly reacted only with the lung carcinoma lines. Other types of cancer cells except a stomach cancer line, MKN-45 which bound MoAb of HF10B4, HF10F7 and HB4G7, had no reactivity with any of the 4 MoAb. None of these 4 MoAb reacted with normal fibroblast lines, including WI-38 and Flow 2000 originating from lung tissue. MoAb of HF10B4 reacted with lung cancer lines more strongly than those of HF10F7, HB4G7 or HB4C5. MoAb of HF10B4 reacted with all six lung carcinoma cell lines except for PC-9, a differentiated adenocarcinoma. IgM from clone H-7, H-9 or human plasma did not react with any cell line.

MoAb of HF10B4 clone was tested for reactivity with lung adenocarcinoma tissues from patients. As shown in Table 2, this MoAb reacted with all seven adenocarcinoma tissues. Reactivity of the MoAb against the normal tissues adjacent to the corresponding cancer tissues was also tested. Most of the normal tissues were negative. Although one normal tissue (patient 5) was judged positive, inclusion of cancer cells in this tissue was suspected. This MoAb did not react against normal tissue of kidney or liver, or erythrocytes of any blood type.

Four hybridoma clones producing IgM specific to lung cancer were examined for growth in serum-free culture by using RDF medium supplemented with ITES. The doubling time of these hybridomas in the serum-free medium was almost twice that in serum-containing medium, as shown in Table 3. Long-term cultivation of these cells was also successful in the serum-free medium. MoAb secreted by the cells showed no difference in the serum-free and serum-containing conditions, indicating that productivity of the IgM by hybridoma cells depended on cell density but not on the presence of serum components. MoAb productivity by HF10F7 cells was the highest among 4 clones, and reached 16 and 41 μg/ml after 3 day culture in the serum-free and serum-containing media, respectively. There was no difference between the two media in the specificity of secreted MoAb to the cell lines tested. Purities of the MoAb were 20 and 0.2% of the total protein in spent media of serum-free and serum-containing conditions, respectively.

DISCUSSION

We first established HGPRT-deficient human lymphoma cells as a fusion partner with human B lymphocytes. Namalwa cells vigorously growing in the serum-free medium were selected, as we expected to grow resultant hybridoma cells in serum-free medium and had known that myeloma cells, which could grow in a serum-free condition, usually gave

TABLE 3

GROWTH OF HYBRIDOMA CLONES IN SERUM-FREE MEDIUM[a]

Clone	Cell density at day 5 (cells/ml × 10^{-5})		Doubling time in logarithmic phase (hr.)	
	serum-free	serum	serum-free	serum
HF10B4	12	23	20	13
HF10F7	10	24	31	19
HB4G7	13	29	20	12
HB4C5	10	17	40	21

[a]Hybridoma clone cells were plated at a density of 10^5 cells per ml of serum-free or serum-containing medium (see text).

hybridomas growing in the same serum-free medium. In addition to this treatment, these vigorously growing cells were screened in the dead Namalwa-containing medium, to obtain stronger cells for fusion. When fusion was carried out with the cells thus obtained, the fusion efficiency was one hybridoma per 4×10^5 NAT-30 cells, which is similar to that with LICR-LON-HMy2 and SKO-007 cells reported by Cote et al. (2).

We obtained 4 hybridoma clones (HF10B4, HF10F7, HB4G7 and HB4C5), which secrete MoAb reacting against a lung adenocarcinoma line, PC-8. Among these MoAb, MoAb from HF10B4 reacted most widely with a variety of lung adenocarcinoma lines (Table 1). Reactivities of these MoAb with other lung cancer lines such as squamous carcinoma, small cell anaplastic carcinoma and large cell carcinoma depended on the cell line tested. These results indicate that these MoAb are predominantly reactive with the lung adenocarcinoma lines but not with other types of cancer cells or normal fibroblast lines except for a stomach cancer line, MKN-45. Though the reason why this stomach cancer line reacted with the MoAb was still ambiguous, this line may have the same kind of cell surface antigen(s) as lung adenocarcinoma cells.

The reactivity of MoAb against lung adenocarcinoma tissues was also positive (Table 2). However, MoAb did not react with normal tissues or any blood type of erythrocytes. These results suggest that MoAb secreted by HF10B4, HF10F7, HB4G7 and HB4C5 hybridomas may be useful for diagnosis and therapy of adenocarcinoma, which comprises almost half of all lung cancers. More extensive study on the reactivity of these 4 MoAb to cancer and normal tissues is currently under way.

As we expected, hybridoma clones proliferated sufficiently in the serum-free medium (Table 3). MoAb production per cell was not different between serum-free and serum-containing conditions. The MoAb in the serum-free spent medium was easily purified with ammonium sulfate precipitation followed by Sepharose 4B column chromatography, and gave a single band on an agarose gel electrophoretogram.

References

1. Colcher, D.; Horan Hand, P.; Nuti, M.; Schlom, J. A spectrum of monoclonal antibodies reactive with human mammary tumour cells. Proc. Natl. Acad. Sci. USA 78:3199-3203; 1981.
2. Cote, R. J.; Morrissey, D. M.; Houghton, A. N.; Beattie, E. J., Jr.; Oettgen, H. F.; Old, L. J. Generation of human monoclonal antibodies reactive with cellular antigens. Proc. Natl. Acad. Sci. USA 80:2026-2030; 1983.
3. Croce, C. A.; Linnenbach, A.; Hall, W.; Steplewski, Z.; Koprowski, H. Production of human hybridomas secreting antibody to measles virus. Nature 288:488-489; 1980.
4. Cuttitta, F.; Rosen, S.; Gazdar, A. F.; Minna, J. D. Monoclonal antibodies that demonstrate specificity for several types of human lung cancer. Proc. Natl. Acad. Sci. USA 78:4591-4595; 1981.
5. Dorreen, M. S.; Habeshaw, J. A.; Wrigley, P. F. M.; Lister, T. A. Distribution of T-lymphocyte subsets in Hodgkin's disease characterized by monoclonal antibodies. Br. J. Cancer 45:491-497; 1982.
6. Glassy, M. C.; Handley, H. H.; Hagiwara, H.; Royston, I. UC 729-6, a human lymphoblastoid B-cell line useful for generating antibody-secreting human-human hybridomas. Proc. Natl. Acad. Sci. USA 80:6327-6331; 1983.
7. Hakomori, S.; Nudelman, E.; Levery, S. B.; Patterson, C. M. Human cancer-associated gangliosides defined by a monoclonal antibody (IB9) directed to sialosyl $\alpha 2 \rightarrow 6$ galactosyl residue: a preliminary note. Biochem. Biophys. Res. Commun. 113:791-798; 1983.
8. Imai, K.; Ng, A. K.; Ferrone, S. Characterization of monoclonal antibodies to human melanoma-associated antigens. JNCI 66:489-496; 1981.
9. Kennett, R. H.; Gilbert, F. Hybrid myelomas producing antibodies against a human neuroblastoma antigen present on fetal brain. Science 203:1120-1121; 1979.
10. Köhler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497; 1975.
11. Larrick, J. W.; Truitt, K. E.; Raubitschek, A. A.; Senyk, G.; Wang, J. C. N. Characterization of human hybridomas secreting antibody to tetanus toxoid. Proc. Natl. Acad. Sci. USA 80:6376-6380; 1983.
12. Mitchell, K. F.; Fuhrer, J. P.; Steplewski, Z.; Koprowski, H. Biochemical characterization of human melanoma cell surfaces: dissection with monoclonal antibodies. Proc. Natl. Acad. Sci. USA 77:7287-7291; 1980.
13. Murakami, H.; Masui, H.; Sato, G. H.; Sueoka, N.; Chow, T. P.; Kano-Sueoka, T. Growth of hybridoma cells in serum-free medium: ethanolamine is an essential component. Proc. Natl. Acad. Sci. USA 79:1158-1162; 1982.
14. O'Hare, M. J.; Smith, C. M.; Edwards, P. A. W. A new human hybridoma system (LICR-LON-HMy2) and its use in the production of human monoclonal antibodies. Peeters, H. ed. Protides of Biological Fluids, Colloquium 30. Oxford:Pergamon Press; 1982:265-268.
15. Olsson, L.; Kaplan, H. S. Human-human hybridomas producing monoclonal antibodies of predefined specificity. Proc. Natl. Acad. Sci. USA 77:5427-5431; 1980.
16. Ritz, J.; Pesando, J. M.; Notis-McConarty, J.; Lazarus, H.; Schlossman, S. F. A monoclonal antibody to human acute lymphoblastic leukemia antigen. Nature 283:583-585; 1980.
17. Ritz, J.; Pesando, J. M.; Notis-McConarty, J.; Schlossman, S. F. Modulation of human acute lymphoblastic leukemia antigen induced by monoclonal antibody *in vitro*. J. Immunol. 125:1506-1514; 1980.
18. Sikora, K.; Alderson, T.; Ellis, J.; Phillips, J.; Watson, J. Human hybridomas from patients with malignant disease. Br. J. Cancer 47:135-145; 1983.
19. Soule, H. R.; Linder, E.; Edgington, T. S. Membrane 126-kilodalton phosphoglycoprotein associated with human carcinomas identified by a hybridoma antibody to mammary carcinoma cells. Proc. Natl. Acad. Sci. USA 80:1332-1336; 1983.
20. Woodbury, R. G.; Brown, J. P.; Yeh, M. Y.; Hellstrom, I.; Hellstrom, K. E. Identification of a cell surface protein, p97, in human melanomas and certain other neoplasms. Proc. Natl. Acad. Sci. USA 77:2183-2187; 1980.

EDITOR'S STATEMENT

Identification of monoclonal antibodies that recognize human lung adenocarcinoma cells with reasonable specificity represents a potentially important development that may prove useful in diagnosis and therapy of neoplastic disease. The selection procedures and methods for propagation of the human-human hybridomas described in this paper also represent some novel approaches that may be of general application.

David W. Barnes