Short communication

Establishment of hybridomas producing cancer specific human antibodies from B cell line derived from PBL of a patient with adult T cell leukemia

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Abstract

Adult T cell leukemia (ATL) is a malignant disease characterized by tumorous proliferation of CD4⁺ T cells infected with retrovirus human T cell leukemia virus Type-I (HTLV-I) and concurs with an autoimmune disease and cancer due to attenuated immune response. In this study, we established ATL patient derived B-cell line TM-1 producing cancer-specific IgM antibodies, and further characterized its antigen specificity by establishing hybridomas fused with human-mouse origin hetero-myeloma cell line RF-S1. We established three hybridoma cell lines termed 2E12, 3E9, and 3E10, which continuously secreted human IgM antibodies. Immunohistochemical staining of formalin-fixed tissue section using antibodies secreted from these hybridomas showed that these antibodies specifically recognized tumor sites of human colon adenocarcinomas. Antibody produced from hybridoma 3E9 bound to some of leukemic cell lines, but not to normal human PBL, which was evidenced by the flow cytometric analysis, indicating that antibody produced from 3E9 recognizes cell surface antigen specifically expressed in the leukemic cells.

Abbreviations: ATL, adult T cell leukemia; EBNA, Epstein Barr virus nuclear antigen; EB virus, Epstein Barr virus; ELISA, enzyme-linked immunosorbent assay; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; HTLV-I, human T cell leukemia virus Type-I; LMP, latent membrane protein; MAb, monoclonal antibody; MNC, mononuclear cells; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PBL, peripheral blood lymphocytes.

Introduction

Human monoclonal antibodies (MAbs) reactive to human tumor associated antigens are useful tools for diagnosis and therapy of various cancers because of their absences of antigenicities. There have been many reports describing about the acquisition of human MAbs specific to several kinds of cancer cells (Murakami et al., 1985; Kjeldsen et al., 1988; Aihara et al., 1988). In some cases, researchers have succeeded in applying antibodies in clinical uses for diagnosis and therapy

(Hashizume et al., 1990; Shoji et al., 1996; Nishimura et al., 1999).

We established the *in vitro* immunization method to generate cancer specific human antibody (Kawahara et al., 1992). In the *in vitro* immunization protocol, we stimulated PBL from the volunteers with human adenocarcinoma cell line A549 cells in the presence of several additives (Kawahara et al., 1992). After cell fusion with A₄H₁₂ cells, a fusion partner cell line originated from Molt-4 and limited dilutional cloning, we could obtain hybridomas producing cancer specific human antibodies (Shoji et al., 1994). However, the

efficiency in establishment of cancer specific human antibodies was found to be very low in the *in vitro* immunization procedure, which had to be overcome by experimental improvements.

In this study, we tried to reconstruct the experimental procedure to obtain cancer specific human antibodies. Based upon the assumption that ATL patients have B cells specific for cancer because of their high occurrence of cancer (Morimoto et al., 1989) and attenuated immune responses, we stimulated PBL of ATL patient with lipopolysaccharide (LPS) to proliferate Epstein Barr virus (EB virus) infected B cells, and established B cell line, TM-1 producing antibodies reactive to several cancer tissues. In order to further characterize antibodies produced from TM-1, we fused TM-1 with human-mouse heteromyeloma cell line of RF-S1 (Tsukazaki et al., 1995), and established hybridomas producing cancer specific monoclonal antibodies after limited dilutional cloning.

Materials and methods

Establishment of TM-1

Mononuclear cells (MNC) derived from peripheral blood of an ATL patient were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 20% fetal calf serum (FCS) and $100 \mu\text{g ml}^{-1}$ of lipopolysaccharide (LPS). Spontaneously immortalized B cell lines was established and named TM-1.

Cell fusion

Cell fusion method was previously described (Kawahara et al., 1992). Briefly, 3×10^6 cells of TM-1 and 1.5×10^6 cells of RF-S1 were mixed in 50 ml polyethylene tube and fused by the addition of 1 ml of 50% (w/v) polyethylene glycol 4000 in phosphate-buffered saline (PBS). The tubes were gently rotated for 90 sec. After the addition of 10 ml of ERDF medium (Gibco), the tubes were incubated at 37 °C for 5 min. Fused cells were selected by ERDF medium containing 20% fetal bovine serum (FBS; HyClone Lab., Logan, UT), 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine (HAT medium). After 2–3 weeks culture, the wells containing vigorously growing cells were counted and cultured in 20% FBS-ERDF medium containing 100 mM hypoxanthine and 16 mM thymidine.

Cells and cell culture

The B cell line TM-1 was cultured in ERDF medium supplemented with 10% FBS. Human-mouse heteromyeloma cell line RF-S1, which lacked in hypoxanthine-guanine phosphoribosyl transferase activity, were used as fusion partner cell line and cultured in 10% FBS-ERDF medium. Human T cell line of Jurkat and Molt-4, human B cell line of Raji, Daudi and Namalwa and human promonocytic cell line U937 were cultured in 10% FBS-ERDF medium. Human basophilic cell line KU812 was cultured in RPMI1640 medium supplemented with 10% FBS. Human peripheral blood lymphocytes (PBL) were isolated from peripheral blood of a healthy volunteer by density gradient centrifugation using Lymphocyte Separation Medium (Organon Teknica Corporation, Durham, NC) and cultured in 10% FBS-ERDF medium.

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six well immuno-plates were coated overnight with goat anti-human IgM (TAGO, Burlingame, CA) or goat anti-human IgG (TAGO) diluted in 50 mM carbonate buffer (pH 9.6) at 4 °C. After washing out the residual antibodies, the wells were blocked with 10 mM PBS (pH 7.2) containing 1% bovine serum albumin and 1% Tween 20 (T-PBS) at 37 °C for 2 hr. After washing the wells with T-PBS, 50 μl of cell culture supernatant were added to wells and incubated at 37 °C for 2 hr. After washing the wells with T-PBS, bound IgM or IgG were captured by a horseradish peroxidase-conjugated goat anti-human IgM (TAGO) or goat anti-human IgG (TAGO). After washing the wells, the substrate solution (0.3 mg ml^{-1} 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Nacalai Tesque, Kyoto, Japan) and 0.003% hydrogen peroxide in 0.1 M citrate buffer (pH 4.0)) was added and the absorbance at 405 nm was measured.

Immunohistochemical staining of tissues

Formalin-fixed paraffin-embedded human colon adenocarcinoma tissue section were deparaffinized in xylene, and rehydrated in ethanol. After rinsing in PBS, the tissue sections were incubated with culture supernatants of TM-1 cells or hybridoma clones for 40 min at room temperature. The slides were washed in PBS and incubated with biotin-mouse anti human IgM (ZYMED, San Francisco, CA) for 30 min.

Figure 1. Immunohistochemical study with the antibody derived from TM-1. The formalin-fixed tissue sections of lymph node containing T cells infected with HTLV-I (A), colon adenocarcinoma (B) and lung small cell adenocarcinoma (C) were immunohistochemically stained with the antibody derived from TM-1 and with hematoxylin-eosin.

After washing again in PBS, the slides were incubated in streptavidin-alkaline phosphatase conjugate (DAKO) for 30 min and the bound antibodies were visualized by using Histofine New Fuchsin Substrate kit (NICHIREI, Tokyo, Japan). The sections were counter-stained with hematoxylin-eosin to facilitate the histological analysis.

Flow cytometric analysis

Single cell suspensions of leukemic cell lines and PBL were incubated with a culture supernatant of hybridoma clones and control human IgM for 1 hr. After washing with 1 mM EDTA-PBS, the cells were stained with FITC labeled goat anti-human IgM (TAGO) for 20 min on ice. After washing twice, the stained cells were resuspended in PBS and a total of 1×10^4 events were collected for each sample by a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon ion laser running at 488 nm, and subsequently analyzed by using the CellQuest software (Becton Dickinson).

Result

Establishment of B cell line TM-1 producing cancer specific antibody

When we stimulated PBL from ATL patient with LPS, vigorously proliferating cells could be obtained after cultivation for several weeks, and named TM-1. Then, we analyzed cancer reactivity of antibody secreted from by TM-1 cells. Immunohistochemical staining study showed that antibody produced from TM-1 specifically recognizes d ATL positive lymphoma (Figure 1A), colon adenocarcinoma (Figure 1B) and lung small cell adenocarcinoma (Figure 1C), but not normal lymphocytes, large intestine tissue, lung tissue, stomach, liver and kidney (data not shown). These results demonstrate that TM-1 cells produce antibody specific for cancerous region of tissue.

Establishment of hybridomas by fusing TM-1 and RF-S1

EB virus infected B cells are known to be unstable in their abilities to secrete antibodies. Thus, we aimed to fuse TM-1 cells with human-mouse hetero-myeloma cells of RF-S1 to establish hybridomas. Because B cell

Figure 2. Immunohistochemical study with MAb derived from hybridomas. The formalin-fixed tissue sections of colon adenocarcinoma were stained with culture supernatants of hybridoma clones (2E12 (A), 3E9 (B) and 3E10 (C)) and control human IgM(D), respectively.

line TM-1 has an ability to synthesize hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and to proliferate infinitely in the HAT medium, we selected fused hybridomas derived from TM-1 by panning method. Hybridoma fusion frequency was calculated by the following equation:

$$\text{Hybridoma fusion frequency} = \text{Nhy}/\text{Nc} \times 10^5,$$

where Nhy is the number of wells containing growing hybridomas, and Nc is the total number of parental cell (TM-1) used in the fusion. Hybridoma fusion frequency was shown to be 12.5.

Antibody production of hybridomas

After 3–4 weeks of fusion, supernatants were collected and antibody production from hybridomas was measured by sandwich ELISA, showing that 10.6% of wells containing vigorously growing hybridomas secreted IgM type antibody. IgG type antibody can not be

detected in the supernatants. Hybridomas producing IgM type antibody were recloned by limiting dilution. Antibody productivity of three hybridoma clones was about $0.8\text{--}3.0 \mu\text{g ml}^{-1} \text{ week}^{-1}$ for 2E12, $4.0\text{--}5.0 \mu\text{g ml}^{-1} \text{ week}^{-1}$ for 3E9 and 3E10 in culture supernatant of 90 mm dish cultivation, as estimated from standard curves of ELISA assays.

Immunohistological reactivity of antibody produced by hybridomas

MAbs secreted from hybridoma clones were applied to immunohistochemical staining of cancer cells. Three of IgM producing hybridoma clones termed 2E12, 3E9 and 3E10 produced antibody specific for the cancerous part of colon adenocarcinoma tissue (Figure 2), while control non-specific IgM showed no positive signals.

Figure 3. Flow cytometric analysis of MAb produced by 3E9 hybridoma clone. Jurkat (A), MOLT-4 (B), Raji (C), Daudi (D), Namalwa (E), KU812 (F), U937 (G) and healthy human PBL (H) were treated with supernatant of hybridoma 3E9 and visualized by treating with fluorescein-conjugated goat anti-human IgM antibody. The filled curve shows the negative isotype control staining using non-specific IgM antibody.

Immunocytochemical reactivity of antibody produced by hybridomas

Cancer specific reactivity of antibody was also tested by using flow cytometry. MAb from hybridoma clone 3E9 was analyzed for its binding ability to surface antigens on several cancer and normal cells such as Jurkat, Molt-4, Raji, Daudi, Namalwa, U937, KU812, PBL from a healthy volunteer. MAb from hybridoma 3E9 exhibited the strong reactivity to Jurkat and KU812 and slightly to U937, but not to Molt-4, Raji, Daudi, Namalwa and normal human PBL (Figure 3).

Discussion

In this report, we established hybridoma clones 2E12, 3E9 and 3E10 from B cell line of TM-1 by LPS stimulation of spontaneously immortalized B cells of ATL patient. LPS is known to be a thymus independent antigen and is a polyclonal activator for B-cells. Activated B-cells by LPS differentiate into mature plasma cells, but does not proliferate. Thus we envisage that LPS stimulation of PBL of ATL patient triggers the proliferation of B cells immortalized by EB virus infection, resulting in the establishment of TM-1 cells. The involvement of EB virus in the immortalization of TM-1 cells was confirmed by the expression of EB virus nuclear antigen (EBNA) and latent membrane protein (LMP), EB virus specific proteins (data not shown), suggesting that EB virus infected B cells contained in the PBL of ATL patient were proliferated by LPS stimulation. But we must answer the question why TM-1 secretes cancer specific antibody. It has been largely known that ATL patients are statistically more likely to develop several types of cancer, and that incident rate for cancer in the digestive tissue reaches about 15% (Moriwaka et al., 1989; Matsuzaki et al., 1990, 1992; Imajo et al., 1993). Furthermore, autoimmune diseases are also known to attack ATL patients at high rate (Saijo et al., 1999; Zucker-Franklin, 2000; Sugata, 2001). These suggest that ATL patients are in the immunodeficiency state because of the chronic stimulation by HTLV-I and subsequent high content of γ globulin in plasma (Tamura et al., 1996; Tsukasaki et al., 1999). These might explain the reason why B cell line TM-1 derived from ATL patient shows cancer specificity.

2E12, 3E9 and 3E10 hybridoma clones produced IgM antibody binding to the tumor site of tissue section. Staining images using these hybridoma clones

were similar with each other and further that using TM-1. These results suggest that we succeeded in the establishment of hybridomas stably producing cancer specific antibodies by cell fusion of TM-1 with RF-S1. Furthermore, the flow cytometric analysis showed that antibody produced from 3E9 hybridoma reacted to several tumorous leukemic cell lines of Jurkat and KU812, and slightly to U937, but not to Molt-4, Raji, Daudi, Namalwa and normal PBL. These results suggest that antibody from 3E9 hybridoma recognizes the cell surface antigen expressed on the certain types of cancer cells, but not on normal human PBL.

By immunohistochemical staining of tissue section and flow cytometric analysis, antigens recognized by these three hybridoma clones were seemed to exist in adenocarcinoma tumor site and cell surface of leukemic cell. Several cancer-related antigens located in the cell surface are known, such as MUC1 (Nakamori et al., 1994), a mucin-like glycoprotein and Sialyl Lewis X (sLex), a terminal structure found on cell-surface glycoprotein and glycolipid carbohydrate groups (Murata et al., 2001). These antigens were aberrantly expressed in tumor site and recognized by immunocompetent cell. Especially, a positive reaction of MUC-1 is reported in the case of not only human colorectal carcinoma but also lymphatic cancers like ATL and multiple myeloma (Takahashi et al., 1994). Existence of these antigens may explain broad-range reactivity of antibodies produced by TM-1 and Hybridoma clones. Cancer cell specific antigen recognized by the antibody from 3E9 must be identified in the future study, which might be a target antigen for cancer diagnosis and therapy.

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