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Development of a complete human IgG monoclonal antibody to transferrin receptor 1 targeted for adult T-cell leukemia/lymphoma

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ABSTRACT

Iron is an essential nutrient for normal cell growth, and reprogramming of iron metabolism is essential to tumor cell survival and progression. HTLV-1-associated adult T-cell leukemia/lymphoma (ATLL) has no effective therapy and high levels of cell surface transferrin receptor 1 (TFR1) expression have been reported in ATLL by us and other groups. In this study, to develop a novel molecular-targeted therapy against TFR1 to modulate iron metabolism, we initially determined the expression pattern of several iron-related genes along with TFR1 and found that ATLL cells presented characteristic of an iron-deficiency state such as high expression of iron-regulatory protein 2 (IRP2) and low expression of its E3 ubiquitin-ligase, FBXL5. Therefore, we developed human IgG monoclonal antibodies to human TFR1 using a phage display method (ICOS method) to block the incorporation of the transferrin (TF)-iron complex into ATLL cells for inhibiting cell growth. One of the mAbs, JST-TFR09, presented its greater affinity to TFR1 on ATLL cells in flow cytometry (FCM) analysis than those of commercially available anti-TFR1 antibodies and identified high expression of TFR1 in most of the acute-type ATLL cells. Moreover, JST-TFR09 could interfere with binding between TFR1 and TF, which resulted in effective blockade of TFR1 internalization and induction of cell apoptosis by the treatment of ATLL cells with JST-TFR09. JST-TFR09 showed dual activities through direct cell cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), and the treatment of JST-TFR09 significantly suppressed cell growth of ATLL cells with induction of apoptosis in *in vitro* and in vivo experiments. Thus, JST-TFR09 described here may become a promising therapeutic antibody for the treatment of ATLL.

1. Introduction

Adult T-cell leukemia/lymphoma (ATLL) is an aggressive malignancy caused by human T-cell leukemia virus type 1 (HTLV-1). HTLV-1 is endemic to certain regions, including Japan, Africa, the Caribbean and South America, and an estimated 10–20 million people worldwide are currently infected with HTLV-1. HTLV-1 infects primarily $CD4^+$ T-lymphocytes through direct cell-to-cell contact, mainly via breast-feeding. ATLL develops in 3–5% of HTLV-1 carriers after a long latency period of 20–60 years during which polyclonal expansion of HTLV-1-infected lymphocytes predisposes them to transformation [1]. ATLL is classified into four clinical types: acute, chronic, lymphoma, and smoldering. Patients with aggressive forms (acute and lymphoma) have a very poor prognosis because of intrinsic chemoresistance and severe immunosuppression. Although new therapeutic options such as allogeneic hemopoietic stem-cell transplantation (allo-HSCT), interferon-α (IFN-α) plus zidovudine (AZT), and the anti-CCR4 monoclonal antibody (mAb) are gradually improving the curability of aggressive ATLL, treatment still remains challenging [2]. Patients with indolent ATLL (chronic or smoldering) have a better prognosis than the aggressive type of ATLL. However, recently, a poor long-term

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outcome has been demonstrated when patients are managed with a watchful waiting policy [3]. Thus, there is an urgent need to develop new strategies to increase the effectiveness of treatments for this disease.

To search for novel cell surface target molecules for ATLL, we previously studied gene expression profiles between acute-type ATLL leukemia cells and control $CD4^+$ T-lymphocytes by DNA microarray analysis and identified cell adhesion molecule 1 (TSLC1/CADM1), interleukin 2 receptor subunit α (IL2RA), tumor necrosis factor receptor superfamily member 6 (TNFRSF6), fibroblast growth factor receptor 1 (FGFR1), and transferrin receptor 1 (TFRC/TFR1/CD71) as significantly differentially expressed genes in ATLL [4]. TFR1 has been suggested to be one of the promising ATLL cell surface target molecules [5]. In this manuscript, we present work on the development of an anti-TFR1 antibody for the treatment of ATLL. TFR1 plays an important role in the maintenance of iron homeostasis by regulating iron uptake through binding and internalizing the iron-transferrin (TF) complex. Because iron is an essential nutrient required to maintain cell growth, elevation of iron metabolism is often associated with cancer cell growth and survival. Indeed, TFR1 expression is frequently up-regulated in various cancer cells including ATLL, which is often associated with poor prognosis [5–9]. On the other hand, in normal cells, TFR1 is expressed at low levels but is expressed at greater levels on some particular cell types, such as erythroid lineage cells [10]. To date, several kinds of anti-TFR1 antibodies have been developed for cancer diagnosis and targeted therapy, and some of them have been tested in clinical trials. A mouse IgA mAb against TFR (42/6) has been evaluated in a phase I clinical trial, and 3 cases among the 27 patients with advanced refractory cancers with hematologic malignancies showed evidence of mixed tumor responses [11]. Recent studies have shown that a mouse mAb against TFR1 (A24) can inhibit proliferation of ATLL cells through induction of apoptosis [5] and that human single-chain antibodies to TFR1 effectively antagonize the growth of hematopoietic tumor cell lines of various lineages [12]. However, there are no available complete human IgG mAbs directed against TFR1 that can be used in the clinic.

To develop complete human IgG mAbs against human TFR1 for use in novel therapies for ATLL, we screened human antibody phage display libraries using a method called isolation of antigen-antibody complexes through organic solvent (ICOS) and isolated several anti-TFR1 mAbs [13,14]. We previously reported that one of the mAbs showed cytotoxic activity against oral squamous cell carcinoma (OSCC) cells [15]. In an effort to obtain additional anti-TFR1 mAbs that are more effective than other mAbs, such as anti-CCR4 and anti-TFR1 A24, in treating ATLL, we further screened the phage antibody library and succeeded in isolating different anti-TFR1 mAbs. Here, we used one of the mAbs, JST-TFR09, which showed the strongest cytotoxic activity against ATLL cells, to characterize its therapeutic potential for ATLL in vitro and in vivo. The results showed that JST-TFR09 presented direct cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) activity to ATLL cells and significantly better antitumor effects in vitro and in vivo compared with that of the anti-CCR4 antibody, suggesting that the anti-TFR1 antibody will become a promising therapeutic agent for ATLL.

2. Materials and methods

2.1. Patient samples

Blood samples were collected from ATLL patients at the time of hospital admission before chemotherapy. The diagnosis of ATLL was based on clinical features, hematological characteristics and the presence of anti-HTLV-1 antibodies in the sera. Peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers and ATLL patients were purified by gradient centrifugation using Histopaque (Sigma-Aldrich, St. Louis, MO). After the purification of $CD4^+$ cells form PBMCs using anti-CD4 magnetic beads from Miltenyi Biotec (Bergich Gladbach, Germany), ATLL cells were collected using the biotin-conjugated CADM1 antibody [16] together with anti-biotin magnetic beads (Miltenyi Biotec, Auburn, CA) after purification of T-cells using the Pan T Cell Isolation Kit (Miltenyi Biotec). ATLL cells were maintained in AIM-V medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 20% FBS, 10 μM 2-mercaptoethanol (Thermo Fisher Scientific) and 0.75 μg/mL recombinant human IL2 (Pepro tech) in a humidified atmosphere of 5% CO_2 at 37 °C. Informed consent was obtained from all patients. This study was approved by the Institutional Review Board of the Faculty of Medicine, University of Miyazaki.

(The remaining materials and methods are presented in the supplementary information.)

3. Results

3.1. TFR1 is overexpressed in ATLL cells

Because analysis of our microarray data revealed up-regulation of TFR1 expression in leukemic cells from ATLL patients (Fig. S1), we performed quantitative RT-PCR analysis for TFR1 using 9 HTLV-1-positive ATLL-related cell lines, 2 HTLV-1-negative T-cell acute lymphoblastic leukemia (T-ALL) cell lines, primary leukemic cells from 9 acute-type ATLL patients, and $CD4^+$ T-lymphocytes from 4 healthy volunteers as controls (Fig. 1A). The majority of the ATLL-related cell lines, T-ALL cell lines, and primary ATLL cell samples showed more than a 5-fold increase in TFR1 mRNA compared to that of controls. In an immunoblot analysis, we observed that the TFR1 protein was abundantly expressed in all of the ATLL-related cell lines and primary ATLL cell samples, while $CD4^+$ T-lymphocytes from healthy volunteers expressed the TFR1 protein at a very low level (Fig. 1B). In addition, we used the human anti-human TFR1 mAb (JST-TFR09), which was the antibody isolated from the antibody phage display library screening that had the highest binding affinity and cytotoxic activity for the development of a better therapeutic antibody than anti-CCR4 or a mouse anti-TFR1 A24 mAb, to perform flow cytometry (FCM) analysis. The results showed that the vast majority of ATLL cells expressed high levels of TFR1 on their surface (Tables S1 and S2 and Fig. 1C). We also found that JST-TFR09 had much greater sensitivity for ATLL cells than the commercial antibodies (Fig. S2).

Because iron metabolism is reprogrammed in cancer cells for enhanced cellular proliferation [17], we next examined whether the genes involved in iron metabolism are differentially expressed in ATLL cells. As shown in Figs. S3 and S4, mRNA levels of the E3 ubiquitin ligase subunit, FBXL5, and the iron exporter, FPN, were significantly decreased in most of the ATLL-related cell lines and primary ATLL cells, whereas levels of the heavy subunit of ferritin (FTH1) and IRP2 mRNA were significantly up-regulated compared to that of the $CD4^+$ T-lymphocytes from healthy volunteers. On the other hand, the ferritin light chain (FTL), IRP1, and DMT1 mRNA levels were not significantly different between ATLL cells and normal $CD4^+$ T-cells. Moreover, we found that the levels of FBXL5 protein were consistently decreased in all of the ATLL-related cell lines and primary ATLL cell samples, and, conversely, levels of the IRP2 protein were highly up-regulated in these cells (Fig. 1B), suggesting that the increased steady-state levels of IRP2 caused by down-regulation of FBXL5 expression could contribute to sustained up-regulation of TFR1 in ATLL cells, resulting in an increase in the labile iron pool that may support cell growth.

Because epigenetic alteration is one of the important

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