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An IgG1 version of the Anti-Transferrin Receptor 1 Antibody ch128.1 Shows Significant Anti-tumor Activity Against Different Xenograft Models of Multiple Myeloma: A Brief Communication

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Abstract

The transferrin receptor 1 (TfR1) is a meaningful target for antibody-based cancer therapy given its overexpression on malignant cells and its central role in cancer pathology. We previously developed a mouse/human chimeric IgG3 targeting human TfR1 (ch128.1), which exhibits significant anti-tumor activity against multiple myeloma (MM) in xenograft models of SCID-Beige mice bearing disseminated ARH-77 or KMS-11 tumors. This activity is observed in early and late disease stages of disseminated KMS-11 tumors and, in this model, the mechanism of anti-tumor activity is Fc-mediated, involving macrophages. Since human IgG1 is the isotype of choice for therapeutic antibodies targeting malignant cells and has several advantages compared to IgG3, including an established manufacturability, we now developed an IgG1 version of ch128.1. A single dose of ch128.1/IgG1 shows significant anti-tumor activity, not only against early and late stages of disseminated KMS-11 tumors (Asian origin), but also against these stages of disseminated human MM cells MM.1S (African American origin) and its variant that is resistant to dexamethasone MM.1R. Treatment with the Fc mutant version of ch128.1/IgG1 (L234A/L235A/P329S) with impaired effector functions fails to confer protection against MM.1S and MM.1R tumors, indicating a crucial role of the Fc fragment in the anti-tumor activity, similar to its IgG3 counterpart. In fact, we found that ch128.1/IgG1, but not the mutant, elicits antibody-

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dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP) in the presence of murine bone marrow-derived macrophages. Our results suggest that ch128.1/IgG1 is a promising therapeutic against human B-cell malignancies such as MM.

Keywords

transferrin receptor 1; multiple myeloma; antibody effector functions; antibody therapy

Introduction

The transferrin receptor 1 (TfR1), also known as CD71, is a type II transmembrane homodimeric protein involved in the internalization of iron and in the regulation of cell growth¹⁻³. It is a meaningful target for antibody-based cancer therapy, given its overexpression on malignant cells and its central role in cancer pathology¹⁻³.

We have previously developed a mouse/human chimeric IgG3 antibody specific for human TfR1 that contains the variable regions of the 128.1 murine IgG1 antibody (known as ch128.1 or anti-hTfR1 IgG3). This antibody is non-neutralizing meaning that it does not inhibit the binding of TfR1 ligands [both transferrin and hemochromatosis protein (HFE)]^{4, 5}. We initially selected the human IgG3 isotype since we aimed to develop an antibody-avidin fusion (ch128.1Av or anti-hTfR1 IgG3-Av) as a universal delivery system of biotinylated anti-cancer agents, and the extended hinge region of human IgG3 provides spacing and flexibility to the molecule, thereby facilitating simultaneous binding of antigen and biotinylated compounds^{4, 5}. However, we found that ch128.1Av alone, and to a greater extent the parental antibody ch128.1, exhibits significant anti-tumor activity against the B-cell malignancy multiple myeloma (MM) in xenograft models of SCID-Beige mice bearing disseminated ARH-77 or KMS-11 cells⁶. Interestingly, ch128.1 exhibits significant anti-tumor activity in early and late stages of disseminated disease (KMS-11 cells) and the mechanism of protection is Fc-mediated with macrophage involvement⁷.

Human IgG1, which shares the Fc effector functions of human IgG3^{8, 9}, has been the isotype of choice for therapeutic antibodies targeting malignant cells^{8, 10} and has several advantages compared its IgG3 counterpart, such as longer half-life (bioavailability) in blood and an established manufacturability^{8, 9}. In addition, there are 4 allotypes of human IgG1 and 13 of human IgG3, which could enhance the immunogenicity of IgG3 in patients¹¹. Moreover, the extended hinge region of human IgG3 makes it more prone to proteolysis¹⁰. Based on these properties we decided to develop the IgG1 version of ch128.1 (ch128.1/IgG1) described in this report and evaluate its anti-cancer effects in various xenograft models of MM including new models not previously studied using ch128.1/IgG3.

Materials and Methods

Cell lines:

We previously described the use of KMS-11 cell line (human MM; Asian origin)^{6, 7}. MM.1S (ATCC® CRL-2974™; human MM African American origin) and its variant resistant to dexamethasone MM.1R (ATCC® CRL-2975™) were obtained from the American Type

Culture Collection (ATCC, Manassas, VA). Cells were cultured in IMDM (KMS-11) or RPMI (MM.1S and MM.1R) supplemented with 10% FBS and penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA) in 5% CO₂ at 37°C.

Antibody construction and *in vitro* characterization:

The mouse/human chimeric antibody ch128.1/IgG3 was previously developed⁴. To construct the human IgG1 version (ch128.1/IgG1) the DNA encoding the heavy chain variable region of the ch128.1 γ 3 expression vector was cloned into an expression vector containing the constant regions of human IgG1 (γ 1)¹². The Fc L234A/L235A/P329S mutations were generated in the γ 1 heavy chain expression vector by GenScript USA, Inc. (Piscataway, NJ). We selected this triple mutant based on our previously created L234A/L235A/P329S version of ch128.1/IgG3 that retains bioavailability but has impaired binding to Fc gamma receptors (Fc γ Rs) and impaired antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP), relevant effector functions in the anti-tumor activity exhibited by the wild type ch128.1/IgG3^{7, 13}. Either the γ 1 or the mutant γ 1 heavy chain construct, together with the same human κ light chain expression vector used to develop ch128.1/IgG3⁴, were used to express the antibodies in murine myeloma NS0/1 cells as previously reported for ch128.1/IgG3^{4, 5}. Transfectomas were grown in roller bottles and the antibodies purified using affinity chromatography as described^{4, 5}. SDS-PAGE under reducing and non-reducing conditions was performed to confirm the antibody molecular weight and assembly^{4, 5}.

Binding to TfR1 was assessed by flow cytometry and ELISA as reported^{5, 6, 13}; however, for ELISA soluble human TfR1 obtained from Kerafast, Inc. (Boston, MA) was used. Binding to Fc γ Rs was assessed by flow cytometry using the J774.2 murine macrophage-like cell line in the presence or absence of human soluble Fc γ RI (sCD64) to confirm binding specificity as reported¹³. sCD64 was purchased from R&D Systems (Minneapolis, MN). ADCC/ADCP was studied by three-color flow cytometry using KMS-11 cells as targets and murine bone marrow-derived macrophages (BMDM) as effectors as described⁷. A mouse/human chimeric IgG1/ κ antibody specific for the hapten dansyl (5-dimethylamino naphthalene-1-sulfonyl chloride)¹⁴, was produced in murine myeloma cells using the same methods and was used as an isotype control antibody (IgG1/ κ).

In vivo efficacy studies:

All experimental protocols were approved by the University of California, Los Angeles Animal Research Committee and all local and national guidelines on the care of animals were strictly adhered to. Female C.B-17 SCID-Beige mice (8–12 weeks old) were obtained and housed in the Defined Flora Mouse Facility in the Department of Radiation Oncology at UCLA. All mice were exposed to 3 Gy total-body sublethal irradiation (MARK 1–30 irradiator [¹³⁷Cs]; J.L. Shepherd & Associates, San Fernando, CA) the day before tumor challenge. To establish disseminated disease, 5 × 10⁶ human MM cells (either KMS-11, MM.1S, or MM.1R) were injected intravenously (i.v.) via the tail vein as described^{6, 7}. Mice were randomized and treated either 2 or 9 days after tumor challenge with a single i.v. (via the tail vein) dose of ch128.1/IgG1, the mutant ch128.1/IgG1, or buffer (PBS) control. Survival was based on the time from tumor challenge to the development of hind-limb

paralysis. Plots, median survival, and statistical analysis (log-rank test) were generated or determined using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA).

Results

As expected, ch128.1/IgG1 and its Fc mutant L234A/L235A/P329S are properly assembled and secreted (data not shown) and bind antigen as shown by ELISA (Figure 1A) and flow cytometry (Figure 1B). Figure 1C shows that, as expected, ch128.1/IgG1 but not the ch128.1/IgG1 mutant binds to J774.2 cells, which express Fc γ Rs, and the addition of human sCD64 blocks the binding of ch128.1/IgG1, confirming the Fc γ R specificity of this binding. Moreover, ch128.1/IgG1 elicits both ADCC and ADCP, while the mutant antibody does not (Figure 1D), consistent with its impaired binding to Fc γ Rs.

The anti-tumor effect of ch128.1/IgG1 was first explored in a disseminated MM xenograft mouse model using human KMS-11 cells previously used to determine the ch128.1/IgG3 anti-tumor activity^{6, 7}. A single low dose of ch128.1/IgG1 administered at either 2 (median survival: 167 days) or 9 days (median survival: 42.5 days) after KMS-11 inoculation significantly prolongs survival compared to the control groups (median survival of both: 37 days) (Figure 2A).

To further explore the anti-MM activity of ch128.1/IgG1, we tested its efficacy along with that of the mutant IgG1 in two other disseminated mouse models of MM using the human MM cell lines MM.1S and MM.1R. Similar to the studies in KMS-11 cells, ch128.1/IgG1 significantly prolongs animal survival when a single dose was administered in MM.1S bearing mice at either 2 days (median survival: 74.5 days) or 9 days after tumor challenge (median survival: 50 days), compared to the buffer control group (median survival: 44 days) (Figure 2B). However, the mutant ch128.1/IgG1 does not prolong survival (median survival: 42 days), indicating that the Fc region of the antibody is crucial for its anti-cancer activity in this model. Similar effects were observed in SCID-Beige mice bearing MM.1R tumors (Figure 2C). Although these cells took substantial time to cause morbidity in the mice, a single dose of ch128.1/IgG1 still significantly prolongs survival when given 2 days (median survival: 184 days) or 9 days (median survival: 155 days) after tumor inoculation. Again, the mutant antibody does not confer protection (median survival: 88.5 days) compared to the control group (median survival: 114.5 days).

Discussion

We found that a single low dose of ch128.1/IgG1 elicits significant anti-tumor activity in a disseminated xenograft mouse model of MM using the human KMS-11 cell line (Asian origin), similar to the previously reported anti-tumor activity mediated by ch128.1/IgG3⁶⁻⁷. As expected, this anti-tumor effect was greater when the antibody was administered 2 days (early stage disease) after tumor challenge, compared to treatment given at 9 days (late stage disease), due to the increased tumor burden at time of treatment.

We have further extended our knowledge using antibodies targeting TfR1 and now show that the ch128.1/IgG1 also has significant anti-tumor activity in xenograft models of MM using two additional human MM cell lines: MM.1S and MM.1R. These MM cell lines, which are

of African American origin, are relevant given the higher MM incidence and mortality of individuals of African ancestry compared with other races¹⁵. In addition, resistance to the glucocorticoid dexamethasone, a common therapeutic in MM, is well known¹⁶. We also found that the Fc mutant L234A/L235A/P329S version of ch128.1/IgG1 fails to confer anti-tumor activity against MM.1S and MM.1R cells, indicating a crucial role of the Fc fragment in protection, which may be attributed to antibody effector functions. These results are consistent with our previous studies showing the lack of anti-tumor activity of this triple mutant in ch128.1/IgG3 in SCID-Beige mice bearing disseminated KMS-11 cells⁷. In this model we found that the anti-tumor activity of ch128.1/IgG3 wild type is affected by macrophage depletion, but not complement depletion⁷. In addition, we also previously found that ch128.1/IgG3 induces ADCC and ADCP against KMS-11 cells in the presence of murine BMDM⁷, as we now show using the ch128.1/IgG1 version, which is expected since both antibody isotypes are capable of binding FcγRs and mediating ADCC/ADCP^{8, 9}.

Taken together, our results suggest that both ch128.1/IgG3 and ch128.1/IgG1 share similar mechanisms of action without regard to the targeted MM cells. Our results also suggest that ch128.1/IgG1 is a promising therapeutic against human B-cell malignancies such as MM. However, a limitation of using mice in our studies is that this species does not adequately address the toxicity of ch128.1 since this antibody does not cross-react with the murine TfR1¹⁷. Further studies are needed to fully define the therapeutic potential and the toxicity profile of ch128.1/IgG1.

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Figure 1. *In vitro* characterization of the ch128.1/IgG1 and its mutant.

A) ELISA analysis showing binding of ch128.1/IgG1 and its mutant to sTfR1. Binding was detected using an alkaline phosphatase (AP)-conjugated goat anti-human κ antibody and AP substrate. Error bars indicate the standard deviation of duplicate samples. B) Flow cytometry analysis showing binding of ch128.1/IgG1 and its mutant to KMS-11 cells that express the TfR1. Black line histogram indicates primary antibodies; gray line histogram indicates incubation with the phycoerythrin (PE)-conjugated goat F(ab')₂ anti-human κ antibody alone. C) Flow cytometry analysis showing binding of ch128.1/IgG1 to J774.2 cells. sCD64

was added to confirm Fc γ R binding specificity. Black line histogram indicates primary antibodies; gray line histogram indicates incubation with the PE-conjugated goat F(ab')₂ anti-human κ secondary antibody alone. D) Three-color flow cytometry analysis showing that ch128.1/IgG1 mediates ADCC and ADCP. Carboxyfluorescein succinimidyl ester (CFSE)-labelled KMS-11 target cells pre-incubated with each antibody were added to murine BMDM (effector cell to target cell ratio of 3:1) at 37°C for 2 hours. A PE-conjugated F4/80 antibody was used to visualize macrophages. All cells were stained with DAPI to detect dead cells. Cells positive for CFSE and DAPI indicate ADCC, while cells positive for F4/80 (PE) and CFSE indicate occurrence of phagocytosis. The error bars indicate standard deviation of duplicate samples. * $p < 0.05$ (Student's t -test). An anti-dansyl IgG1 ("IgG1 control") antibody was used as an isotype control for studies in panels A, B, and D. Results shown in the four panels of this figure are representative of at least two independent experiments.

Figure 2. *In vivo* efficacy of ch128.1/IgG1 in disseminated xenograft models of MM.

(A) SCID-Beige mice were challenged i.v. with 5×10^6 KMS-11 cells and 2 or 9 days later treated i.v. with 100 μ g ch128.1/IgG1. Mice were also challenged i.v. with 5×10^6 (B) MM.1S or (C) MM.1R cells. Two days after tumor challenge, mice were treated i.v. with 100 μ g ch128.1/IgG1 or the ch128.1/IgG1 Fc mutant L234A/L235A/P329S. For each cell line (MM.1S and MM.1R), one group of mice was treated with 100 μ g ch128.1/IgG1 9 days after tumor challenge. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (log-rank test).