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Efficacy of an Anti-transferrin Receptor Antibody Against AIDS-related non-Hodgkin Lymphoma: A Brief Communication

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Summary

The transferrin receptor 1 (TfR1), also known as CD71, is a target for antibody-based cancer immunotherapy due to its high expression levels on the surface of cancer cells and its ability to internalize. We have previously developed a mouse/human chimeric IgG3 specific for human TfR1 genetically fused to avidin, as a vector to deliver biotinylated anticancer agents into malignant cells. However, we found that this fusion protein (ch128.1Av), and to a lesser extent the same antibody without avidin (ch128.1), exhibits direct cytotoxic activity *in vitro* against certain malignant hematopoietic cells through the induction of TfR1 degradation and lethal iron starvation. Importantly, both ch128.1 and ch128.1Av have also shown significant anti-cancer activity in two xenograft models of the B-cell malignancy multiple myeloma. It is interesting to note that ch128.1 exhibited superior anticancer activity in both models compared with ch128.1Av, even against malignant cells that show no sensitivity to ch128.1 *in vitro*. In the present study, we evaluated the efficacy of ch128.1 against an AIDS-related human Burkitt lymphoma cell line (2F7) to determine if ch128.1 can eliminate these cells *in vitro* and in an *in vivo* model of AIDS-related non-Hodgkin lymphoma (AIDS-NHL). Even though 2F7 cells expressed high TfR1 levels, these cells lacked sensitivity to the cytotoxicity induced by ch128.1 *in vitro*. However, ch128.1 showed significant anticancer activity against these AIDS-NHL cells *in vivo* by significantly

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prolonging the survival of immunodeficient mice bearing 2F7 tumors. Therefore, ch128.1 warrants further study as a potential candidate for the treatment of AIDS-NHL and other B-cell malignancies.

Keywords

transferrin receptor; antibody-mediated therapy; cancer; non-Hodgkin lymphoma; AIDS-related malignancies

Introduction

The transferrin receptor 1 (TfR1), also known as CD71, is a type II transmembrane glycoprotein involved in iron uptake and the regulation of cell growth [1,2]. This receptor has been used extensively as a target of antibody-mediated cancer therapy due to its increased expression in malignancies, its extracellular accessibility, and its ability to internalize therapeutic agents through receptor-mediated endocytosis [1,2]. We have previously developed a mouse/human chimeric IgG3 specific for human TfR1 containing avidin genetically fused to the carboxy-terminus of the C_H3 domains [3–5]. This fusion protein, known as ch128.1Av, was designed and has been shown to deliver biotinylated agents into cancer cells [3,4,6]. However, we also found that ch128.1Av, and to a lesser extent the same antibody without avidin (ch128.1), exhibits direct cytotoxic activity *in vitro* against certain malignant hematopoietic cells through the induction of TfR1 degradation and lethal iron starvation [4–8]. Neither ch128.1 or ch128.1Av inhibit the binding of transferrin to the TfR1 and the affinity of ch128.1 for TfR1 was found to be high (K_D = 5.7 nM)[3,9]. Importantly, both ch128.1 and ch128.1Av have also shown significant anti-cancer activity in two xenograft models of the B-cell malignancy multiple myeloma, in which either ARH-77 (human B-lymphoblastoid cells) or KMS-11 (human multiple myeloma cells) were systemically inoculated into immunodeficient mice [7]. It is interesting to note that ch128.1 exhibited superior anticancer activity in both models compared with the fusion protein, despite its lower *in vitro* cytotoxicity in ARH-77 compared to ch128.1Av and the fact that KMS-11 cells are not sensitive to ch128.1 *in vitro*.

To further explore the anticancer activity of ch128.1, we sought to determine its efficacy against other B-cell malignancies. AIDS-related non-Hodgkin lymphomas (AIDS-NHL) are heterogeneous B-cell lymphomas that develop in individuals infected with HIV; the incidence of NHL in HIV-positive subjects not treated with combination antiretroviral therapy (cART) is much higher than the incidence of NHL in the uninfected population [1]. Even though the overall incidence of AIDS-NHL has decreased during the cART era, HIV positive persons continue to be at risk and AIDS-NHL is the most common AIDS-related cancer in developed countries where poor survival rates remain problematic [11,12]. The clinical use of rituximab, an anti-CD20 IgG1, in combination with chemotherapy has improved the survival of many NHL patients; however, limited responses and the development of resistance to the treatment remain a challenge [13]. In this report we evaluated, for the first time, the anti-cancer activity of ch128.1 against an AIDS-NHL cell line both *in vitro* and in an animal model.

Materials and Methods

Cell Lines

2F7 (human AIDS-associated Burkitt lymphoma) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). 2F7 cells are Epstein Barr virus positive, HIV negative, and express the B-cell markers: CD19 and CD20.[14,15] ARH-77 (human Epstein Barr virus-transformed lymphoblastoid) cells were also purchased from ATCC, and KMS-11 (human multiple myeloma) cells were a kind gift from Dr. Lawrence Boise (Emory University). All cell lines were cultured in Iscove's Modified Dulbecco's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and antibiotics in 5% CO_2 at 37°C.

Recombinant antibody production

The ch128.1 antibody containing the variable regions of the murine antibody 128.1 (formerly known as anti-hTfR IgG3) and the fully human anti-HER2/*neu* IgG3 antibody (IgG3) used as an isotype control for the proliferation and *in vivo* studies have been described [5,7]. Both antibodies have kappa light chains and were expressed in murine myeloma cells, expanded in roller bottles, and purified from cell culture supernatants using affinity chromatography as described [5,7].

Cell surface TfR1 expression and ch128.1 binding

2F7 cells (2.5×10^5) were incubated for 30 minutes on ice with either phycoerythrin (PE)-conjugated mouse IgG2a isotype control or PE-conjugated mouse anti-human CD71 (TfR1) monoclonal antibodies (both from BD Biosciences, San Jose, CA) according to the instructions of the manufacturer. For ch128.1 binding, 2 μg of ch128.1 or a humanized anti-human HER2/*neu* IgG3/kappa (previously described [16] and used as an isotype control) were incubated with the cells (2×10^5) on ice for 1 hour. An anti-human kappa-PE antibody (Thermo Fisher Scientific) was used for detection. After staining, all cells were washed, fixed, and analyzed on a BD FACS/Scan Analytical Flow Cytometer. Ten thousand events were collected per sample. The FCS Express V3 software (De Novo Software, Los Angeles, CA) was used to create the histograms.

Proliferation assay

2F7, ARH-77, or KMS-11 cells were seeded in 96-well plates at a density of 10,000 cells per well. Cells were treated with the IgG3 isotype control or ch128.1 at various concentrations ranging from 25–500 nM for a total of 96 hours. Control cells for each cell line were incubated with an equal volume of buffer alone. Inhibition of cell proliferation was monitored using the [^{3}H]-thymidine incorporation assay as described [6]. Significant differences in proliferation were determined using the Student's *t*-test.

In vivo efficacy study

Immunodeficient female non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mice, 8–12 weeks old, were purchased from The Jackson Laboratory (NOD.CB17-*Prkdc*^{scid}/J; stock # 001303; Bar Harbor, ME). Mice were injected on day 0 with 2F7 cells

(10^6) by intra-peritoneal (i.p) injection as described [17]. Mice were treated by i.p. injection with 200 μg of ch128.1, 200 μg of isotype control (IgG3), or buffer alone [Hank's balanced salt solution (HBSS)] on days 1, 8, and 22. Animals were monitored for tumor development and euthanized when moribund or when the abdomen was distended. Survival was recorded as the number of days from tumor challenge to euthanasia. Survival plots were generated and statistical analysis (log-rank test) was performed using GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA).

Results

Cell surface TfR1 (CD71) expression was verified using flow cytometry. 2F7 cells showed homogeneous and high expression of TfR1 (Fig. 1A, top). ARH-77 and KMS-11 cells have previously been reported to express TfR1 [7]. Binding of ch128.1 to 2F7 was also verified by flow cytometry (Fig. 1A, bottom). 2F7 cells were then tested for their sensitivity to the direct cytotoxic effects of ch128.1 using a proliferation assay. ARH-77 and KMS-11 cells were tested simultaneously since these cells are sensitive and resistant to ch128.1-induced cytotoxicity, respectively [5,7]. Figure 1B, shows the expected response of ARH-77 and KMS-11 cells under these conditions. The proliferation of 2F7 cells was not affected by exposure to different concentrations of ch128.1 (data not shown), even at the highest concentration of 500 nM (Figure 1B). These data show that even though 2F7 cells express high levels of the TfR1, they are not vulnerable to the direct cytotoxic effects of ch128.1 *in vitro*.

We used an AIDS-associated Burkitt lymphoma mouse model developed by our group in which 2F7 cells are inoculated i.p. into NOD-SCID mice [17]. Mice develop palpable tumors in the peritoneal cavity that are similar to human Burkitt lymphoma by histopathology [17]. Importantly, animals also develop metastases in the spleen, thymus, and mesenteric lymph nodes. Mice bearing 2F7 tumors treated with ch128.1 showed prolonged survival with a median survival of 80 days, compared to 67 days for mice treated with the isotype control antibody (Figure 2; $p = 0.0015$, log-rank test). There was no statistical difference in survival between the group treated with buffer alone and the isotype control-treated group (data not shown). Survival of both the isotype-control and buffer only groups were within the range previously described [17]. This *in vivo* experiment was replicated with similar results using buffer as control (data not shown). These studies show that even though ch128.1 has no direct cytotoxic effects *in vitro* against 2F7 cells, the antibody shows anti-cancer effects *in vivo* in an animal model of AIDS-NHL that uses the 2F7 cell line.

Discussion

We previously demonstrated the anti-cancer effect of ch128.1 in two xenograft models of multiple myeloma in SCID-Beige mice, where ch128.1 showed dramatic protection, including 100% survival, even against cells that are not sensitive to the direct cytotoxic effects of this antibody *in vitro* [7]. In this report we showed that the 2F7 AIDS-NHL cell line expresses TfR1 and that ch128.1 strongly binds to these cells. In addition, like KMS-11 cells, 2F7 cells are not sensitive to the cytotoxic effects of ch128.1 *in vitro*. Despite this fact, ch128.1 significantly delayed formation of 2F7 tumors *in vivo* in NOD-SCID mice.

Although the level of anti-tumor protection was not as dramatic as that previously reported in the multiple myeloma models, this discrepancy may be explained by the difference in the animal models, in the targeted tumors, in the route of tumor inoculation, and/or in the treatment strategy.

The mechanism of anti-tumor protection against this AIDS-NHL model is unclear at this moment. Even though no direct cytotoxicity was observed *in vitro*, it is possible that the tumor microenvironment makes the 2F7 cells particularly sensitive to the induction of iron starvation. An alternative and non-exclusive explanation is the induction of Fc-effector functions, such as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), or antibody-dependent cell-mediated phagocytosis (ADCP). However, NOD-SCID mice contain multiple immunological defects, including the lack of functional B cells, T cells, and complement activity as well as reduced natural killer (NK) cell activity and functionally less mature macrophages [18]. In contrast, these mice have increased percentages of monocytes and neutrophils [18], which could potentially interact with ch128.1 and mediate tumor cell killing. It is interesting to note that previous studies have shown that F4/80 positive cells of murine origin that are potentially tissue macrophages (histiocytes) infiltrate 2F7 tumors in this model [17], which raises the possibility that these cells may be involved in the anti-cancer activity of ch128.1. Further studies are needed to investigate the anti-cancer activity of ch128.1 in this model of AIDS-NHL. Our studies suggest that ch128.1 is a potential therapeutic for AIDS-NHL and further studies are warranted to explore this possibility.

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CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

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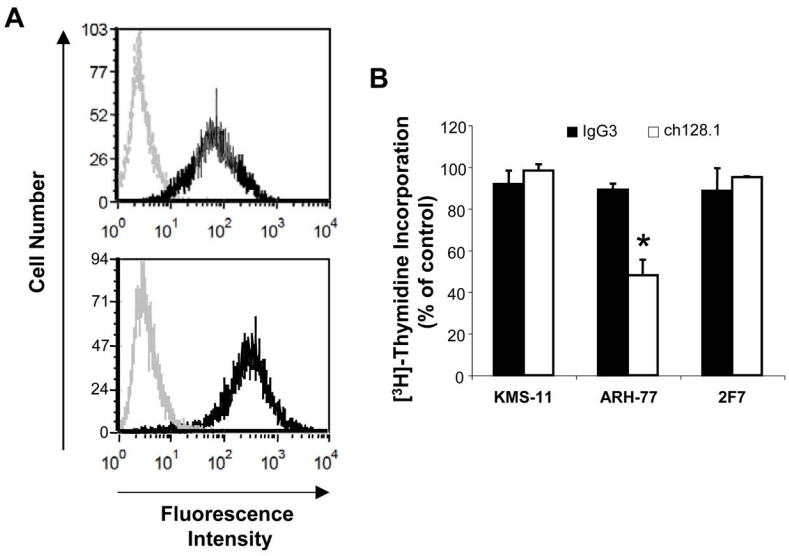

Figure 1. Cell surface TfR1 expression, ch128.1 binding, and *in vitro* sensitivity of 2F7 cells to ch128.1

A) Cells were incubated with for 1 hour on ice with either top panel: PE-conjugated mouse anti-human CD71 (black line) or PE-conjugated mouse IgG2a isotype control antibody (gray line) or bottom panel: 2 μg ch128.1 (black line) or an isotype IgG3 control (gray line) followed by an anti-human k antibody-PE conjugate. All cells were analyzed by flow cytometry. Data are representative of 2 independent experiments. B) 2F7, ARH-77, and KMS-11 cells were incubated with 500 nM ch128.1 or the isotype control (IgG3) for 96 hours. Proliferation was monitored using the [^{3}H]-thymidine incorporation assay. The rate of proliferation in treated cells is reported as a percentage of [^{3}H]-thymidine incorporated into control cells. Data are the averages of triplicate wells and the error bars represent the standard deviation (* $p < 0.05$ compared to either IgG3-treated cells or control cells, Student's *t*-test). Data are representative of 2 independent experiments.

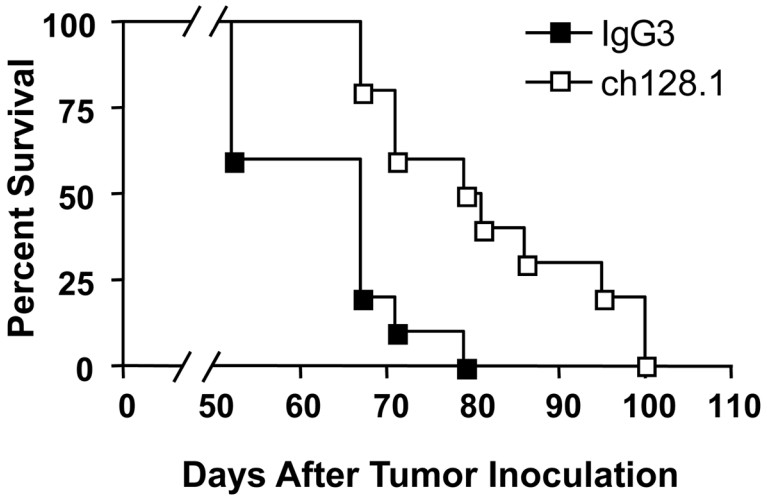

Figure 2. ***In vivo*** **efficacy of ch128.1 in a model of AIDS-associated Burkitt lymphoma**

Survival curve of NOD-SCID mice inoculated on day 0 with 2F7 cells by i.p. injection. On days 1, 8, and 22, mice were treated i.p. with 200 μg ch128.1 (n = 10) or IgG3 (n = 10).