Macrophages contribute to the antitumor activity of the anti-

CD30 antibody SGN-30

Ezogelin Oflazoglu, 1 Ivan J. Stone, 1 Kristine A. Gordon, 2 Iqbal S. Grewal, 3 Nico van Rooijen, 4 Che-Leung Law, 2 and Hans-Peter Gerber 1

Departments of 1 Translational Biology, Seattle Genetics, Inc., Seattle, Washington, USA; 2 Molecular Oncology and Immunology, Seattle Genetics, Inc., Seattle, Washington, USA; 3 Preclinical Therapeutics, Seattle Genetics, Inc., Seattle, Washington, USA; and 4 Department of Molecular Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands.

Running title: ROLE OF MACROPHAGES IN SGN-30 ACTIVITY

Correspondence: Hans-Peter Gerber., Seattle Genetics, Inc., 21823 30th Drive SE, Bothell, WA 98021, USA. Phone (425)-527-4910; Fax: (425)-527-4911; E-mail: hgerber@seagen.com.
ABSTRACT

Increased expression of CD30 is associated with a variety of hematologic malignancies, including Hodgkin’s disease (HD) and anaplastic large cell lymphoma (ALCL). The anti-CD30 monoclonal antibody SGN-30 induces direct antitumor activity by promoting growth arrest and DNA fragmentation of CD30 positive tumor cells. In this study, we investigated the contributions of Fc-mediated effector cell functions to SGN-30 activity. We determined that antibody-dependent cellular phagocytosis (ADCP), mediated by macrophages, to contribute significantly to antitumor activity in vitro. To delineate the identity of the host effector cells involved in mediating anti-tumor activity in vivo, we studied the effects of effector cell ablation in a disseminated model of HD (L540cy). Depletion of macrophages markedly reduced efficacy of SGN-30, demonstrating that macrophages contribute significantly to SGN-30 efficacy in this model. These findings may have implications for patient stratification or combination treatment strategies in clinical trials conducted with SGN-30 in HD and ALCL.
INTRODUCTION

Increased expression of CD30 was observed on Reed-Sternberg cells in HD\(^1\), on neoplastic cells in ALCL and on a subset of non-Hodgkin’s lymphomas (NHLs)\(^2\). Expression of CD30 on normal cells is restricted to activated T and B cells, as CD30 is not expressed by resting lymphocytes. Such highly restricted expression of CD30 to activated lymphocytes and neoplastic tumor cells led to the investigation of this cell surface antigen as a target for immunotherapy in hematologic malignancies\(^3\). Previous reports demonstrated that SGN-30, a therapeutic antibody binding to CD30, elicits direct effects on tumor cell intrinsic signaling by promoting growth arrest and DNA fragmentation of CD30+ tumors including HD and ALCL, ultimately resulting in cell death\(^4,5\). However, it remained unknown whether SGN-30 engages Fc-mediated effector cell activities. In this report, we demonstrate that macrophages contribute significantly to the antitumor activity of SGN-30 \textit{in vitro}, by inducing cellular cytotoxicity via ADCP. \textit{In vivo}, depletion of macrophages reduced survival of tumor bearing mice treated with SGN-30. In contrast, ablation of natural killer (NK) cells did not significantly impact on efficacy in this model. Combined, these data suggest that the interaction between SGN-30 and host macrophages contributes significantly to the mechanisms by which SGN-30 interferes with tumor growth. The newly defined role of macrophages in mediating antitumor activity of SGN-30 identified here may be relevant for the clinical activity of SGN-30, which is currently being developed for HD and ALCL.
MATERIALS AND METHODS

Cells and Reagents

CD30-positive HD lines: L540cy was provided by Dr. Phil Thorpe (University of Texas, Southwestern Medical School, Dallas, TX), HDLM-2 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The CD30 positive lymphoblast B cell line WIL2-S and CD30 negative acute promyelocytic leukemia line HL60 were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI (Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). For in vivo depletion studies, rabbit anti-asialo-GM-1 antibody was obtained from Wako Pure Chemical Industries, Ltd. (Richmond, VA), rat anti-mouse-Gr-1 antibody was obtained from BD Biosciences (San Diego, CA). Liposome-encapsulated clodronate (CEL) was prepared as previously described6. Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany). SGN-30 was engineered at Seattle Genetics, Inc.4 and does not cross-react with rodent CD30.

Antibody-Dependent Cellular Phagocytosis Assay

This assay was performed as previously described7. Briefly, once CD30 positive target cells (TCs) were labeled, they were pre-coated with SGN-30 and then incubated with monocyte derived macrophages. The monocytes represent the adherent cell components of PBMCs cultured for 10-15 days in X-VIVO 15 medium (Cambrex BioScience, Walkersville, MD) containing 500 U/mL rhGM-CSF (PeproTech, Rocky Hill, NJ). Monocytes harvested from these cultures were
CD3/CD19 negative and expressed CD14, CD11b, CD16, CD32 and CD64 as determined by flow cytometry or by fluorescence microscopy (Zeiss microscope, Thornwood, NY). The purity of the macrophage preparations was routinely > 95%.

**Xenograft Models**

C.B-17 SCID mice were injected with 5 x 10^6 L540cy cells i.v. on day 0. Mice were monitored at least twice weekly and were terminated when exhibiting signs of disease as described previously ⁴. Tumor-bearing mice were depleted of effector cells using specific antibody or CEL as described previously ⁶-⁸. Natural killer (NK) cells were depleted by i.p. injection of anti-asialo-GM 1 (1.25 mg/kg). Mice were given a total of 3 doses once every 5 days, beginning the day of tumor cell implantation. Macrophages were depleted by i.p. injection of CEL (100µl/10gr) on the day of tumor injection and every 3 days thereafter for a total of 5 doses. Cell depletion was confirmed by flow cytometric analysis of splenocytes, lymph nodes and blood (data not shown). Statistical analysis was conducted using the log-rank test provided in the Graphpad Prism Software Package version 4.01 (Graphpad, San Diego, CA). All animal experiments were conducted under Seattle Genetics’ IACUC guidelines and approval.
RESULTS AND DISCUSSION

In this report, we investigated the ability of SGN-30 to engage Fc-mediated effector cell functions in vitro and in vivo. When tested in an ADCP assay described previously, SGN-30 induced ADCP against a panel of cell lines including L540cy, HDLM-2, and WIL2-S (Fig 1A, B). As expected, ADCP activity was not observed in the presence of an F(ab')2 fragment of SGN-30 lacking the Fc domain or when a CD30 negative tumor cell line (HL-60) was tested. These findings demonstrate that anti-tumor activity of SGN-35 is antigen specific that Fc-Fc receptor interactions are necessary for phagocytic uptake of antibody labeled target cells (Fig 1C). The degree of ADCP induced by SGN-30 was comparable with the levels obtained for similar human IgG1 subtype antibodies, targeting cell surface antigens expressed on hematologic tumor cells, including an anti-CD70 antibody. SGN-30 was further tested for its ability to induce cell lysis of CD30+ tumor cells via complement fixation, a process termed complement dependent cytotoxicity (CDC) and/or via antibody dependent cellular cytotoxicity (ADCC), mediated by natural killer (NK) cells. For the determination of ADCC, a previously described assay using PBMCs as a source for NK cells was employed. In neither assay, we were able to detect significant levels of CDC or ADCC by SGN-30 when tested on a large variety of CD30+ cell lines (data not shown). These findings support the notion that therapeutic antibodies can vary in their potency to engage effector cells, depending on their IgG isotypes and other, less well characterized characteristics, including antigen binding.
Having demonstrated that SGN-30 induces tumor cell killing via ADCP in vitro, we sought to define the nature of the FcγR-expressing cells that are responsible for SGN-30 targeted cell clearance in vivo. For this purpose, control or SGN-30 treated mice were depleted of NK cells or macrophages as described in Materials and Methods. As shown in Figure 2, depletion of either NK cells or macrophages in control treated mice did not significantly affect survival of mice implanted with L540cy tumors (Fig. 2A, B). In SGN-30 treated mice, macrophage depletion reduced survival of mice significantly, resulting in a median survival of 81 days compared to a 90% survival at 143 days in the control, non-macrophage depleted group (Figure 2B). However, despite the profound level of macrophage depletion in most organs achieved in our experiments (data not shown), SGN-30 treated mice survived significantly longer when compared to control, untreated mice. These observations are consistent with our previous observations, suggesting that SGN-30 can induce direct tumor cell killing in the absence of effector cells via direct effects on tumor cell signaling, resulting in apoptosis.

As demonstrated here, Fc-dependent effector-cell functions contribute significantly to the antitumor activity of SGN-30 when tested in a preclinical model of HD. Additional evidence for ADCP contributing to the effects of some therapeutic monoclonal antibodies targeting cell surface markers on hematopoietic cells was provided by other reports. Even though the role of macrophages in tumor progression is controversial, it is tempting to speculate that therapeutic strategies aimed at enhancing host immune cell functions, in particular myeloid cell lineages, may improve anti-tumor activity.
Response to SGN-30 treatment in patients may be less affected by the Fc-gamma polymorphism if efficacy is predominantly mediated by cells of macrophages/monocyte origin. In contrast, the naturally occurring sequence polymorphisms in the Fc gamma receptor IIIa (FcγRIIIA;158V/F) is shown to correlate with therapeutic efficacy of rituximab in clinical trials in NHL\textsuperscript{12,13}, supposedly by affecting ADCC activity mediated by NK cells\textsuperscript{14-16}. Interestingly, the efficacy of the same therapeutic antibody may not correlate with FcγRIIIA polymorphism in CLL trials, indicating the different mechanism of action may be operative in different indications\textsuperscript{17,18}. Macrophages express all 3 subtypes (FcγRIIIa/CD16, FcγRIIa/CD32 and FcγRI/CD64) and FcγRI is involved in ADCP activity whereas NK cells, which are predominantly involved in ADCC activities, express exclusively FcγRIIIa\textsuperscript{14-16}. For FcγRI, no sequence polymorphisms were reported so far. Based on these observations, it is plausible that ADCP may not be affected by the sequence polymorphisms within the FcγRIIIa alleles. These findings may have implications for patient stratification or combination treatment strategies in clinical trials conducted with SGN-30 in HD and ALCL.
Authorship:

Contribution: E.O. designed and conducted research, analyzed data, and wrote the manuscript; K.A.G. and I.S., conducted research., N.v.R., contributed vital reagents; I.S.G. and C.-L.L contributed to the design of research; H. G. designed research and wrote the manuscript.

REFERENCES


16. Shields RL, Namenuk AK, Hong K, et al. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J Biol Chem. 2001;276:6591-6604.


FIGURE LEGENDS

Figure 1 SGN-30 mediates ADCP activity in vitro. (A) Representative flow cytometry analysis and fluorescence microscopy of SGN-30-mediated phagocytosis. For flow cytometry, L540cy, HDLM-2, Wil-2S and HL-60 target cells were labeled with PKH26 lipophilic dye for tracking purposes, and treated with SGN-30 or non-binding control IgG and mixed with monocyte-derived macrophages (MΦ). MΦ were stained with PE-conjugated anti-CD11b. Cells present in the upper right quadrant (PKH26+CD11b+) are MΦ that internalized tumor cells. For microscopy, tumor cells were labeled with PKH67 (green) and the macrophages were detected with Alexa fluor 568-conjugated antibody specific for CD11b (red). (B) Tumor targets were treated with varying concentrations of SGN-30 prior to incubation with MΦ. The levels of MΦ that engulfed tumor cells were determined by flow cytometry (C) WIL2-S cells were incubated with SGN-30, SGN-30 F(ab’)2, or non-binding control IgG prior to incubation with MΦ, and analyzed by flow cytometry.

Figure 2 Macrophages are involved in antitumor activity of SGN-30 in vivo. L540cy-bearing mice were depleted of (A) NK cells, (B) macrophages (MΦ), as described in Materials and Methods. Mice were either left untreated (control) or treated with 10 mg/kg SGN-30 on day 1 following L540cy challenge (n = 10 per group). Panel (A), untreated vs. SGN-30 (p = 0.0007), -NK vs. SGN-30 – NK (p < 0.0001), SGN-30 vs. SGN-30 – NK (p = 0.2450). Panel (B), untreated vs. SGN-
30 (p = 0.0007), –Mac vs. SGN-30 – MΦ (p = 0.0002), SGN-30 vs. SGN-30 – MΦ (p < 0.0001).
Figure 1

A

IgG

SGN-30

CD11b

PKH

B

% Tumor Positive Mφ (background subtracted)

mAb (ng/mL)

HL-60

L540cy

HDLM2

C

% Tumor Positive Mφ

IgG

SGN-30

SGN-30 F(ab')2
Figure 2

A

B

Days

Percent survival

Untreated
SGN-30
-NK
SGN-30-NK

Days

Percent survival

Untreated
SGN-30
-Mac
SGN-30-Mac
Macrophages contribute to the antitumor activity of the anti-CD30 antibody SGN-30