Antibody-Based Delivery of IL2 and Cytotoxics Eradicates Tumors in Immunocompetent Mice

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Abstract

Antibody–drug conjugates are increasingly being used for cancer therapy, but little is known about their ability to promote anticancer immunity, which may lead to long-lasting remissions. We investigated the therapeutic effect of antibody-based pharmacodelivery of cemadotin, a cytotoxic drug, and IL2, a strong proinflammatory cytokine. Using the F8 antibody, which selectively localizes to the tumor neovasculature, combination treatment led to tumor eradication, in a process dependent on CD8⁺ T cells and natural killer cells in the C1498 syngeneic mouse model of acute myelogenous leukemia. The clinical combination of antibody–drug conjugates and antibody–cytokine proteins should be facilitated by their orthogonal toxicity profiles.

Introduction

There is a growing interest in pharmaceutical biotechnology to "arm" tumor-targeting antibodies with bioactive payloads, such as cytotoxic drugs and cytokines, to increase activity at the site of disease and spare normal tissues (1–4). Antibody–drug conjugates (ADC) and antibody–cytokine fusion proteins (immunocytokines) represent two advanced classes of armed antibodies.

ADCs are on the market. Adcetris (brentuximab-vedotin) is approved for the treatment of refractory Hodgkin lymphoma and anaplastic large-cell lymphoma, whereas Kadcyla (trastuzumab-emtansine) is applied for the treatment of metastatic Her2⁺ breast cancer. Over 30 novel ADCs against hematologic and solid tumors are in clinical development.

Immunocytokines, carrying IL2 as immunostimulatory payload, have shown selective tumor accumulation and anticancer activity, allowing these agents to progress to phase II clinical trials for the treatment of metastatic solid tumors (5). The most advanced candidates at present are immunocytokines directed against the melanoma and neuroblastoma cellular target GD2 disialoganglioside (h14.18-IL2) or the splice-isoform A1 of tenascin-C (F16-IL2) and extradomain B of fibronectin (L19-IL2), which are markers of the tumor neovasculature (5). Recently, we have shown that neovascular-targeting immunocytokines can be efficient for the treatment of hematologic malignancies, including lymphomas and certain acute leukemias (6, 7).

ADCs are typically studied in immunocompromised mice, due to a lack of interspecies cross-reactivity for many antibodies. As a consequence, preclinical studies with ADCs focus on cytotoxic activity, without addressing the impact of immune-mediated anticancer mechanisms. However, anticancer immunity (e.g., as a consequence of IL2 treatment) can lead to cures (8, 9) and it is known that certain cytotoxic agents may display immunomodulatory properties that justify their therapeutic application beyond chemotherapy (10, 11).

To explore the possibility of improving the therapeutic potential of both ADCs and immunocytokines, we treated immunocompetent mice bearing acute myelogenous leukemia (AML) tumors with a combination of armed antibody products, featuring the cytotoxic drug cemadotin thiol (12) or IL2 as payloads. We chose the F8 antibody as delivery vehicle of proven tumor-homing performance, which recognizes the alternatively spliced extradomain A (EDA) of fibronectin, a marker of angiogenesis, with identical affinity in mouse and man (13). As negative control antibody of irrelevant specificity in the mouse, we used the anti-hen egg lysozyme antibody KSF (14).

Materials and Methods

Cell lines, animals, and xenograft models

The murine AML cell line C1498 was purchased from the ATCC in March 2012 and cultured according to the supplier’s recommendations. Six- to 8-week-old female C57BL/6 mice were purchased from Elevage Janvier. For generation of the localized xenograft model, 10⁶ C1498 cells were injected into the flank of 8- to 10-week-old C57BL/6 mice. All animal experiments were performed on the basis of project license (42/2012) administered by the “Veterinäramt des Kantons Zürich” and approved by all participating institutions.
Antibodies

F8 is a human monoclonal antibody specific to the EDA domain of fibronectin (13). KSF is specific to hen egg lysozyme and does not show any specificity toward human antigens. The expression and characterization of the F8-IL2 immunocytokine as a recombinant protein has previously been described (14). SIP(KSF) was expressed previously been described (15), whereas SIP(F8) was provided by Philogen.

Preparation of ADCs

Site-selective ADCs were prepared by disulfide linkage of cemadotin thiol (CemCH₂-SH) to a cysteine residue at the C-terminus of SIP(F8) or SIP(KSF) as previously described in detail (12). In summary, the covalent C-terminal disulfide bond of antibodies in the small immunoprotein (SIP) format was reduced with TCEP (tris(2-carboxyethyl)phosphine), allowing activation of the resulting cysteine residues with Ellman reagent 5,5'-dithio-bis(2-nitrobenzoic acid). The resulting antibody product was purified over a HiPrep desalting column (GE Healthcare) and incubated with CemCH₂-SH to generate the homogeneous mixed disulfide ADC.

Characterization of the armed antibody products

Recombinant F8-IL2 and chemically modified F8-SS-CH₂Cem and KSF-SS-CH₂Cem were characterized by SDS-PAGE, electrospray–mass spectrometry (ESI–MS), and gel filtration, as previously described (12). Immuno-reactivity of the antibody products to EDA of fibronectin was measured by surface plasmon resonance real-time interaction analysis on a CM5 microsensor chip coated with recombinant EDA on a Biacore3000 instrument (GE Healthcare).

Combination therapy and tumor rechallenge in the localized C1498 model

C1498 cells were subcutaneously injected into the flank of 6- to 8-week-old female C57BL/6j mice. When tumors were established (50–100 mm³), mice were grouped to maximize uniformity (n = 5) and injected into the lateral tail vein with either saline, F8-IL2 (1 mg/kg), F8-SS-CH₂Cem (10 mg/kg), KSF-SS-CH₂Cem (10 mg/kg) or a combination of F8-IL2 and F8-SS-CH₂Cem (at same dosages). The immunocytokine was administered every third day for three injections (days 7, 10, and 13), whereas the ADCs were injected on the days in between for a total of four injections (days 8, 9, 11, and 12).

Mice that were cured after combination treatment were rechallenged by subcutaneous injection with 10⁶ C1498 cells on day 125 and monitored for an additional 120 days.

The mice were monitored daily for body weight loss, and tumor growth was measured every second day with a digital caliper using the following formula: volume = length × width × width × 0.5. Animals were sacrificed when the tumor reached a volume of >1,200 mm³. The data were displayed as average values ± SE. The differences in tumor volume between therapeutic groups were compared with the two-tailed Student test with α = 0.05.
Taking the Bonferroni correction into account, significance was determined when $P \leq 0.002$.

**In vivo depletion of natural killer cells, CD4$^+$ T cells, and CD8$^+$ T cells**

Subcutaneous C1498 tumors were established in C57BL/6 as described above. The mice ($n = 5$) were injected intraperitoneally with either saline, anti-asialo GM1 (Wako Chemicals), anti-CD4 (Bio X Cell), or anti-CD8 (Bio X Cell) antibodies on days 6, 9, 12, 15, and 19 as recommended by suppliers. Saline or the combination of F8-IL2 and F8-SS-CH$_2$Cem was administered as described above. The mice were monitored as described above. The data were displayed as average values ± SE.

**Results**

Site-selective ADCs were generated by disulfide linkage of cemadotin thiol (CemCH$_2$-SH) to the C-terminal cysteine of antibodies in SIP format, as previously described, yielding products F8-SS-CH$_2$Cem and KSF-SS-CH$_2$Cem (12, 15). As immunostimulatory product, we used the immunocytokine F8-IL2 in homobivalent format (7). Figure 1 shows a schematic representation of the structure of the armed antibody products and their characterization by SDS-PAGE, gel filtration analysis, mass spectrometry, as well as Biacore on microsensor chips coated with recombinant EDA. All products exhibited a high level of chemical purity. Both F8-IL2 and F8-SS-CH$_2$Cem bound to the cognate EDA target with high affinity, indicating that antibody functionalization strategies had not altered antigen binding. By contrast, the negative control KSF-SS-CH$_2$Cem product did not bind to EDA, as expected.

Immunocompetent C57BL/6j mice, bearing subcutaneously grafted murine C1498, were treated with saline, F8-SS-CH$_2$Cem, or KSF-SS-CH$_2$Cem (10 mg/kg; days 8, 9, 11, and 12). Significant tumor growth retardation was observed after day 10 in mice treated with F8-SS-CH$_2$Cem compared with KSF-SS-CH$_2$Cem. Furthermore, the combined administration of F8-SS-CH$_2$Cem (as above) and F8-IL2 (1 mg/kg; days 7, 10, and 13) promoted complete and long-lasting tumor eradication in 4 of 5 mice (Fig. 2A). Monitoring of the body weight showed no weight loss during therapy (Fig. 2B). The cured mice were rechallenged with C1498 cells on day 125 and did not develop neoplastic lesions, indicating that they had acquired protective immunity (Fig. 2C). In vivo depletion of either natural killer (NK) cells or CD8$^+$ T cells led to the complete abrogation of the combinatorial therapeutic effect of F8-IL2 and F8-SS-CH$_2$Cem, whereas CD4$^+$ T-cell depletion did not have any detrimental effect on therapeutic performance (Fig. 3A and B). Four of five of the control mice and 5 of 5 of the CD4$^+$ T-cell-depleted mice displayed complete tumor eradication after combination treatment. However, CD4$^+$ T-cell-depleted mice developed disseminated disease symptoms after day 39, whereas control mice remained disease free (Fig. 3C).

**Discussion**

In this study, we show, for the first time, that ADCs and immune-stimulatory immunocytokines synergize, boosting a curative anticancer immunity in an immunocompetent mouse model of leukemia.

We used ADCs, which release their cytotoxic payload in the subendothelial extracellular space at tumor sites (15). This strategy is attractive (3), as the payload can potentially diffuse and affect both tumor endothelial cells and cancer cells. The tumor-homing properties of vascular-targeting ADCs may be easier to be studied both in terms of quantitative biodistribution and microscopic
localization, as previously shown by PET studies in patients with cancer (16) and by ex vivo microscopic analysis (7, 13). Furthermore, the SIP format may be preferable to the more conventional IgG antibody format, as it exhibits better tumor-to-organ ratios at earlier time points (17, 18). Obviously, internalizing ADCs and other antibody formats could also be considered, in particular, as novel ADCs may gain marketing approval.

The observation that cancer cures were dependent on both CD8+ T cells and NK cell was in line with previous reports on long-term complete remissions (7, 19, 20), but remains intriguing from a mechanistic point of view. The therapeutic benefit of the immunocytokine plus ADC combination treatment is completely lost if one of the two cell populations is absent, suggesting an interplay between CD8+ T cells and NK cells. The anticaner activity of CD8+ cytotoxic T cells is normally attributed to their recognition of tumor-rejection antigens, while activating receptors on NK cells are able to recognize proteins that are expressed in conditions of cellular stress. CD4+ T cells did not contribute to eradication of the primary tumor, but their depletion led to a late onset and dissemination of the disease (Fig. 3).

Different cytokines are present in blood at concentrations, which may vary by as much as three orders of magnitude (e.g., from 10 pg/mL to 10 ng/mL). Similarly, various cytotoxic anticancer drugs have been described, which display in vitro cell killing activity in the micromolar, nanomolar, or picomolar concentration range. By judiciously choosing payloads of comparable potency, it would thus be conceivable to incorporate both cytokine and cytotoxic drug into one armed antibody molecule, rather than using combinations of immunocytokines and ADCs.

The strong therapeutic effect observed by combining F8-IL2 with F8-SS-CH2Cem may find direct therapeutic applications, because the EDA of fibronectin is expressed in the majority of human solid tumors, lymphomas, and in acute leukemias. We have previously reported a potent anticaner effect of antibody-based IL2 delivery in AML in combination with cytarabine (7). It is likely that ADCs may represent a better combination partner for F8-IL2 and other proinflammatory immunocytokines compared with cytarabine, as this drug represents the backbone of induction therapy and may give rise to resistance in patients with relapsed or refractory leukemia.

Disclosure of Potential Conflicts of Interest

D. Neri has employed as a member of the board by, has ownership interest in, is a consultant/advisory board member for Philogen. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K.L. Gutbrodt, D. Neri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Gutbrodt, D. Neri
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.L. Gutbrodt, D. Neri
Writing, review, and or revision of the manuscript: K.L. Gutbrodt, G. Casi, D. Neri
Study supervision: D. Neri

Grant Support

D. Neri received funding from ETH Zürich, Federal Commission for Technology and Innovation (CTI; grants ADC and Medtech), the Maiores Foundation, and the Swiss National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 6, 2014; revised April 3, 2014; accepted April 4, 2014; published OnlineFirst April 23, 2014.
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