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Potentiating the activity of rituximab against mantle cell lymphoma in mice by targeting interleukin-2 to the neovasculature

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ABSTRACT

There is increasing interest in the site-directed pharmacodelivery of therapeutic payloads to the tumor site using antibodies as transport vehicles. Here, we investigated the efficacy of L19-IL2, an antibody–cytokine fusion protein that specifically delivers IL-2 to the tumor site by homing to the extra-domain B of fibronectin (EDB-Fn) expressed on tumor-associated blood vessels, against mantle cell lymphoma (MCL) in mice. L19-IL2 was shown to selectively localize at lymphoma lesions in vivo and to mediate significant lymphoma growth retardation, which was potentiated by co-administration of the anti-CD20 antibody rituximab. When co-injected with rituximab, L19-IL2 induced complete remissions of localized MCL xenografts in 6/8 mice (75%), whereas the combination of rituximab and equivalent doses of non-targeted IL-2 only slightly delayed tumor growth. In disseminated MCL, combination therapy with L19-IL2 and rituximab exhibited a significant survival benefit over treatment with IL-2 and rituximab and completely eradicated the disease in 2/7 cases (28.6%). Mechanistically, histological analyses of post-therapeutic lymphoma tissues revealed a strong intratumoral accumulation of macrophages and natural killer cells after a single dose of the immunocytokine, whereas L19-IL2 had no significant impact on microvessel density or on tissue penetration of co-injected rituximab. Collectively, these results provide the scientific rationale for the clinical evaluation of L19-IL2 in combination with anti-CD20 immunotherapy in patients with MCL.

1. Introduction

Mantle cell lymphoma (MCL) is a non-Hodgkin lymphoma genetically characterized by the *t*(11;14)(q13;q32) translocation. MCL represents 2–10% of all NHL cases in adults [1,2] and is often associated with a clinically aggressive course of the disease, but in contrast to most aggressive B cell lymphomas does not respond well to conventional cytotoxic chemotherapy. Even though recent studies have shown that median survival of MCL patients almost doubled in the past three decades, now being 4–6 years, it still comes with one of the worst prognoses among all B cell lymphomas [3]. One of the reasons for improved therapeutic outcomes in MCL has been the introduction of the monoclonal anti-CD20 antibody rituximab, which improves response rates when added to standard chemotherapy [4] and also proved effective in maintenance therapy following chemotherapy induction [5].

In recent years, there is increasing biotechnological interest in the arming of therapeutic antibodies with potent bioactive payloads, to increase activity at the site of disease while sparing normal organs [6,7]. Whereas most antibody derivatives in clinical development and all currently approved armed antibody products are directed against targets expressed on the cancer cell surface (e.g., CD30 or Her2), we have made good experiences with the targeting of extracellular antigens expressed in and around the tumor-associated neovasculature. This approach is termed "vascular targeting" and aims at the selective pharmacodelivery

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of bioactive molecules (e.g., drugs, pro-inflammatory cytokines, radionuclides) to the tumor site by their conjugation to carrier antibodies, which specifically home to tumor blood vessels [8–10]. The approach benefits from the inherent accessibility of vascular targets to antibody therapeutics coming from the bloodstream and circumvents many of the difficulties associated with the direct targeting of cancer cells deeply embedded in bulky tumor masses. In addition, whereas cancer cell surface proteins are often shared by cells of corresponding normal tissues (e.g., CD20 by normal B cells, EGFR by skin cells), certain vascular targets are nowadays available, which allow a stringent discrimination between cancer tissues and normal organs [11,12]. While initially being neglected in the context of hematological malignancies, vascular targeting antibody derivatives are now increasingly being considered for the treatment of leukemias and lymphomas as well, with encouraging pre-clinical and clinical efficacy [13–16].

The human monoclonal antibody L19 [17] specifically recognizes the alternatively spliced extra-domain B of fibronectin (EDB-Fn), a well-characterized marker of angiogenesis which is expressed in the sub-endothelial extracellular matrix of angiogenic blood vessels in almost all solid and hematologic malignancies but is virtually absent in the vasculature of normal organs (exception made for some angiogenic vessels in the ovaries and in the placenta) [11,12,18,19]. L19 has demonstrated a remarkable ability to selectively accumulate at the tumor site in tumor-bearing animals and in cancer patients, both by quantitative biodistribution studies [20–23] and by nuclear medicine imaging studies [14,15,24].

We have previously shown that the L19-based vascular targeting fusion protein L19-IL2, carrying the immunostimulatory cytokine interleukin-2 (IL-2) as therapeutic payload, exerts potent activity against Burkitt and follicular lymphoma xenografts in mice, especially when used in combination with anti-CD20 immunotherapy [13].

L19-IL2 belongs to a novel class of therapeutic antibodies ('immunocytokines'), which genetically fuse immunomodulatory cytokines to tumor-homing antibodies, in order to improve the therapeutic index of the cytokine by its targeted delivery to the site of disease [25]. Indeed, there is a strong preclinical and clinical rationale suggesting that IL-2-based immunocytokines can display a superior dose-dependent anticancer activity compared to equivalent doses of non-targeted IL-2 either in monotherapy or in combination with standard antineoplastic agents [16,25–28]. Accordingly, when evaluated against Burkitt and follicular lymphoma xenografts in mice, free IL-2 was not able to enhance the efficacy of rituximab, while the therapeutic action of rituximab was dramatically potentiated whenever IL-2 was carried directly to the lymphoma tissue using L19-IL2 [13]. These promising results prompted us to investigate the efficacy of L19-IL2 against MCL, a subtype of NHL for which current therapeutic options still remain unsatisfactory.

2. Materials and methods

2.1. Animals and cell lines

Six to 8 week old female CB17 SCID mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and were kept in groups of five under standard conditions. Experimental design was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany (2011-04-26). Cell lines used in experiments were GRANTA-519 (DSMZ; Braunschweig, Germany), Z-138 (ATCC; Manassas, VA, USA) and JEKO-1 (DSMZ), all characterized by the mantle cell lymphoma-inherent translocation *t*(11;14)(q13;q32) and overexpression of Cyclin-D1. JEKO-1 cells were maintained in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS), GRANTA-519 cells in DMEM + 10% FCS and Z-138 cells in IMDM + 10% FCS. All cell lines were cultured in 5% CO_2 at 37 °C and periodically assessed for the presence of mycoplasma.

2.2. Antibodies and therapeutic reagents

L19 is a human monoclonal antibody specific to the EDB-Fn [17]. The expression and characterization of the L19-IL2 immunocytokine has previously been described [26]. The fusion of the L19 antibody to IL-2 does not interfere with the activity of the cytokine and equimolar amounts of L19-IL2 and free IL-2 show identical biological IL-2 activities in a T cell proliferation assay [26]. KSF is specific to hen egg lysozyme and does not show any specificity toward human antigens [29]. Antibodies in SIP (small immunoprotein) format were biotinylated and carried a comparable number of biotin molecules. Rituximab (MabThera) was purchased from Roche (Grenzach-Wyhlen, Germany) and recombinant IL-2 (Proleukin) from Novartis (Basel, Switzerland).

2.3. Analyses of EDB expression

MCL xenografts were analyzed for expression of EDB-Fn using immunofluorescence. Six micrometer-thick cryo-sections of GRANTA-519, Z-138 and JEKO-1 tumors were stained with a biotinylated version of L19-SIP as primary antibody (2 μg/ml). Biotinylated KSF-SIP (2 μg/ml) was used as negative control. Detection of the primary antibodies was performed using the streptavidin Alexa Fluor 488 conjugate (Invitrogen, Darmstadt, Germany). Endothelial cells were visualized using rat anti-mouse CD31 antibody (BD Pharmingen, Heidelberg, Germany) and a goat anti-rat IgG TRITC conjugate (Dianova, Hamburg, Germany). In another set of experiments, the fusion protein L19-IL2 (6 μg/ml) was applied to frozen 6 μm sections of MCL xenografts and visualized using goat anti-human IL-2 antibody (R&D Systems, Minneapolis, MN) and Alexa Fluor 594 donkey anti-goat (Invitrogen).

The Institute of Pathology Kiel kindly provided human MCL cryo-samples for analysis of EDB-Fn expression. The use of frozen lymphoma material was made necessary by the fact that the L19 antibody does not work in paraffin [18], which prevented the use of larger collections of formalin-fixed, paraffin-embedded specimens. Biotinylated L19-SIP, specific to both murine and human EDB-Fn, was used as described in the previous paragraph. Endothelial cells were outlined with a mouse anti-human CD31 antibody (DAKO, Glostrup, Denmark) and the Alexa Fluor 594 goat anti-mouse conjugate (Invitrogen) as secondary antibody. Images were captured using a Nikon Eclipse 50i microscope equipped with a Nikon DS-Qi1Mc camera.

2.4. In vivo targeting of L19-IL2

Lymphoma cells (GRANTA-519 or Z-138; 1×10^7) were injected subcutaneously into the flank of 6–8 week old SCID mice. When tumors averaged 400 mm^3, animals were treated with a single injection of 30 μg L19-IL2 into the lateral tail vein. As a control, the equivalent dose of recombinant IL-2 (9.9 μg) was administered. After 24 h, animals were euthanized and frozen sections of the excised lymphomas and normal organs were incubated with goat anti-human IL-2 antibody and Alexa Fluor 594 donkey anti-goat secondary antibody to detect L19-IL2 bound to its target in situ. Endothelial cells were outlined using rat anti-mouse CD31 antibody (BD Pharmingen) and Alexa Fluor 488 rabbit anti-rat IgG (Invitrogen). Nuclei were stained with DAPI.

2.5. Therapy experiments with localized MCL xenografts

GRANTA-519 lymphoma cells (1×10^7) were injected subcutaneously into the flank of SCID mice. On day 14 after injection, all mice had developed a visible tumor and were assigned to 6 therapy groups of 6–8 mice, each group having an average tumor volume of 50–100 mm^3. Therapy was administered by intravenous injection into the lateral tail vein on days 14, 17, 20, 23 and 26 after inoculation of tumor cells. Mice received 30 μg of L19-IL2, the corresponding dose of non-targeted IL-2 (9.9 μg), 200 μg rituximab, 200 μg rituximab and 9.9 μg IL-2, 200 μg rituximab and 30 μg L19-IL2, or saline. Tumor growth was measured every second day, using a caliper. Tumor volumes were estimated by the formula (width^2 × length)/2. Animals were euthanized when the tumor reached a volume greater than 2000 mm^3 or developed critical ulceration. Responses were defined as partial remission (PR: at least 50% reduction of tumor volume) or complete remission (CR: no visible or palpable tumor).

2.6. Therapy experiments with disseminated MCL xenografts

To model systemic MCL, SCID mice were injected intravenously with GRANTA-519 lymphoma cells (1×10^6). Mice were randomly divided into three groups of 7–8 mice, receiving either saline, 30 μg of L19-IL2 or the equivalent dose of IL-2 (9.9 μg), both of the latter in combination with 200 μg of rituximab. Sufficient dissemination and engraftment of lymphoma cells was allowed before therapy was administered intravenously on days 10, 13, 16, 19 and 22 after injection of tumor cells. Animals were monitored every second day for the presence of hind-leg paralysis or weight loss of 20% or more, whereupon mice were euthanized and scored as dead for Kaplan–Meier plots.

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