Novel FGFR4-Targeting Single-Domain Antibodies

Subjects: Oncology

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FGFR4, member of the Fibroblast Growth Factor Receptor family, is a promising therapeutic target for rhabdomyosarcoma, the most frequent pediatric soft tissue sarcoma. FGFR4 single domain Antibodies specific for FGFR4 were selected, characterized, and showed promising results against rhabdomyosarcoma cell lines as single agents, as targeting moieties for liposomes, and as antigen binding domain in Chimeric Antigen Receptor modified T cells.

Keywords: FGFR4 ; Rhabdomyosarcoma ; targeted drug delivery ; nanobodies ; single domain antibodies ; CAR T cells ; chimeric antigen receptor

1. Introduction

1.1. Rhabdomyosarcoma

Approximately 50% of children and adolescents diagnosed with soft tissue sarcoma suffer from rhabdomyosarcoma (RMS), a striated-muscle lineage malignancy with variable pathologies[1]. The two major subtypes of the tumor are embryonal RMS (ERMS) and alveolar RMS (ARMS), accounting for 60% and 20% of all cases, respectively. ARMS is more aggressive and is characterized by a chromosomal translocation resulting in a *PAX3-FOXO1* gene fusion, whereas ERMS is associated with different tumor-promoting mutations and chromosome number aberrations[2]. Surgery, radiation, and multi-drug chemotherapy composed of vincristine (VCR), actinomycin D and cyclophosphamide are the standard treatments for RMS [1]. The overall survival rates for RMS patients have improved within the last few decades but the prognostic outcome is still very poor for high-risk patients, including those presenting metastatic diseases, ARMS subtype, or diagnosis in adulthood[3][4]. The treatment goes along with high toxicity and many who survive RMS will experience long-term adverse effects as adults[5]. Therefore, there is an urgent need for new targeted therapies to improve overall survival rates, and to overcome long-term side effects.

1.2. Liposomal Drug Delivery - Passive

Nanovesicle-mediated chemotherapeutic drug delivery offers the possibility to increase the therapeutic effect in the tumor and to decrease side effects in healthy tissues[6][7][8][9]. Passive accumulation of nanoparticles in the tumors has been attributed to the so-called “enhanced permeability and retention” (EPR) effect[10]. Fast-growing solid tumors display a leaky vascular architecture and a lack of functional lymphatics, enabling the size-dependent passive extravasation and accumulation of nanoparticles in the interstitial space of the tumor[11]. Most recently, this dogma has been challenged by findings showing that the great majority of nanoparticles enter tumors using an active process through endothelial cells[12]. Liposomal formulations of chemotherapeutic drugs have demonstrated the safety and improved pharmacokinetic properties of the drug[13]. Prominent examples are liposomal doxorubicin (Doxil), daunorubicin (DaunoXome), and VCR (Marqibo) which have contributed to reducing side-effects compared to the free drug[14][15][16]. However, liposomal formulations have not, so far, been able to increase the therapeutic effect of the encapsulated drug.

1.3. Liposomal Drug Delivery - Active

One possibility to increase the therapeutic effect of the encapsulated drug is to modify the liposomal surface with tumor targeting-ligands, such as peptides[17], antibodies or antibody fragments[18], for active targeting to cancer cells. Single-domain antibodies (sdAb), first discovered in camelids[19], are the smallest possible antibody fragments (15 kDa) derived from heavy-chain antibodies. They are characterized by affinities comparable to conventional bivalent antibodies, as well as by high solubility, tissue penetration, and stability[20]. Previously, we developed the optimal formulation of liposomal VCR[21], and we investigated its pharmacokinetic and biodistribution in a mouse model engrafted with human RMS cells, revealing longer plasma circulation time and enhanced tumor accumulation of the liposomal drug compared to free VCR. Now, to further improve tumor accumulation of the liposomes in RMS by active targeting, we selected and investigated novel RMS-targeting sdAb.

1.4. Fibroblast Growth Factor Receptor 4 (FGFR4)

FGFR4 belongs to the family of receptor tyrosine kinases and is involved in myogenesis and muscle regeneration by promoting cell survival and differentiation[22][23]. FGFR4 is absent in normal differentiated muscles and is specifically overexpressed in RMS[24], as well as in other tumors, such as hepatocellular carcinomas, head and neck squamous cell carcinomas and basal-like breast cancer[25][26][27][28]. Therefore, FGFR4 represents a promising candidate for targeted therapies in RMS.

1.5. Chimeric Antigen Receptor (CAR) T cell

Another approach that can benefit from specific tumor targeting and that may improve the therapeutic outcome for RMS patients, is represented by chimeric antigen receptor (CAR) T cells. These cytolytic T cells are engineered with an extracellular antigen-binding domain recognizing specifically surface antigens on tumor cells. The intracellular part of the receptors is composed of T cell receptor signaling and costimulatory domains[29]. Tremendous clinical success has been achieved in the treatment of hematological malignancies with CAR T cells targeting CD19[30][31], CD22[32] and the B cell maturation antigen (BCMA)[33]. The application of CAR T cells for solid tumors has been more challenging, due to the lack of ideal tumor-specific target molecules, and also due to the strong immunosuppressive tumor microenvironment (TME) of solid tumors. Nevertheless, preclinical studies of CD276 (B7-H3) CAR T cells in pediatric solid tumors demonstrated good activity[34], and encouraging results have been reported for RMS CAR T cells targeting HER2 led to remission in a child with refractory metastatic RMS[35].

2. Strategies for RMS Therapy by Targeting FGFR4 with sdAb

Researchers selected four FGFR4-binding sdAb and tested them *in vitro* for (a) inhibitory activity of FGFR4 signaling; (b) active delivery as liposome conjugates, and (c) cell-mediated immunotherapy as CAR constructs. Surface plasmon resonance spectroscopy of sdAb binding to FGFR4 revealed strong affinities in the order of nano- to picomolar.

2.1. sdAb Inhibitory Activity on FGFR4 Signaling

The four selected sdAb A8, B1, B5 and F8 not only bind to FGFR4 expressed on RMS cells but are also able to block the FGF19-FGFR4-MAPK signaling axis. In ARMS, FGFR4 is a direct target gene of the fusion protein PAX3-FOXO1 [41], and in ERMS FGFR4 is frequently amplified with 12% of the tumors harboring activating mutations of the receptor[42][43][44]. In RMS, besides overexpression, FGFR4 has been shown to harbor activating mutations in over 6% of all tumors, resulting in constitutive tumor-promoting signaling within the cells[2][45][46]. Eventhough we did not observe a toxic effect on cultured RMS cells, it is tempting to speculate that FGFR4 signaling could still represent a therapeutic target for sdAb in RMS. Moreover, FGFR4 is not only implicated in RMS tumorigenesis, but drives tumor progression in FGF19 expressing hepatocellular carcinomas, head and neck squamous cell carcinomas, and basal-like breast cancer[25][26][27][28]. It is also estimated that 0.5% of all tumors display abnormalities in FGFR4[47]. The selected sdAb could therefore also serve as possible therapeutic approach for cancers other than RMS.

2.2. Active Delivery of Liposomes-sdAb Conjugates

Both free and liposome-conjugated sdAb bound specifically to Rh4-FR4wt cells and showed, except for uncoupled B5 sdAb, no binding to Rh4-FR4ko cells. Nevertheless, recombinant B5 binding to Rh4-FR4ko cells in FACS experiments was only 0.25 times higher than mCh control sdAb, whereas on Rh4-FR4wt it was 2 times higher. Affinity measurements revealed binding of only A8 and B5 another FGFR-member, FGFR2. The binding affinity of A8 to FGFR2 was in the micromolar range and therefore very low when compared to the binding affinity to FGFR4. The fast koff rates further highlighted its weak binding to FGFR2. In contrast, B5 showed high affinities to FGFR2 in the low nanomolar range, thus similar to its affinity for FGFR4. Rh4 cells do express FGFR2, but protein levels are lower compared to FGFR4. Moreover, the Rh4-FR4ko cells have reduced FGFR1 and FGFR2 protein levels compared to Rh4-FR4wt. The reasons for the lower expression level of FGFR1 and FGFR2 in Rh4-FR4ko are not completely clear but could be due to a clonal effect or to regulatory loops. Therefore, it is well possible that the binding of sdAb A8 and B5 to cell surface FGFR2 would be only detectable above a certain expression level.

The formulation of liposomal VCR was modified from the previously established one[21]by the introduction of DSPE-PEG-maleimide at 1 mol%. As expected, the resulting physico-chemical properties of the liposomes and the drug loading efficiency were comparable. SdAb coupling to the surface was performed as described by Oliveira and colleagues[48]with 0.4 nmol sdAb per µmol of total lipids and it resulted in high coupling efficiencies. Among various conditions of the

coupling reaction tested, we also tested higher sdAb-to-lipid ratios, but this resulted in precipitation of the liposomes. The fraction of uncoupled sdAb in the liposome suspension was negligible and it did not apparently interfere with binding on cells.

Confocal microscopy of Rh4-FR4wt cells incubated with the fluorescent FGFR4-targeting liposomes showed a very specific internalization after 2 h of incubation, represented by dot-like structures within the cells, which were absent in Rh4-FR4ko cells. This indicates a rather fast internalization process which can represent an advantage for a drug delivery platform to highly vascularized tumors.

2.3. Cell-Mediated Immunotherapy as CAR Constructs

Importantly, researchers were able to verify the selective cell-mediated cytotoxicity of sdAb-based FGFR4 CAR T cells towards Rh4-FR4wt. Although some differences in cytotoxic efficiencies between three CD8+ T cell donors were observed, all FGFR4-CAR Ts showed the same specific trend. Real-time cell analysis revealed no or lower effects of FGFR CAR Ts on Rh4-FR4ko, comparable to that of control CD19 CAR Ts.

The immune-based treatment of RMS with FGFR4 CAR Ts holds promising potential, since RMS tumors display aberrantly high FGFR4 expression compared to healthy tissues[42]. It has been shown that high antigen densities above a certain threshold level are required for effective CAR T cell activation, offering a therapeutic window for RMS treatment[49][50]. Further studies will be required to test FGFR4 CAR Ts efficiency in a RMS *in vivo* model.

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