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RESEARCH ARTICLE

Recombinant Antibody Fragments for Neurodegenerative Diseases

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Abstract: ***Background*:** Recombinant antibody fragments are promising alternatives to full-length immunoglobulins and offer important advantages compared with conventional monoclonal antibodies: extreme specificity, higher affinity, superior stability and solubility, reduced immunogenicity as well as easy and inexpensive large-scale production.

***Objective*:** In this article we will review and discuss recombinant antibodies that are being evaluated for neurodegenerative diseases in pre-clinical models and in clinical studies and will summarize new strategies that are being developed to optimize their stability, specificity and potency for advancing their use.

***Methods*:** Articles describing recombinant antibody fragments used for neurological diseases were selected (PubMed) and evaluated for their significance.

***Results*:** Different antibody formats such as single-chain fragment variable (scFv), single-domain antibody fragments (VHHs or sdAbs), bispecific antibodies (bsAbs), intrabodies and nanobodies, are currently being studied in pre-clinical models of cancer as well as infectious and autoimmune diseases and many of them are being tested as therapeutics in clinical trials. Immunotherapy approaches have shown therapeutic efficacy in several animal models of Alzheimer´s disease (AD), Parkinson disease (PD), dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), Huntington disease (HD), transmissible spongiform encephalopathies (TSEs) and multiple sclerosis (MS). It has been demonstrated that recombinant antibody fragments may neutralize toxic extra- and intracellular misfolded proteins involved in the pathogenesis of AD, PD, DLB, FTD, HD or TSEs and may target toxic immune cells participating in the pathogenesis of MS.

***Conclusion*:** Recombinant antibody fragments represent a promising tool for the development of antibody-based immunotherapeutics for neurodegenerative diseases.

Keywords: Recombinant antibody fragments, nanobody, intrabody, prion protein, alzheimer´s disease, parkinson disease, Huntington disease.

INTRODUCTION

Since the discovery of the therapeutic potential of the serum from animals exposed to attenuated forms of the pathogen more than a century ago, enormous progress in our understanding as well as in our ability to design protective antibodies (Abs) has been made. Many Abs are currently used for the treatment of different types of cancer, multiple sclerosis, rheumatoid arthritis or asthma [1-5]. Recombinant Ab fragments are promising alternatives to full-length immunoglobulins and offer important advantages compared with conventional monoclonal Abs: extreme specificity, higher affinity, superior stability and solubility, reduced immunogenicity as well as easy and inexpensive large-scale production [6-16]. Different antibody formats such as single-chain fragment variable (scFv), single-domain antibody fragments (VHHs or sdAbs), bispecific antibodies (bsAbs), intrabodies and nanobodies, are currently being studied in pre-clinical models of cancer as well as infectious and autoimmune diseases and many of them are being tested as therapeutics in clinical trials [4, 16-22].

Immunotherapy approaches, both active immunization and passive transfer of protective Abs, have shown therapeutic efficacy in several animal models of Alzheimer´s disease (AD), Parkinson disease (PD), dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), Huntington disease (HD), transmissible spongiform encephalopathies (TSEs) and multiple sclerosis (MS) [23-32]. Recombinant antibody fragments containing only variable regions or complementarity determining regions (CDRs) of the antibody heavy and/or light chains represent a promising tool for the development of antibody-based immunotherapeutics

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for neurodegenerative diseases. It has been demonstrated that these fragments may neutralize toxic extra- and intracellular misfolded proteins involved in the pathogenesis of AD, PD, DLB, FTD, HD or TSEs and may target toxic immune cells participating in the pathogenesis of MS [24, 25, 27, 33-38].

Below, we will review and discuss recombinant Abs that are being evaluated for neurodegenerative diseases in pre-clinical models and in clinical studies (Table **1**) and will summarize new strategies that are being developed to optimize their stability, specificity and potency for advancing their use.

Alzheimer´s Disease

The accumulation of extracellular and intracellular amyloid-beta (Aβ) peptide aggregates and neurofibrillary tangles, consisting of hyperphosphorylated microtubule-associated protein tau, in the human brain has been hypothesized to play a central role in the neuropathology of AD [39-41]. The first clinical trial of active immunization with Aβ peptide has been halted because of the development of symptoms of aseptic meningoencephalitis in 6% of immunized patients, and efforts towards the generation of antibody-based reagents for passive immunotherapy were multiplied [42-45]. However, it has been demonstrated that passive immunization using full-length immunoglobulin molecules led to adverse side effects such as vasogenic edema, microhemorrhages and meningoencephalitis in APP Tg mice [46-48]. Therefore, efforts have been made to generate recombinant antibody fragments for targeting Aβ and tau aggregates without serious adverse events.

First anti-Aβ scFv antibody, designated 508F(Fv), was constructed based on variable regions of heavy and light chain genes of a protective monoclonal IgM 508 antibody. This recombinant antibody retained the specificity and protective properties of the parental antibody and, in addition, showed an increased stability and higher affinity [49]. Moreover, authors demonstrated that scFvs, displayed on the surface of filamentous phage, enter the CNS [50].

Table 1. Summary of preclinical studies using recombinant antibody fragments in animal models of neurodegenerative disorders.

Recombinant Ab Fragment	Target/Disease	Key Findings	Refs.
Phage-displayed scFv-508F(Fv)	Aβ, Alzheimer´s disease	Enters the CNS	[50]
Phage-displayed VHH-C1.27	Aβ, Alzheimer´s disease	Efficiently clears amyloid deposits in Tg2576 mice	[53]
Purified or rAAV-expressed scFv59	Aβ, Alzheimer´s disease	Reduces amyloid deposits in Tg2576 and APPswe/PS1dE9 mice	[56, 62, 67]
rAVV-expressed scFvs	Aβ, Alzheimer´s disease	Attenuates amyloid pathology and cognitive impairment in CRND8 and APPswe/PS1dE9 mice	[61, 66]
rAVV-expressed scFvs	Aβ, Alzheimer´s disease	Decreases amyloid and tau pathology and improves cognitive function in 3xTg-AD mice	[63, 64]
Purified scFv-h3D6	Aβ, Alzheimer´s disease	Attenuates amyloid pathology and cognitive impairment and protects DCN neurons from death in 3xTg-AD mice	[75, 76]
VH with grafted Aβ18-21 or 34-42 peptide in CDR3 (gammabody)	Aβ, Obesity, Alzheimer´s disease	Reverses cognitive deficits in an animal model of obesity	[84]
VL1-VL2 catabody	Aβ, Alzheimer´s disease	Reduces Aβ burden in 5xFAD mice	[91]
Purified scFv fused to LDL receptor-binding domain of ApoB	Oligomeric α-synuclein, Parkinson disease	Reduces the accumulation of α-synuclein in neurons and ameliorates behavioral deficits in mice	[113]
rAAV-expressed scFv-C4 intrabody	Mutant htt, Huntington disease	Delays the accumulation of mutant htt in B6.HDR6/1 Tg mice	[120]
rAAV-expressed scFv-EM-48	Mutant htt, Huntington disease	Suppresses mutant htt accumulation and ameliorates neuronal dysfunction in R6/2 and N171-82Q Tg mice	[126]
rAAV-expressed Happ1 intrabody	Proline-rich domain of htt, Huntington disease	Ameliorates the neuropathology and cognitive deficits and prolongs the lifespan in four HD Tg mouse strains	[125]
rAVV-expressed scFvs	PrP^{Sc}, Prion diseases	Decreases PrP^{Sc} burden in the CNS and improves clinical signs in scrapie-infected mice	[141, 142]
RA2 micloglial cell line expressing scFv-3S9	PrP, Prion diseases	Prolongs survival time of scrapie-infected mice	[143]
Purified or rAVV-expressed scFv S18 and scFv N3	LRP/LP, Prion diseases	Reduces peripheral PrP^{Sc} propagation but does not prolong survival time of scrapie-infected mice	[144, 145]

Another recombinant Fab antibody, developed by cloning the heavy and light chain variable domains of the parent monoclonal antibody, was shown to share similar properties with the parental monoclonal antibody and to bind plaques in AD brain samples [51]. We have constructed the first immune scFv and VHH antibody libraries displayed on M13 phage using spleen cells from mice immunized with human Aβ and selected Aβ-specific antibody fragments with protective potential binding to different regions of the peptide [52, 53].

Subsequently, a naïve human scFv library was used to select Aβ-specific antibody fragments, and it has been demonstrated that these fragments were capable of inhibiting Aβ aggregation and preventing Aβ-induced toxicity both *in vitro and in vivo* [53-59]. Furthermore, we have demonstrated that a synthetic peptide, based on the CDR3 sequence of the variable region of the heavy chain of the clone with the highest specificity, inhibited Aβ-induced toxicity in primary rat hippocampal neurons [55].

An interesting novel gene-based passive vaccination strategy for delivery of anti-Aβ scFvs was proposed almost simultaneously by two groups [60, 61]. An adeno-associated virus (AAV) constructs encoding various scFvs were injected into the brain of APP Tg mice and were shown to induce intraneuronal expression of corresponding scFvs without causing neurotoxicity [60, 61]. Importantly, a decreased Aβ deposition in the brain was observed although cerebral hemorrhages were also found in the latter study [60-62]. Moreover, intracerebral administration of Aβ-specific scFv with an endoplasmic reticulum (ER)-targeting signal sequence, expressed in AAV vector, in 3xTg-AD mice resulted in reduced Aβ accumulation in neurons, and, interestingly, in reduced tau pathology as well [63]. Another study in 3xTg-AD mice showed reduced Aβ and tau accumulation and improved cognitive performance after intrahippocampal administration of a human anti-Aβ scFv expressed in AAV vector [64]. These observations together with other studies further suggest that inhibition of intra-neuronal accumulation of Aβ may lead to reduced tau pathology [see a recent review 65]. Interestingly, AAV constructs coding for anti-Aβ scFvs had a protective effect in APP Tg mice after intra-muscle injection, probably by increasing Aβ efflux from the brain due to peripheral clearance of the peptide [66, 67]. Although rAAV-based gene therapy has been approved recently for clinical use, a single study, that demonstrated hepatic genotoxicity, raised concerns over the clinical use of AAV vectors [68-70]. Careful design of safer AAV vectors and development of novel methods of recombinant antibody delivery merit further research [69]. Thus, naked DNA plasmids encoding protective Abs may have advantages because they do not represent a risk of genome integration observed with AAV [71-73].

Another efficient strategy for delivery of recombinant immunoglobulin fragments to the brain was described by Poduslo and collaborators [74]. Authors demonstrated that a polyamine-modified F(ab´)2 fragment of a monoclonal anti-fibrillar Aβ antibody has increased blood-brain barrier (BBB) permeability after intravenous administration [74]. Such modifications of recombinant antibody fragments may have potential diagnostic as well as immunotherapeutic applications.

Intraperitoneal administration of an anti-Aβ scFv-h3D6 has been shown to reduce amyloid deposits in the cortex and olfactory bulb but not in the hippocampus and to ameliorate learning and memory deficits in 3xTg-AD mice [75]. In addition, a single low dose of scFv-h3D6 protected deep cerebellar nuclei (DCN) neurons from death after intra-peritoneal administration in 3xTg-AD mice [76]. Chronic intranasal treatment with a scFv targeting C-terminus of Aβ ameliorated amyloid pathology in APPswe/PS1dE9 mice [77]. These studies give us hope that recombinant antibody fragments may be applied as therapeutics for AD by different routes, however, improved delivery strategies still need to be explored for future use in humans.

Numerous scFvs specifically targeting morphologically different Aβ aggregates were developed. Thus, a scFv A4, isolated from a human library using phage display technology and atomic force microscopy (AFM), was shown to bind to oligomeric Aβ but not to monomeric or fibrillar forms and to inhibit Aβ toxicity *in vitro* [78]. Such Abs represent useful tools for identification and targeting of pathological Aβ oligomers in human brain [79]. Another anti-oligomeric scFv antibody specifically binding to a smaller earlier stage oligomeric form of Aβ was isolated and shown to stabilize non-toxic low-n Aβ forms [80]. Finally, anti-oligomeric Aβ scFv 11A5 decreased cerebral amyloid burden and improved behavioral performance in the Morris water maze in APP/PS1 mice after intracerebroventricular injection [81].

An interesting Ab format consisting of a single VH domain with grafted Aβ hydrophobic region (residues 18-21 or residues 34-42) in CDR3 and referred to as grafted amyloid-motif antibody (gammabody) was described [82-84]. Authors demonstrated that Aβ18-21 bearing gammabodies bind selectively to Aβ fibrils while Aβ34-42 gammabodies bind to oligomeric and fibrillary forms [82]. Importantly, these studies showed that oligomer-binding gammabodies inhibit Aβ toxicity *in vitro*, block oligomeric hippocampal Aβ and attenuate or reverse cognitive impairment in an animal model of diet-induced obesity [83, 84].

An alternative approach for reducing amyloid accumulation in the brain is to promote Aβ proteolysis, and scFvs with increased catalytic activity towards Aβ were obtained after affinity maturation of a corresponding parental antibody fragment [85]. A proteolytic scFv, Asec-1A, prevented Aβ aggregation and reduced Aβ-induced cytotoxicity in human neuroblastoma cells [86]. It has been demonstrated that the levels of two Aβ-degrading proteases, insulin-degrading enzyme (IDE) and neprilysin (NEP), are reduced in the hippocampus of APP Tg mice and humans as function of age, and this may explain increased Aβ accumulation [87]. Various IDE- and NEP-based therapeutic strategies, aimed to up-regulate their expression and/or restore their activity to normal levels were proposed [reviewed in 88]; however, these enzymes participate in other biological processes and caution should be taken. Thus, specifically targeting Aβ proteolysis by catalytic scFvs represents a promising approach for reducing amyloid deposits in the brain without potential adverse effects. On the other hand,

one may reduce amyloid load by selectively inhibiting amyloidogenic processing of APP and thus reducing Aβ production. The iBSEC1 scFv isolated from human scFv yeast display library was shown to reduce both intracellular and extracellular Aβ levels by around 50% in Chinese hamster ovary (CHO) cells overexpressing APP [89]. Furthermore, authors showed that a bispecific tandem scFv, combining iBSEC1 with the Asec-1A, simultaneously inhibits amyloidogenic processing of APP and increases Aβ proteolysis [90]. Recently, another catalytic antibody fragment was described by Planque and collaborators [91]. Authors demonstrated that the catalytic IgV construct 2E6 composed of VL1 and VL2 domains reduces brain Aβ deposits in 5xFAD mice after intravenous injection [91].

Although much of the research on AD immunotherapy has been focused on Aβ, recent studies are developing both active and passive immunization strategies targeting hyperphosphorylated, aggregated and insoluble toxic forms of tau as well [reviewed in 65, 92, 93]. Similar approaches have been applied to isolate scFvs that selectively bind toxic tau aggregates [94]. These anti-tau scFvs detected oligomeric tau at earlier stages when neurofibrillary tangles are not observed yet [94]. Potential biomarkers based on such recombinant antibody fragments may be developed for early detection of AD. Thus, a peripheral injection of phosphor-tau-specific scFv resulted in a strong brain signal in Tg mice but not in wild type animals [95]. However, there is an urgent need for expansion of research on therapeutics targeting different forms of phosphorylated and/or truncated tau aggregates involved in AD pathology [93, 96, 97].

Parkinson's Disease

The accumulation of pathological aggregates of α-synuclein in the Lewy bodies and Lewy neurites with the subsequent progressive loss of dopaminergic neurons in the brain is linked to the pathology of PD [reviewed extensively elsewhere 98-100]. Immunotherapy approaches inhibiting α-synuclein aggregation and preventing their toxic effects on cells were broadly studied, and a number of protective specific anti-C- and anti-N-terminus Abs were discovered and tested in animal models [reviewed in 28, 29, 101]. Importantly, α-synuclein, a cytosolic neuronal protein, participates in the regulation of synaptic vesicle trafficking, fusion and neurotransmitter release [see a recent review 98]. However, at high concentrations and in aggregated form α-synuclein acquires toxic properties leading to a number of pathological pathways and neurodegeneration, hence specific immunotherapeutics targeting toxic forms without interfering with physiological function of the protein should be developed [98]. All above discussed properties of recombinant antibody fragments and the possibility of intracellular expression make them suitable candidates for future therapies for PD.

Using phage display technology alone or in combination with atomic force microscopy, various human scFvs specifically binding to morphologically distinct oligomeric and/or fibrillary forms of α-synuclein were described [102-109]. Interestingly, while some of these scFvs were binding only to early oligomeric forms of α-synuclein, others recognized larger later stage oligomers, however, all of them inhibited *in vitro* aggregation and toxicity of α-synuclein [105, 108]. In addition, selected scFvs specifically recognized naturally occurring aggregates in PD brain [108]. Importantly, one of these anti-oligomeric α-synuclein scFv antibodies also blocked the formation of fibrillary huntingtin (htt) aggregates involved in HD, but stabilized cytotoxic oligomeric forms [107]. Moreover, another scFv selected against the fibrillar α-synuclein and targeting misfolded htt, also increased htt aggregation and cytotoxicity [109]. Therefore, caution should be taken when proposing immunotherapy strategies for targeting different misfolded proteins using the same immunogen or antibody/antibody fragment.

It is worth mentioning that one of the possible drawbacks of intrabodies may be their limited cytoplasmic solubility, and an interesting approach for increasing the solubility and for simultaneously enhancing the degradation of α-synuclein was proposed recently [110]. Authors showed that fusion of a proteasome-targeting PEST motif to a set of four diverse, poorly soluble anti-α-synuclein intrabodies increases their solubility and significantly enhances degradation of the target protein [104, 110].

Some common mutations in the gene coding for leucine-rich repeat kinase 2 (LRRK2) have been linked to early-onset familial and late-onset sporadic PD, and a number of small-molecule kinase inhibitors with improved specificity, pharmacokinetics and brain penetration were tested in pre-clinical models [111, 112]. Similar to anti-α-synuclein intrabodies, LRRK2-specific recombinant antibody fragments may have potential therapeutic value.

Despite numerous promising pre-clinical and clinical studies on passive immunotherapy using anti-α-synuclein full length monoclonal and polyclonal antibodies [reviewed in 101], there are few reports on *in vivo* evaluation of α-synuclein-specific recombinant antibody fragments. In an interesting study by Spencer *et al*., anti-oligomeric α-synuclein scFv, fused to the low-density lipoprotein (LDL) receptor-binding domain of apolipoprotein B (ApoB), showed enhanced brain penetration, reduced the accumulation of pathogenic α-synuclein accumulation in neurons and ameliorated behavioral deficits in a mouse model of PD/DLB [113]. We think that many of scFvs mentioned above and shown to target α-synuclein and/or LRRK2 warrant further *in vivo* evaluation and may represent a promising therapeutic approach for PD. Importantly, recombinant antibody fragments targeting α-synuclein may also be applied for the treatment of other synucleinopathies, such as multiple system atrophy (MSA) and DLB.

Huntington´s Disease (HD)

Misfolded and aggregated N-terminal fragments of mutant huntingtin (mhtt) accumulate in the neuronal nuclei and processes and lead to neurodegeneration, although the exact pathways involved in this process remain unclear [reviewed in 114]. Intrabody-mediated modulation of toxic htt aggregates represent an alternative therapeutic approach for HD [24, 35, 115]. Thus, it has been shown that the anti-N-terminal htt (residues 1-17) C4 scFv intrabody inhibits aggregate formation in cell cultures and prevents toxicity in

an organotypic slice culture model of HD [116-118]. Subsequently, it has been demonstrated in a *Drosophila* model of HD that C4 intrabody slows the progression of neurodegeneration and formation of htt aggregates, increases survival to adulthood and significantly prolongs adult lifespan [119]. In addition, C4 intrabody reduced pathological features in B6.HDR6/1 transgenic mice after intracranial delivery using adeno-associated viral vectors [120]. To enhance efficacy, C4 intrabody was fused to a proteasome-targeting PEST motif and promising results were obtained in cell cultures [121]. Finally, the crystal structure of the complex of C4 intrabody and htt1-17 was determined and provided important insights into the mechanism of pathogenic htt aggregates formation [122].

Importantly, anti-htt antibodies or their fragments of various specificities showed different effect on mutant htt aggregation and toxicity: intrabodies binding to the proline-rich domains of htt were protective while antibody fragments recognizing the polyglutamine (polyQ) sequence stimulated htt aggregation and apoptosis [123, 124]. Subsequently, it has been demonstrated that VL12.3 intrabody, recognizing the N terminus of htt, has no beneficial effect in YAC128 HD Tg mice after intracranial injection and, moreover, increases mortality in R6/2 HD Tg mice [125]. On the other hand, Happ1 intrabody recognizing the proline-rich domain of htt strongly ameliorated the neuropathology and cognitive deficits and significantly prolonged the lifespan in multiple HD Tg mouse strains [125]. Finally, Wang and collaborators engineered an intrabody (scFv-EM48) that binds to a unique epitope in mutant htt and selectively reduces its toxic effect without interfering with normal htt functions [126]. In addition, authors showed that scFv-EM48 suppresses mutant htt accumulation in the neuronal processes and ameliorates neuronal dysfunction in a mouse model of HD [126].

Interestingly, VL single-domain antibodies more effectively blocked aggregation and toxicity of htt in a cellular model of HD compared with a parental scFv, and their affinity and function were further improved by deletion of a disulfide bond [127, 128].

To the best of our knowledge, none of these antibody fragments are being tested in clinical trials yet.

Prion Diseases

Prion diseases are lethal neurological disorders caused by the pathological scrapie-associated form (PrP^{Sc}) of the normal cellular prion protein (PrP^{c}) [129]. Previous *in vitro* and *in vivo* studies suggested that immunotherapy may represent a realistic strategy against prion diseases [130-133]. Although several groups demonstrated that anti-prion nanobodies and intrabodies (scFvs, VHH or their CDRs) prevent PrP^{C} conversion to its toxic PrP^{Sc} form in cell cultures [134-140], there are very few studies in animal models. Thus, it has been demonstrated that rAAV vector-expressed anti-PrP^{Sc} scFvs significantly extended incubation periods, decreased CNS PrP^{Sc} burden and improved clinical signs and rotarod performance in scrapie-infected mice without inflammatory or neurotoxic effects [141, 142]. An interesting approach has been reported recently by Fujita and collaborators [143]. Authors established a Ra2 microglial cell line expressing anti-PrP 3S9 scFv antibody and demonstrated that intracerebral injection of these cells before or at an early time point after scrapie infection significantly prolongs survival times of mice [143].

An alternative promising target for the prevention/ treatment of prion diseases is the non-integrin 37kDa/67kDa laminin receptor (LRP/LR), a cell surface receptor involved in PrP^{Sc} propagation in scrapie infected cells [144, 145]. It has been demonstrated that a scFv antibody (S18) directed against LRP/LR reduced by 40% PrP^{Sc} levels in the spleen of scrapie infected C57BL/6J mice after intraperitoneal administration [144]. However, authors didn't observe a significant prolongation of the incubation and survival times in S18 treated mice, and proposed that higher amounts of scFv for a longer period of time might be required to achieve a regression of the disease [144]. Subsequently, authors used *in vivo* gene delivery system based on rAAV vectors expressing anti-LRP/LR scFvs, but did not observe protective effect against disease progression after intracerebral administration of antibody fragments despite the reduction of peripheral PrP^{Sc} propagation [145].

CONCLUSION

Therapeutic antibodies represent one of the fastest growing segments in the pharmaceutical industry. The development of new tools for a successful delivery of Ab fragments into the brain and the design of fusion peptides/ proteins for targeting them to the cells/cell compartments of interest might enhance immunotherapy efficacy [146, 147]. Also, novel methods for optimizing the properties of Ab fragments such as affinity, stability and solubility merit further research [110, 148]. Finally, application of elegant tools such as the use of cellular implants for controlled and continuous delivery might be of interest [149, 150]. All above mentioned approaches may hopefully someday prevent or slow the progression of neurodegenerative diseases with known shared pathology-extra-and intracellular accumulation of misfolded proteins.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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