Delivery of single-chain antibodies (scFvs) directed against the 37/67 kDa laminin receptor into mice via recombinant adeno-associated viral vectors for prion disease gene therapy

Chantal Zuber, Gerda Mitteregger, Natascha Schuhmann, Clémence Rey, Stefan Knackmuss, Wolfgang Rupprecht, Uwe Reusch, Claudia Pace, Melvyn Little, Hans A. Kretzschmar, Michael Hallek, Hildegard Bünig and Stefan Weiss

Correspondence
Stefan Weiss
weiss@mb.uni-muenchen.de

1Laboratorium für Molekulaire Biologie - Genzentrum - Institut für Biochemie der LMU München, Feodor-Lynen-Str. 25, D-81377 München, Germany
2Zentrum für Neuropathologie und Prionforschung der LMU München, Feodor-Lynen-Str. 23, 81377 München, Germany
3Universität zu Köln, Klinik I für Innere Medizin, Kerpener Str. 62, 50937 Köln, Germany
4Affimed Therapeutics AG, Technologiepark, Im Neuenheimer Feld 582, 69120 Heidelberg, Germany
5Zentrum für Molekulare Medizin Köln, Universität zu Köln, Joseph-Stelzmann-Str. 52, 50931 Köln, Germany

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The 37/67 kDa laminin receptor (LRP/LR) acts as a receptor for prions providing a promising target for the treatment of prion diseases. Recently, we selected anti-LRP/LR single-chain antibodies (scFvs) and proved a reduction of the peripheral PrPSc propagation by passive immunotransfer into scrapie-infected mice. Here, we report the development of an in vivo gene delivery system based on adeno-associated virus (AAV) vectors expressing scFv-S18 and -N3 directed against LRP/LR. Transduction of neuronal and non-neuronal cells with recombinant (r)AAV serotype 2 vectors encoding scFv-S18, -N3 and -C9 verified the efficient secretion of the antibodies. These vectors were administered via stereotactic intracerebral microinjection into the hippocampus of C57BL/6 mice, followed by intracerebral inoculation with 10% RML at the same site 2 weeks post-injection of rAAV. After 90 days post-infection, scFv-S18 and -N3 expression resulted in the reduction of peripheral PrPSc propagation by approximately 60 and 32%, respectively, without a significant prolongation of incubation times and survival. Proof of rAAV vector DNA in spleen samples by real-time PCR strongly suggests a transport or trafficking of rAAV from the brain to the spleen, resulting in rAAV-mediated expression of scFv followed by reduced PrPSc levels in the spleen most likely due to the blockage of the prion receptor LRP/LR by scFv.

Prion diseases are fatal lethal neurodegenerative diseases affecting humans and animals (for review see Weissmann, 2004; Zuber et al., 2007a). None of the affected individuals can be treated or cured effectively (Ludewigs et al., 2007; Vana et al., 2007; Weissmann & Aguzzi, 2005). The abnormal form of the prion protein, PrPSc, is frequently associated with infectivity and propagates mainly in the brain and the lymphoreticular system (LRS). Accumulation of the aggregated PrPSc leads to neuronal death. PrPSc is distinct from the host protein PrPc by its biochemical properties such as proteinase K sensitivity and insolubility, but harbours the same amino acid sequence. The generation of PrPSc from PrPc involves conformational changes accompanied by modifications in the secondary structure of the protein (for review see Aguzzi & Weissmann, 1998; Prusiner, 1998; Weissmann, 2004).

The 37/67 kDa laminin receptor (LRP/LR) is a multifunctional protein (i) playing an important role in cell adhesion, movement and growth of many cell types, (ii) acting as a receptor for some subtypes of adeno-associated
virus (AAV), alphaviruses and dengue virus, and (iii) playing an important role in cancer progression and metastasis (for review see Gauczynski et al., 2001a; Nelson et al., 2008; Rieger et al., 1999). We recently showed that blockage or downregulation of LRP/LR in neoplastic cells prevents invasion of these cells, suggesting that LRP/LR plays a major role in cancer metastasis (Zuber et al., 2008b) and (iv) finally, LRP/LR represents a key player in prion infection (Ludewigs et al., 2007; Vana et al., 2007; Zuber et al., 2007a). LRP has been shown to act both as the PrPc receptor (Gauczynski et al., 2001b) and PrPsc receptor (Gauczynski et al., 2006) and is responsible for bovine PrPsc internalization by human enterocytes (Morel et al., 2005). The fact that LRP levels are increased in organs of the LRS and central nervous system (CNS), such as spleen and brain, of infected animals (Rieger et al., 1997) strongly suggests that this receptor is not only essential for prion uptake after oral infection but also plays an important role for PrPsc propagation and prion pathogenesis in the peripheral nervous system, including the LRS and CNS. Additionally, several laminin receptor isoforms have been found in mouse brain all binding to PrP (Simoneau et al., 2003). LRP/LR, plays a key role as a cell surface receptor for prions, was also recently found to interact with PrP in the perinuclear compartment and in part with a mutated PrP lacking the signal sequence in the nucleus (Nikles et al., 2008). LRP/LR attracts more and more attention as a target for therapy in prion diseases and cancer. Multiple strategies on LRP inactivation have been shown to be successful by inhibiting PrPsc propagation in vitro: (i) downregulation of LRP via antisense or siRNA strategies completely blocks PrPsc propagation (Leucht et al., 2003) and delays the incubation time in scrapie-infected mice (H. Ludewigs and others, unpublished data), (ii) a trans dominant-negative LRP mutant interferes with PrPsc propagation in ScN2a cells (Vana & Weiss, 2006), (iii) polysulfated glycans block the PrPsc-LRP/LR interaction and strongly reduce PrPsc binding (Gauczynski et al., 2006) and (iv) the anti-LRP antibody, W3, abrogates PrPsc accumulation in scrapie-infected cells (Leucht et al., 2003) and prevents binding and internalization of PrPsc prions (Morel et al., 2005). W3 reduces peripheral PrPsc propagation significantly by 66 % and prolongs the survival in scrapie-infected mice by 1.8-fold (Zuber et al., 2007b). Many of these anti-LRP/LR tools particularly antibodies, siRNAs and polysulfated glycans interfere with the laminin–LRP/LR interaction, which results in a reduced invasive potential of neoplastic cells, recommending these tools as powerful therapeutic agents in the treatment of cancer, especially metastasis formation (Zuber et al., 2008b).

Monoclonal antibodies are attractive therapeutic agents and at least 21 of them obtained FDA approval for therapeutic use in patients (Reichert et al., 2005; Waldmann, 2003). Nevertheless immunotherapy is limited by the immunogenicity of murine-derived antibodies and the restricted tissue penetration. Single-chain antibodies (scFv) have been developed as an alternative system to circumvent such problems. In contrast to entire immunoglobulins, scFv are much smaller in size, which allows them to penetrate into tissues and lacking the Fc part they do not provoke an immune response (for review see Sanz et al., 2005). We recently described the selection of anti-LRP scFvs termed S18 and N3 from a human antibody library by phage-display (Zuber et al., 2008a). Employing a passive immunotransfer approach, scFv-S18 reduced PrPsc deposition in the spleen of infected mice by approximately 40 % (Zuber et al., 2008a). However, intraperitoneal injection of the antibody did not significantly prolong the incubation times and survival (Zuber et al., 2008a), most likely due to the short half-life of scFvs in the blood (approx. 12 h or less) and probably due to insufficient amounts that had been administered (1 mg per week). In addition, due to the low stability, scFvs might have failed to cross the blood–brain barrier and therefore have failed to reach the brain where most of the prion agent propagates. To circumvent these limitations, we exploited a gene therapeutic approach based on the recombinant (r)AAV vector system. Due to its non-inflammatory and non-pathogenic nature, we chose the AAV system for in vivo delivery of scFvs-S18 and -N3 to achieve a permanent expression of the antibodies from neuronal cells. Up to now 12 serotypes have been identified named AAV type 1–12 (for review see Wu et al., 2006; Schmidt et al., 2006). AAV serotype 2 is the best characterized one and is conventionally utilized as a gene therapy vector. This serotype offers a series of advantages including, e.g. transduction of a wide variety of cell types and low immunogenicity after in vivo application (Tal, 2000). Furthermore, the vector genome persists for extended periods of time enabling long-term transgene expression.

AAV is receiving increasing attention as a promising candidate for gene therapy and at least 13 gene therapeutic approaches are currently under investigation in clinical trials worldwide (see http://www.clinicaltrials.gov). Applications of AAV to treat Parkinson’s disease are actively studied in experimental models (Hayashita-Kinoh et al., 2006; Luo et al., 2002). AAV was efficiently used to target the Prn-P gene (Hirata et al., 2004) and PrP was overexpressed by adenovirus-mediated gene targeting (Shyu et al., 2005).

rAAV2-mediated delivery of PrPsc-specific scFvs targeting the prion protein delayed the onset of prion pathogenesis in mice (Wuertzer et al., 2008). Here, we provide the proof of principle for a successful AAV-mediated gene therapy targeting the prion receptor LRP/LR by anti-LRP/LR scFvs. To examine which AAV serotype is suitable for the transduction of neuronal cells, we determined transduction efficiencies for serotypes 2, 3 and 5 based vectors in two neuronal cell lines, N2a and GT1, using the green fluorescent protein as the marker gene. The highest transduction efficiency was achieved by AAV-2 (data not shown). Consequently, we constructed rAAV-2 vectors encoding anti-LRP scFv-S18, -N3 and -C9, respectively.
The cDNAs encoding anti-LRP scFv-N3 and -S18 and the anti-preS1 (coat protein of the hepatitis B virus) scFv-C9 were subcloned from the expression vector pSKK2-N3, -S18 or -C9 (Le Gall et al., 2004) into the mammalian expression vector pSecTag2B (Invitrogen) to attach the Igκ leader sequence (Coloma et al., 1992) for antibody secretion, a carboxy-terminal myc tag for immunodetection, a polyhistidine tag and a CMV promoter, resulting in the vectors pSecTag2B-N3, -S18 and -C9, respectively. The cDNA sequences were then cloned into the XbaI restriction site of the AAV vector plasmid pSub/CEP4 (Wendtner et al., 2002), resulting in the vector plasmids pSub/CEP4-N3, -S18 or -C9, respectively. Transfection of N2a cells with these vector plasmids confirmed that all recombinant scFvs-N3, -S18 and -C9 were expressed and secreted into the medium (Fig. 1a). Detection of scFvs was achieved by using a murine anti-c-myc tag antibody. rAAV-2 vectors from HeLa cells. Supernatants were analysed by immunoblotting 72 h post-transduction. Detection was performed with an anti-c-myc antibody.

Fig. 1. (a) Expression and secretion of scFvs-N3, -S18 and -C9 in N2a cells after transfection with AAV vector plasmids pSub/CEP4-N3, -S18 or -C9, respectively. Mock, transfection with the pSub/CEP4 vector plasmid only. After transfection (48 h), medium and N2a cell lysate were immunoblotted and analysed with an anti-c-myc antibody (Santa Cruz Biotechnology) for the presence of scFvs. (b) Transduction of N2a and GT1 cells with rAAV-S18 results in the secretion of scFv-S18. Depicted are supernatants collected 3 and 6 days post-transduction, respectively. (c) Secretion of scFv-S18, -N3 and -C9 after rAAV-N3, -S18 and -C9, respectively, transduction with the corresponding rAAV vectors from HeLa cells. Supernatants were analysed by immunoblotting 72 h post-transduction. Detection was performed with an anti-c-myc antibody.

To investigate the therapeutic feasibility, scrapie-infected mice were microinjected with rAAV-N3, -S18 and -C9, respectively. PrPSc accumulates mainly in the CNS and particularly high amounts have been detected in the hippocampus. For that reason, we decided to target this region of the brain by stereotactic microinjection. 5 x 10⁴ genomic particles (volume 5 μl) were injected intracerebrally (i.c.) into each mouse and the presence of the secreted scFv-N3 was confirmed 30 days post-injection by immobilized metal ion affinity chromatography (IMAC) purification (Fig. 2a). Briefly, homogenates were diluted in 6 M guanidium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄ pH 8, sonicated and incubated with 100 μl Ni²⁺ beads (Probond resin; Invitrogen) in the presence of 10 mM imidazole. After extensive washing, beads were eluted in SDS sample buffer and analysed by Western blotting.

A volume of 10 % RML scrapie homogenate was administered into the same area of the brain 2 weeks after rAAV microinjection to allow mice to recover from the application procedure. Neither the intracerebral microinjection of the rAAV nor the viral particles themselves had any obvious effect on the behaviour of the mice. To examine the peripheral PrPSc propagation, spleen samples were analysed 90 days post-infection (p.i.) by Western blotting. Spleens were homogenized and 200 μg total protein was digested with proteinase K (final concentration of 20 μg ml⁻¹) for 30 min at 37 °C. We observed a reduced PrPSc content in mice treated with rAAV-N3 by approximately 32 % compared with the control group (rAAV-C9). Furthermore, rAAV-S18-injected mice displayed a significant PrPSc reduction by approximately 60 % (Fig. 2b, c), suggesting that both antibodies hampered peripheral PrPSc propagation. In addition, the presence of the scFv-S18 and -C9 encoding DNA sequences in the spleen were verified by real-time PCR (Fig. 2d). The reduced PrPSc level might therefore be due to the presence of the scFvs encoded by the rAAV-2s, which had trafficked or had been transported from the brain to the spleen. Since heparan sulfate proteoglycans (HSPGs) have been reported to act as initial attachment receptors for AAV-2
(Summerford & Samulski, 1998) concomitant with the fact that the spleen exhibits high HSPG levels (Murdoch et al., 1994; Wrenshall & Platt, 1999), we speculate that the administered rAAVs might have crossed the blood–brain barrier and targeted the spleen followed by transgene expression, resulting in the hampering effect on peripheral PrP$_{Sc}$ propagation by the expressed scFvs. In addition to the primary receptor HSPG, the administered AAV-2 might have also used the LRP/LR as a receptor, since it has been reported that LRP/LR can act as a receptor for AAV serotype 2 (Akache et al., 2006).

Although we observed a significant reduction in the PrP$_{Sc}$ level in the spleen of mice after i.c. RML inoculation post-microinjection with rAAV-S18, incubation times and survival were not significantly affected (Table 1). This correlates with an earlier study describing an unaltered incubation period in splenectomized hamsters intracerebrally infected with ‘Chandler’ scrapie strain (Kimberlin & Walker, 1977). These hamsters lacking spleens displayed a prolongation in the incubation time only if they were infected intraperitoneally. We conclude therefore, that a reduction in the peripheral PrP$_{Sc}$ propagation observed in...
the spleen does not necessarily implicate a prolonged survival or incubation time after intracerebral inoculation with RML prions.

Furthermore, the rAAVs might not have reached all the relevant brain cells supporting prion propagation. AAV-2 infects a restricted region near the injection site of the brain, which is believed to result from rapid HSPG-mediated uptake of AAV-2 particles by neurons (Bartlett et al., 1998; Wang et al., 2003). Multiple injection approaches into both hippocampal hemispheres might increase the expression of the scFvs. It is also possible to combine intracerebral treatment with systemic delivery to increase the expression of the scFvs. It is also possible to employ rAAVs encoding scFv sequences targeting LRP/LR. Single microinjections of rAAV carrying scFv sequences directed against LRP/LR into the brain resulted in the expression of anti-PrP scFvs, mice were infected intraperitoneally. We might therefore have observed an effect on the incubation time or survival if we had used this route of infection.

The fact that we observed a reduction in PrPSc levels in the spleen without a prolongation of incubation times or survival, provides further evidence for the assumption that PrPSc levels in spleen and brain do not correlate with infectivity. Several reports discuss the absent link between infectivity or disease progression and high titres of proteinase K-resistant PrPSc (Lasmezas et al., 1997). Manson and colleagues demonstrate in a 101TG mouse model that infectivity is not automatically linked with the presence of PrPPrPRes (Manson et al., 1999). Although no or low levels of the disease-associated PrP were found, Gerstmann-Straussler-Scheinker syndrome infection was followed by the development of clinical transmissible spongiform encephalopathy (TSE) signs. Moreover tissues containing little or no proteinase K-resistant PrP can be infectious with high titres of TSE infectivity (Barron et al., 2007).

Taken together, the load of PrPSc does not automatically predict disease progression. To examine whether also the infectivity is reduced concomitant with the observed reduction in PrPSc levels in the spleen of rAAV-treated mice expressing scFvs directed against LRP/LR, a potential infectivity titre of the spleen has to be determined by employing bioassays.

We describe here a promising gene therapeutic approach employing rAAVs encoding scFvs targeting LRP/LR. Single microinjections of rAAV carrying scFv sequences directed against LRP/LR into the brain resulted in the expression of the therapeutic antibody followed by a significant reduction by approximately 60% of the PrPSc level in the spleen of rAAV-S18-treated mice. This result is in line with our previously reported studies: passive immunotransfer of the polyclonal anti-LRP/LR antibody W3 (Zuber et al., 2007b) and the scFv-S18 (Zuber et al., 2008a) both resulted in a reduction of the PrPSc content in the spleen, indicating that anti-LRP/LR antibodies reduce peripheral PrPSc propagation. Despite the significant reduction of the PrPSc content in the spleen achieved by all three delivery approaches, a prolongation of the survival of scrapie-infected mice was only achieved by the treatment with the polyclonal antibody W3 (Zuber et al., 2007b). This might be explained by the higher stability of full-length IgGs in the organism (half life up to 21 days in blood) compared with scFvs (half life less than 12 h in blood). Both half life and stability of the scFvs have to be improved to achieve an even more pronounced therapeutic effect for the treatment of prion diseases or the availability has to be improved by stable expression from a vector genome.

In summary, the AAV system used either for expression of scFvs directed against PrP (Wuertzer et al., 2008) or LRP/LR represents a powerful delivery system targeting the prion protein or its LRP/LR receptor for the treatment of prion disorders.

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Table 1. Incubation times and survival of scrapie-infected C57BL/6 mice treated with rAAV-C9, -S18 and -N3

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Incubation times plus survival (days, mean ± SD)</th>
<th>No. mice (affected/inoculated)</th>
</tr>
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<tbody>
<tr>
<td>rAAV-C9</td>
<td>125.3 ± 1.9</td>
<td>6/6</td>
</tr>
<tr>
<td>rAAV-S18</td>
<td>128.6 ± 3.7</td>
<td>7/7</td>
</tr>
<tr>
<td>rAAV-N3</td>
<td>126.7 ± 7.4</td>
<td>4/4</td>
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Incubation time represents the time span from the day of RML inoculation until one of the four characteristic TSE symptoms: ataxia of gait, tremor, difficulty righting from a supine position or rigidity in the tail, occurs. Survival represents the time span from the day one of the four symptoms occurs until the day mice showed two of the four TSE-related symptoms over 3 days (Sethi et al., 2002). At this time point mice were sacrificed.

http://vir.sgmjournals.org
microinjection, mice were anaesthetized and placed in a stereotoxic apparatus (SR-6N Narishige).

References


