PERSPECTIVE

Targeting the transferrin receptor to develop erythropoietin for Alzheimer's disease

Alzheimer's disease (AD) is the sixth leading cause of death in the United States with approximately 5.8 million Americans currently living with AD. Due to the lack of a disease modifying treatment for AD and the aging baby boomer generation, this number is projected to grow to 13.8 million by 2050 (Gaugler et al., 2019). Amyloid-beta (Aβ) plaque accumulation, one of the major pathological hallmarks of AD, can begin > 20 years before clinical symptoms of AD. By the time AD is clinically diagnosed, neuronal loss and neuropathological lesions (Aβ plaques and tau tangles) have already occurred in many brain regions (Gaugler et al., 2019). AD dementia correlates highly with neuronal loss, and therefore, reduction of neuropathological lesions in the AD brain at the time of clinical diagnosis alone cannot reverse AD dementia. We propose that a therapy that combines a reduction of neuropathological lesions of AD along with neuronal repair and neurogenesis may be required to treat AD dementia.

Erythropoietin (EPO), a class I cytokine and growth factor, is the primary regulator of erythropoiesis and is widely used for the treatment of anemia and diseases associated with low plasma EPO (Rey et al., 2019; Sun et al., 2019a). Besides its role in hematopoiesis, the presence of EPO and its receptor in non-erythroid tissues, including the brain, suggested a role apart from its hematopoietic effects (Rey et al., 2019; Sun et al., 2019a). Today, numerous studies show the protective effects of EPO in various neurodegenerative conditions including AD (Rey et al., 2019; Sun et al., 2019a). In a recent clinical study, EPO significantly improved neuropsychological test scoring in chronic kidney disease patients with cognitive deficits (Rey et al., 2019). The mechanisms underlying neuroprotective effects of EPO in AD are pleiotropic (Sun et al., 2019a), as discussed below. In experimental AD, Lee et al. (2012) showed that EPO (5000 IU/kg per day, intraperitoneally (i.p.) for 5 days; equivalent to ~350 μg/kg per week) reduced Aβ plaque by modulating the blood-brain barrier (BBB) influx transporter for Aβ, increased synaptophysin expression and angiogenesis in the brain, and improved contextual memory in AD transgenic mice. Similarly, Li et al. (2015) observed that EPO (1000 IU/kg per day, i.p. for 2 weeks; equivalent to ~70 μg/kg per week) ameliorated $A\beta_{42}$-induced mitochondrial dysfunction, neuronal apoptosis, tau hyperphosphorylation through the regulation of glycogen synthase kinase-3β at multiple AD-related sites, and overall memory impairment in mice. Maurice et al. (2013) administered EPO for 4 days (125–500 μg/kg per week, i.p.) and found a reduction in $A\beta_{25-35}$-induced: lipid peroxidation, Bax level, tumor necrosis factor α and interleukin-1β, hippocampal synaptic loss and learning deficits in a non-transgenic mouse model of AD. The work by Armand-Ugon et al. (2015) showed that EPO (2500 IU/kg, 3 days a week for 4 weeks, i.p.; equivalent to ~75 μg/kg per week) improved memory and reduced Aβ load in AD transgenic mice. Similar therapeutic effects of EPO have also been demonstrated in rat AD models wherein EPO reduced memory deficits by attenuating neuronal loss, neuroinflammation and cholinergic deficits and enhanced hippocampal neuronal proliferation (Rey et al., 2019). Overall, the protective effects of EPO include enhanced neurogenesis and angiogenesis, reduced neuroinflammation, oxidative stress and mitochondrial dysfunction, and modulation of AD-hallmark pathology (Aβ and tau phosphorylation; Sun et al., 2019a). The typical dose of EPO used to treat anemia is between 100–400 IU/kg per dose, which is, many-fold lower than the dose of EPO used in the aforementioned AD experimental studies. Large EPO doses were used in experimental AD because the large molecular weight (30.4 kDa) and polarity of EPO limit its entry into the brain across the BBB (Boado et al., 2010). This is the first obstacle to the development of EPO for a central nervous system disease including AD.

The second obstacle to the development of this neurotrophin for AD is the unwanted hematopoietic adverse effects associated with high EPO doses. This is particularly relevant while trying to develop EPO for AD due to chronicity of AD treatment and the potential for continuous EPO receptor stimulation. There have been limited studies reporting the hematopoietic effects of chronic EPO dosing in experimental AD, and a recent study showed marked elevation in hematocrit and reduction in reticulocytes with chronic EPO dosing in AD transgenic mice (Armand-Ugon et al., 2015). Alternate non-hematopoietic variants of EPO that retain neuroprotective effects have been developed and studied (Sun et al., 2019a), however we have taken a different approach to offset the two aforementioned obstacles to develop EPO for AD, which will be discussed below.

A BBB-penetrable EPO has been engineered by fusing human EPO to a chimeric monoclonal antibody against the mouse transferrin receptor (TfRMAb), and the fusion protein is designated as TfRMAb-EPO (Zhou et al., 2010). Human EPO was fused to the mouse TfRMAb since human EPO (reference: P01588) shares high (78.8%) amino acid identity with the mouse EPO (reference: P07321), binds mouse EPO receptor with high affinity and is what will be further developed for treatment in humans. Engineering a TfRMAb-EPO fusion protein offers dual advantages both of which are TfR mediated: 1) the TfRMAb domain of the fusion protein acts as a molecular Trojan horse to drive the EPO into the brain by binding to the BBB TfR and subsequent receptor mediated transcytosis, and 2) the TfRMAb domain also binds to the peripheral TfR to aid in systemic clearance of the TfRMAb-EPO fusion protein. The latter accelerates the peripheral clearance, reduces the systemic plasma exposure, and is expected to alleviate the hematopoietic adverse effects of EPO. The brain and plasma pharmacokinetics of TfRMAb-EPO show high brain uptake (2% injected dose/g brain in the mouse following intravenous dosing) and a 10-fold higher systemic clearance compared with EPO alone (Zhou et al., 2010).

Based on the above, in our recent work we investigated the effect of the BBB-penetrating TfRMAb-EPO fusion protein, in comparison with EPO, in a transgenic mouse model of AD. The overall aim was to determine if TfRMAb-EPO offers better therapeutic and hematologic indices compared with EPO alone, as would be expected based on the TfR-mediated BBB uptake and peripheral clearance of TfRMAb-EPO (Sun et al., 2019b). We treated male APP/PS1 transgenic mice (strain B6C3-Tg APPswe, PSEN1dE9, 85Dbo/Mmjax; 9.5 months of age at the start of the study), 2 days a week with either saline, TfRMAb-EPO (3 mg/kg per dose) or EPO (0.6 mg/kg per dose since the TfRMAb-EPO fusion protein is 20% EPO based on amino acid content) subcutaneously for 6 weeks (Sun et al., 2019b). Both TfRMAb-EPO and EPO significantly reduced brain Aβ load. It has been shown that EPO can reduce the expression of receptor for advanced glycation end products which is involved in the influx of Aβ into the brain, and both TfRMAb-EPO and EPO can modulate the expression of receptor for advanced glycation end products given its luminal location at the BBB. Interestingly, only the BBB-penetrating TfRMAb-EPO reduced brain $A\beta_{1-42}$ which is the more pathologic and aggregation prone isoform of Aβ. BBB-penetrating TfRMAb-EPO also increased brain synaptophysin, a marker of synaptic function, in treated mice. EPO alone did not alter synaptophysin levels in the brain. Memory deficits in the mice correlated with synaptic function in our study, and mice treated with TfRMAb-EPO did not show impaired memory, however, mice treated with the EPO alone had significant memory impairment (Sun et al., 2019a).

With respect to the hematopoietic effects of chronic EPO dosing, we observed marked differences in the hematologic indices of the mice treated with the BBB-penetrating TfRMAb-EPO and those treated with EPO alone. AD transgenic mice treated with EPO alone had elevated red blood cell indices (hematocrit, hemoglobin and red blood cell count) following 4-week treatment. This increase in the red blood cell indices is expected given the erythropoietic effects of EPO. However, TfRMAb-EPO treated mice showed no alteration in hematocrit, hemoglobin or red blood cell count throughout the study, and this can be explained by the lower plas-

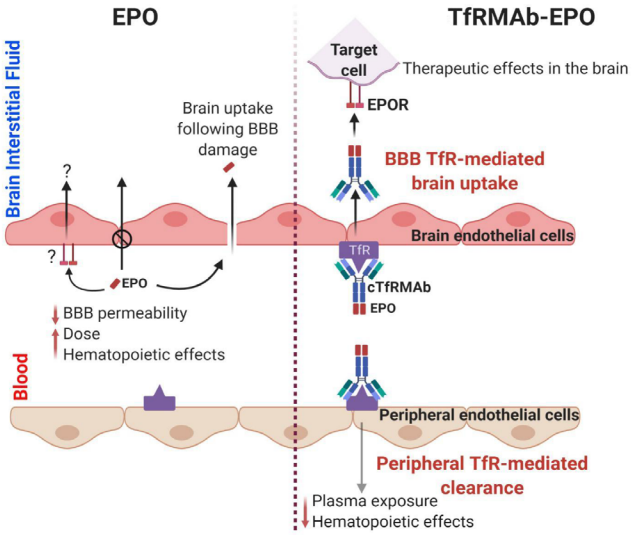

Figure 1 Transferrin receptor (TfR) mediated brain uptake and peripheral clearance of erythropoietin (EPO) fused to a monoclonal antibody against the TfR (TfRMAb).
TfRMAb-EPO binds to blood-brain barrier (BBB) TfR enabling BBB penetration and to peripheral TfR resulting in low plasma exposure and a favorable hematologic profile. EPO on the other hand does not cross the BBB in the absence of BBB disruption. Another suggested route of EPO entry into the brain is via the BBB EPO receptors (EPOR). This route of EPO entry into the brain is currently debated. Low EPO BBB penetration necessitates high EPO doses for central nervous system diseases increasing the potential for hematologic adverse effects, as seen in our recent study (Sun et al., 2019b). The figure was created using BioRender.com.

ma exposure of TfRMAb-EPO and thus reduced hematopoietic effects of the fusion protein. The EPO analogs however had opposing effects on the reticulocyte levels in our study. While EPO alone significantly reduced reticulocytes at 4 weeks, which remained low at 8 weeks, TfRMAb-EPO treatment resulted in reticulocyte elevation at 4 weeks, which normalized at 8 weeks. Acute reduction in circulating reticulocytes in mice following treatment with similar TfRMAb-based therapies is known, and the increase in circulating reticulocytes at 4 weeks seen in our study may be a repair response to the acute reduction in reticulocytes observed with the TfRMAb-based therapy (Couch et al., 2013; Pardridge et al., 2018). These TfRMAb mediated transient effects on reticulocytes observed in mice are less pronounced in primates at the same 3 mg/kg dose, given that the number of TfR-positive reticulocytes is lower in the blood circulation in the primates compared with mice (Couch et al., 2013; Pardridge et al., 2018). What really stood out in the study was the pronounced effect of chronic EPO (not TfRMAb-EPO) dosing on hematologic indices at the end of the study (8 weeks). Chronic treatment with EPO suppressed hematocrit, hemoglobin, red blood cell count and reticulocytes. Though the mechanism behind this chronic suppression of the hematologic indices is unclear, one hypothesis is that chronic treatment with high-dose EPO (higher than the doses used to treat anemia), may lead to EPO resistance, which is characterized by anemia along with low reticulocytosis in the presence of chronic elevated circulating EPO levels.

Taken together, the results from our recent work (Sun et al., 2019b; **Figure 1**) show that while both the BBB-penetrating TfRMAb-EPO and EPO (non-BBB-penetrating) reduce total Aβ load in the brains of AD transgenic mice, only the BBB-penetrating TfRMAb-EPO reduced brain $A\beta_{1-42}$ and increased synaptophysin. Further, EPO significantly altered multiple hematologic indices following chronic treatment, effects that were not seen with TfRMAb-EPO. These favorable therapeutic and hematologic effects of TfRMAb-EPO can be attributed to the TfR-mediated BBB penetration and TfR-mediated peripheral clearance that drive the EPO into the brain and away from the peripheral circulation.

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