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Clearance of amyloid-beta in Alzheimer's disease: progress, problems and perspectives

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Alzheimer's disease (AD) is the most common form of senile dementia and the fourth highest cause of disability and death in the elderly. Amyloid-β (Aβ) has been widely implicated in the etiology of AD. Several mechanisms have been proposed for Aβ clearance, including receptor-mediated Aβ transport across the blood–brain barrier and enzyme-mediated Aβ degradation. Moreover, pre-existing immune responses to Aβ might also be involved in Aβ clearance. In AD, such mechanisms appear to have become impaired. Recently, therapeutic approaches for Aβ clearance, targeting immunotherapy and molecules binding Aβ, have been developed. In this review, we discuss recent progress and problems with respect to Aβ clearance mechanisms and propose strategies for the development of therapeutics targeting Aβ clearance.

Alzheimer's disease (AD) is the most common senile dementia of later life and a major cause of disability and death in the elderly. Amyloid plaques are one of the pathological hallmarks of AD. Amyloid-β peptide (Aβ) appears to play a pivotal role in the pathogenesis of AD. A relatively small number (<5%) of AD patients (familial cases) might have increased Aβ production in the brain because of inherited mutations in the amyloid protein precursor (APP) gene or presenilins 1 or 2 genes. However, the majority of patients with so-called sporadic or late-onset AD do not have an increased Aβ production or APP overexpression in the brain. The steady levels of Aβ are determined by the balance between its production and clearance (Figure 1). Dysfunction in Aβ clearance is crucial for the accumulation of Aβ in AD brains. In this review, we discuss recent progress and problems with respect to Aβ clearance mechanisms and propose strategies for the development of therapeutics targeting Aβ clearance.

Receptor-mediated Aβ transport across blood–brain barrier (BBB)

Soluble Aβ can be removed slowly, via interstitial fluid (ISF) bulk flow, into the bloodstream [1]. However, this is responsible for the clearance of only 10–15% of the total Aβ in the brain and circulating Aβ can also influx into the brain from plasma. Receptor-mediated transport of Aβ is principally responsible for the transport of Aβ across the BBB (Table 1).

Efflux of Aβ from brain to blood

Lipoprotein receptor-related protein (LRP)-mediated Aβ efflux

Low-density LRP mediates the efflux of Aβ from the brain into blood. The interaction between Aβ and LRP mediates Aβ brain capillary binding, endocytosis and transcytosis across the BBB into blood [2,3]. Dysfunction of LRP leads to reduced efflux of Aβ from the brain and thus increased Aβ deposition in the mouse brain [3–5]. LRP has been shown to be genetically linked to AD in epidemiological studies [6]. In AD reduced expression of brain endothelial LRP is associated with positive Aβ staining of vessels [3]. The expression of LRP is negatively regulated by Aβ levels [3,7]. This might explain previous observations of relatively low LRP activity in brain microvessels in AD patients and mutant APP mouse models.

P-glycoprotein-mediated Aβ efflux

ATP-binding cassette transporter p-glycoprotein (p-gp) has been suggested to be involved in Aβ clearance as an Aβ efflux pump at the BBB [8]. Increased levels of Aβ in the temporal lobe of

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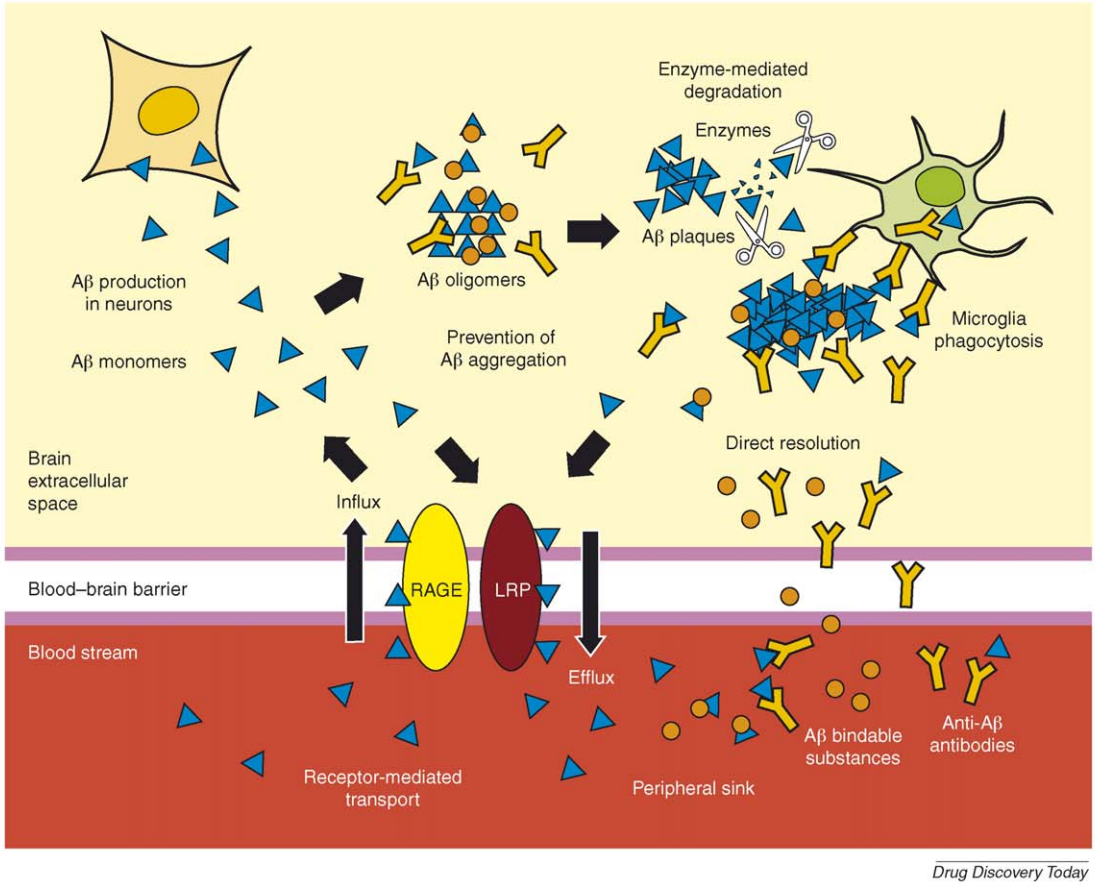

FIGURE 1

Mechanisms of Amyloid-β (Aβ) clearance. The steady-state level of Aβ depends on the balance between production and clearance. The transport of Aβ across the blood–brain barrier (BBB) is mainly mediated by receptors [i.e. receptor for advanced glycation end products (RAGE) and lipoprotein receptor-related protein (LRP)] on endothelial cells. Aβ in the extra- and intra-cellular space can be degraded by enzymes [i.e. neprilysin (NEP) and insulin-degrading enzyme (IDE)]. Peripheral anti-Aβ antibodies and Aβ-bindable substances are able to enter the brain at low levels, where they prevent Aβ aggregation and resolve Aβ fibrils. By binding to peripheral Aβ they also exert as a peripheral sink to promote the efflux of Aβ from the brain and disrupt the Aβ equilibrium between the brain and the blood, resulting in the clearance of Aβ from the brain. These mechanisms of Aβ clearance become potential targets for drug development for Alzheimer's disease.

the brain of non-demented elderly people are inversely correlated with p-gp expression levels in cerebral vessels [9]. However, the significance of p-gp in the development of amyloid accumulation and Aβ clearance in AD remains to be determined.

Influx of Aβ from blood to brain

Receptor for advanced glycation end products (RAGE)-mediated Aβ influx

RAGE, a multi-ligand and cell surface receptor, binds soluble Aβ in the nanomolar range [10], and mediates transport of

TABLE 1

Receptors that mediate Amyloid-β (Aβ) transport across the blood–brain barrier

Receptor	*Function*	*Evidence for involvement in* **in vitro** *or animal models*	*Evidence for involvement in human Alzheimer's disease (AD)*
Lipoprotein receptor-related protein (LRP)	Transport of Aβ from brain into blood	[3–5]	Low level of LRP is associated with positive staining of vessels for Aβ [3]; linkage studies [6]
P-glycoprotein	Transport of Aβ from brain into blood	[8]	Levels in cerebral vessels reduced with increased Aβ level in AD brain [9]
Receptor for advanced glycation end products (RAGE)	Transport of Aβ from blood into brain	[10,11]	Not established
gp330/megalin	Transport of Aβ from blood into brain	[12]	Not established

pathophysiologically relevant concentrations of plasma Aβ across the BBB [11]. Downregulation of RAGE can inhibit the influx of Aβ [11]. A feature of RAGE is its unusual sustained juxtaposition with its ligand in tissues. In contrast to suppression of receptors observed with LRP in an Aβ-rich environment [3], RAGE expression is upregulated and sustained at an elevated level by excess amounts of Aβ in AD brain through a positive-feedback mechanism. Given that Aβ efflux appears compromised during aging and in AD [3], this mechanism might exacerbate cellular dysfunction because of RAGE–Aβ interaction, as increasing expression of the receptor allows for more profound RAGE-mediated influx of Aβ.

gp330/megalin-mediated Aβ influx

Although gp330/megalin has also been reported to transport circulating plasma Aβ in a complex with ApoJ back into the brain across the BBB [12], it is normally saturated by high levels of plasma ApoJ, which precludes significant influx of Aβ into the brain under physiological conditions. Thus, RAGE is the most likely receptor responsible for the transport of Aβ back into the brain [13].

Enzyme-mediated Aβ degradation

Aβ is degraded by several peptidases, principally two zinc metalloendopeptidases referred to as neprilysin and insulin-degrading enzyme (IDE) (Table 2).

Neprilysin

Neprilysin is a rate-limiting Aβ-degrading enzyme in the brain [14]. The catalytic site of neprilysin is exposed extracellularly, making it a prime candidate for peptide degradation at extracellular sites of Aβ deposits. Intracerebral human neprilysin gene transfer leads to a remarkable decrease in amyloid deposits in an AD mouse brain [15], and inhibition of neprilysin protein or disruption of the neprilysin gene results in a defect in Aβ degradation [16,17]. In AD brain, the level and activity of neprilysin decrease in the cortex and hippocampus but not in other brain areas or peripheral organs [18–20]. A clear inverse correlation between neprilysin and Aβ peptide levels has been found in the vasculature of AD patients [21]. These findings suggest that the deficient degradation of Aβ caused by low levels of neprilysin might contribute to AD pathogenesis.

IDE

IDE is another major enzyme for Aβ degradation in the brain. The levels of IDE in the brain decrease during aging. It has a distinct distribution in the AD brain, with lower levels and being more oxidized in the cortex and hippocampus than in the cerebellum [18]. In animal models, deficits in IDE function lead to the impairment of Aβ degradation in the brain [22–24], whereas overexpression of IDE reduces Aβ levels, and retards or completely prevents amyloid plaque formation in the brain [25]. Defect in Aβ proteolysis by IDE also contributes to Aβ accumulation in the cortical microvasculature of AD cases with cerebral amyloid angiopathy [26].

Epidemiological studies suggest that chromosome 10q encompassing the gene encoding IDE has genetic linkage for both late-onset AD (LOAD) [27,28] and type 2 diabetes mellitus (DM2) [29]. Within the region, the gene for IDE represents a strong positional and biological candidate for LOAD, DM2, and for the epidemiological relationships among hyperinsulinemia, DM2, and AD [30]. In this regard, sequence variants of IDE have recently been shown to be associated with LOAD [31] and extent of Aβ deposition in the AD brain [32].

Other enzymes associated with Aβ degradation

Other metalloendopeptidase candidates, such as endothelin-converting enzyme (ECE) and angiotensin-converting enzyme (ACE), also degrade Aβ. ECE-1 and a closely related enzyme, ECE-2, can hydrolyze Aβ in the brain [33,34]. Consistent with the relationship between ACE and AD as revealed in epidemiological studies [35], ACE has recently been found to be capable of degrading Aβ [36].

Anti-Aβ autoantibodies

Recently, endogenous autoantibodies against Aβ have been found in AD patients and healthy individuals [37–39]. These autoantibodies exist in very low levels, tend to be reduced in AD patients, and appear to be harmless. Some studies have raised concerns with regard to their functions. Autoantibodies against the neurotoxic oligomeric Aβ species have been found to be depleted in AD plasma and correlated with age at onset for AD [40]. In a small-size pilot study, monthly treatment for six months with intravenous immunoglobulins containing autoantibodies against Aβ significantly lowered cerebrospinal fluid (CSF) levels of total Aβ and improved the cognitive performance in AD patients [41]. Autoantibodies isolated from immunoglobulin preparations also strongly blocked Aβ fibril formation, disrupted formation of fibrillar structures and almost completely prevented Aβ neurotoxicity [42]. In addition, some naturally occurring proteolytic antibodies have also been found to cleave Aβ [43,44]. These findings make it

TABLE 2

Enzymes that degrade Amyloid-β (Aβ)

Enzyme	*Function*	*Evidence for involvement in* in vitro *and animal models*	*Evidence for involvement in human Alzheimer's disease (AD)*
Neprilysin	Degrades Aβ	[15–17]	Levels and activity decreased in aging and AD brains [18–21]
Insulin-degrading enzyme	Degrades Aβ	[23–25]	Levels reduced in AD cases with cerebral amyloid angiopathy [26]; some linkage studies [27,28]
Endothelin-converting enzyme	Degrades Aβ and synthetic Aβ40 and Aβ42	[33,34]	Not established
Angiotensin-converting enzyme	Degrades Aβ	[36]	Epidemiological studies [35]

tempting to speculate that naturally occurring autoantibodies against Aβ might be beneficial to Aβ clearance. Although levels of these autoantibodies are normally very low, their persistence for many years in serum might be sufficient to protect against AD.

Therapeutic clearance of Aβ

Immunotherapy-mediated Aβ clearance

Immunological approaches intended to reduce Aβ load in the brain by either active or passive immunization, have shown concomitant improvement in neuritic dystrophy and cognitive deficits in animal models [45–50]. Clinical trials also suggested that the active immunization with Aβ peptide is therapeutically effective, as demonstrated by eliciting amyloid plaque clearance, attenuating plaque-associated pathology (reduction in dystrophic neurites or reactive astrocytes compared with unimmunized controls), decreasing CSF tau level and slowing patients' cognitive decline [51–54]. However, a significant number of patients developed autoimmune meningoencephalitis, caused primarily by the infiltration of autoreactive T lymphocytes into the brain in response to active immunization [51,52]. T lymphocytes are activated by T-cell epitopes mapped to the Aβ15–42, which is segregated from the dominant B-cell epitopes identified in Aβ1–15 [55]. In addition to meningoencephalitis, cerebral haemorrhage might be another potential risk of immunotherapy. Postmortem examinations showed severe small cerebral blood vessel disease and multiple cortical haemorrhages [51]. A recent study suggested that the occurrence of microhaemorrhage requires the presence of cerebral amyloid angiopathy and antibody recognition of deposited forms of Aβ [56].

Currently several basic hypotheses have been proposed on the mechanism of Aβ-plaque clearance by immunotherapy, including Aβ phagocytosis by microglia, disruption of Aβ aggregates, neutralization of oligomers and peripheral sink hypothesis. These mechanisms are not necessarily mutually exclusive and could act in concert.

Aβ-bindable substance-mediated Aβ clearance

According to the peripheral sink hypothesis, Aβ-bindable substances sequester plasma Aβ, leading to clearance of Aβ by promoting a net efflux of a rapidly mobilized soluble pool of Aβ (Figure 1). Peripheral treatment with gelsolin or GM1, an agent that has high affinity for Aβ, reduced the level of Aβ in the brain, probably because of a peripheral action [57].

Penetration of Aβ-bindable substances into the brain provides a chance for them to inhibit the aggregation of soluble Aβ and/or resolubilization of Aβ fibrils, then shift brain equilibrium between soluble and aggregated Aβ species towards soluble ones and finally facilitate Aβ clearance. The phenolic, yellow pigment, curcumin, found naturally in turmeric, a spice used extensively in Indian cookery, directly binds small Aβ species to block the formation of oligomer and fibril as well as to disaggregate Aβ aggregates *in vitro* and *in vivo*. When administered peripherally, curcumin can cross the BBB, bind plaques, and reduce amyloid levels and plaque burden in aged transgenic AD mice [58]. Another Aβ-bindable substance, enoxaparin (a low-molecular-weight heparin), when administered peripherally, significantly lowered the number of, and the area occupied by, cortical Aβ deposits and the total Aβ40 cortical concentration, possibly by either impeding the structural changes in Aβ necessary for fibril formation in the brain, or by sequestering the plasma Aβ peripherally [59].

Perspectives for drug discovery of Aβ clearance

The molecular pathways responsible for transport of Aβ across the BBB and the mechanisms involved in the proteolytic degradation of Aβ (Figure 1), suggest an array of potential therapeutic strategies for the clearance of brain Aβ. Clearance of Aβ via BBB transport and cellular degradation reduce brain plaque burden, but in the AD brain these mechanisms are either impaired or overloaded and might even contribute to disease progression. Based on the mechanisms of Aβ transport and degradation, some therapeutic strategies could be developed for the clearance of Aβ from the brain.

Promoting receptor-mediated Aβ efflux

RAGE and LRP play opposing roles in the regulation of Aβ transport across the BBB. In AD patients and in APP transgenic models of AD, RAGE is significantly upregulated at the BBB, whereas LRP is downregulated [3]. One potential strategy would be to develop new drugs that regulate the activity or expression of Aβ transport receptors in the vascular system. The downregulation of RAGE and upregulation of LRP at the BBB might readjust the transport equilibrium for Aβ by promoting its net efflux from the brain into the bloodstream. Two statins (simvastatin and lovastatin) which upregulate LRP on BBB endothelial cells, might facilitate the clearance of Aβ from the brain [60]. It is worth noting that blockade of RAGE, using RAGE-specific IgG, can also increase the expression of LRP in human brain endothelial cells exposed to an Aβ-rich environment [60]. This interesting finding not only implies a close link between the two receptors, but also suggests the potential of this strategy to promote the LRP-mediated Aβ efflux and inhibit RAGE-mediated Aβ influx.

Another strategy is to block the interaction between Aβ and RAGE, and thus prevent the RAGE-mediated influx of Aβ and block detrimental responses induced by the Aβ–RAGE interaction. As RAGE activation by Aβ could take place at an early stage of AD and result in early neuronal dysfunction [61,62], the prevention of RAGE–Aβ interaction at very early stages of AD might be a useful strategy. The antibodies to RAGE and soluble RAGE have been shown to block the Aβ-RAGE ligation-induced cell-type-specific consequences [63,64]. Peripheral administration of soluble RAGE can significantly reduce the Aβ levels in the brain of transgenic APP mice by either preventing the RAGE-mediated influx of Aβ or via the peripheral sink mechanism [11]. Interestingly, the amino acid residues of Aβ involved in the interaction with RAGE are from 17 to 20, which are also involved in Aβ–Aβ binding. Therefore, drugs targeting this region might prevent Aβ–RAGE interaction and arrest the Aβ aggregation.

Upregulating enzyme-mediated Aβ degradation

Experimental and epidemiological studies suggest that a decrease in activities of the Aβ-degradating enzymes because of genetic mutations, and age- or disease-related alterations in gene expression or proteolytic activity, might increase the risk for AD. Enhancement of Aβ-degradation enzymes through gene therapy, transcriptional activation or even pharmacological activation of the Aβ-degrading enzymes represents a novel therapeutic strategy

for the prevention and treatment of AD [65]. In animal models, gene transfer of neprilysin and IDE reduces the accumulation of Aβ in the brain [15,25,66]. A small synthetic peptide substrate has been shown to increase the activity of IDE with respect to the hydrolysis of Aβ without affecting its activity towards insulin, suggesting that small-molecule peptide analogs can be used to increase the rate of Aβ clearance without affecting insulin levels [67]. Somatostatin can also regulate the metabolism of Aβ in the brain through enhanced proteolytic capacity as a result of upregulation of neprilysin. Aging-induced downregulation of somatostatin expression might, therefore, be a trigger for Aβ accumulation leading to LOAD, suggesting that somatostatin receptor agonists might be useful in the prevention and treatment of AD. Neprilysin gene promoters can be transactivated by amyloid precursor protein intracellular domain (AICD) produced from gamma-secretase cleavage of APP-like proteins [68]. This presenilin-dependent regulation of neprilysin provides a physiological means to modulate Aβ levels with varying levels of gamma-secretase activity [68]. A very recent study has shown that the enzymatic activity of neprilysin is elevated in mouse brain and inversely correlated with amyloid burden after exposure of transgenic mice to an 'enriched environment', suggesting the role of brain activity and exercise in the prevention and treatment of AD [69]. It should be kept in mind that upregulation of neprilysin and IDE might affect physiological functions of other endogenous substrates, such as neuropeptides.

Overcoming adverse effects of immunotherapy

Several alternative strategies might be considered for the future development of a safer immunotherapy. Because full-length Aβ1–42 peptide contains both B-cell epitopes mapped in Aβ1–15 and T-cell epitopes in Aβ15–42 [55], immunization with the full-length Aβ would be expected to result in extensive T-cell activation. New vaccines composed of parts of the Aβ molecule, specifically excluding the epitope that might provoke abnormal T-cell reactions, are currently under development. Recent studies indicate that immunization with Aβ1–15 is effective to generate anti-Aβ antibodies in the absence of a T-cell response against full-length Aβ and leads to a reduction of cerebral-plaque burden and cognitive deficits in AD animal models [50,70]. Antibodies generated against N-terminal of Aβ are able to inhibit Aβ fibrillogenesis and cytotoxicity, disaggregate pre-existing Aβ fibrils, and are most effective in clearance of amyloid plaque [45,71–73]. Recent data from the clinical Phase IIa study suggest that the predominant antibodies generated after immunization with Aβ42 (AN1792) are primarily N-terminal (1–8) specific, independent of the presence of meningoencephalitis seen in a subset of immunized patients [74]. These preclinical and clinical data provide the basis for an improvement of immunization antigens by selecting epitopes of eliciting beneficial immune response and avoiding a potentially deleterious cellular immune response.

DNA vaccination is an attractive alternative to direct peptide and adjuvant approaches for inducing a humoral response to Aβ. DNA immunization offers significant advantages over peptide/protein-based immunization, including ease of production, the stability of episomal DNA and the eradication of time-consuming procedures needed for the purification of subunit proteins. Active vaccination with DNA vaccine encoding full-length Aβ peptide alone can effectively induce anti-Aβ antibody and reduce brain Aβ burden [75]. An important feature of DNA immunization is that it offers the capability of modifying genes coding for desired antigens, and to target the desired type of immune response using the appropriate immunostimulatory and immunomodulatory sequences, such as construction of DNA vaccine encoding B-cell epitope of Aβ alone or with Th2-type immune response favouring immunostimulatory and immunomodulatory sequences. Immunization of a DNA vaccine expressing cholera toxin B subunit and Aβ42 fusion protein induced a prolonged, strong production of Aβ-specific serum IgG and resulted in improved ability of memory and cognition, and decreased Aβ deposition in the brain of transgenic AD mice [76]. DNA vaccines encoding N-terminal sequence of Aβ (i.e. 11 tandem repeats of Aβ1–6 or Aβ1–21) alone are able to induce an anti-inflammatory Th2-type immune response, with no inflammation-related pathology detected in the brain of immunized mice [75,77]. A DNA vaccine with the mouse interleukin-4 fused to Aβ42 as a molecular adjuvant generates enhanced Th2-type immune responses. The antibodies generated are primarily of IgG1 and IgG2b subtype and are predominantly directed against the N-terminal sequence (1–15) [78]. Co-immunization of adenovirus vector encoding granulocyte-macrophage colony stimulating factor (GM-CSF) with Aβ42 DNA vaccine also favors a Th2 response [79]. Compared with peptide vaccination, gene-gun delivery of Aβ DNA vaccines offers the advantage of higher efficiency in breaking self-tolerance and for inducing beneficial Th2-based immune responses to reduce possible adverse effects related to Th1 adverse responses seen with Aβ42 peptide.

Mucosal immunization via oral or nasal routes is a desirable strategy because of its convenience and high tolerability [80]. By combining Aβ immunogens selective for the B-cell epitopes with appropriate immune-response-directed adjuvants and routes of administration, it is possible to develop a safer and effective Aβ vaccine [81].

Based on the peripheral sink hypothesis, it is possible to reduce brain Aβ burden without the need for antibodies to actually cross the BBB. Passive immunotherapy was effective in reducing the Aβ burden in animal models. This approach has the potential to be much safer than current active immunization. A Phase I clinical trial with passive immunotherapy is already under way in the United States [82]. The intravenous use of antibodies against Aβ resulted in a reduction in the Aβ concentration in the CSF and stabilization, or even a mild improvement, in cognitive function in AD patients [41].

Single-chain antibody provides another alternative potentially noninflammatory approach to facilitate Aβ clearance. Currently some single-chain antibodies specific against Aβ have been developed with different functions. Single-chain antibodies are able to inhibit Aβ aggregation and disaggregate pre-existing Aβ fibrils *in vitro*, prevent toxic effect of Aβ on cultured cells, and even reduce the Aβ burden after being injected into the brain of AD mouse [83–86]. Two single-chain antibodies have been found to possess α-secretase-like activity, providing a novel use of immunotherapy [87]. Intracellular expression of single-chain antibodies raised to an epitope adjacent to the β-secretase cleavage site of human APP drastically inhibited or almost abolished the Aβ production [88]. Single-chain antibody also provides an opportunity for developing a gene therapy-based non-inflammatory approach to Aβ clearance. Our

experiment shows that adeno-associated virus-mediated intracranial and intramuscular delivery of a single-chain antibody gene, isolated from a human single-chain antibody library [89], can effectively reduce the brain Aβ burden without activating microglia and T lymphocyte (unpublished). Because single-chain antibodies inherit some properties of their parental antibodies, it could be of interest to develop an alternative non-inflammatory approach by mimicking the current active immunotherapy without evoking the detrimental T-cell response and Fc-mediated inflammation.

Another important approach to avoid adverse effects is to select appropriate patients for immunotherapy. A recent study examined the preimmunization gene expression patterns of peripheral blood mononuclear cells of patients participating in the AN1792 study, suggesting that genes related to apoptosis and proinflammatory processes, and the tumor necrosis factor pathway in particular, were associated with the occurrence of meningoencephalitis, and upregulation of genes relating to protein synthesis, protein trafficking, DNA recombination, DNA repair, and cell cycle were associated with immunoglobulin responsiveness to immunization [90]. This key observation provides evidence of the power and precision of using genomic analysis to predict both the patients most at risk of meningoencephalitis, and by so doing, potentially prevent it, and those most likely to give a favorable response to immunization in advance.

Searching for new high-affinity Aβ-bindable substances

Based on the peripheral sink hypothesis, therapeutic agents are not limited by the need to penetrate the BBB or evoke an anti-Aβ immune response. Several Aβ-bindable substances have been found to effectively facilitate Aβ clearance [57–59]. A search for high-affinity Aβ-bindable compounds from Aβ receptors, functional foods or herbs could be a very attractive and promising strategy to shift the Aβ transport equilibrium towards plasma.

Conclusion

The steady-state level of Aβ depends on a balance between production, clearance and influx. Recently, pathologic, genetic and transgenic evidence has suggested that physiological receptor-mediated BBB transport and enzyme-mediated degradation of Aβ are impaired in AD. Immunotherapy is effective in reducing the Aβ load, attenuating AD-like pathology and improving cognitive deficits. Although clinical trials were halted, immunotherapy still holds promise as the first definitive treatment for AD. Several Aβ-bindable substances have been shown to be able to remove Aβ from the brain. Promoting receptor-mediated Aβ efflux from the brain, suppressing the Aβ influx across the BBB, upregulating the enzyme-mediated degradation, overcoming the adverse effect of the immunotherapy and searching for new high-affinity Aβ-bindable agents are some of the many promising approaches for future treatments for AD.

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