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Microglial degeneration in the aging brain – bad news for neurons?

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1. ABSTRACT

We have long promulgated the idea that microglial cells serve an entirely beneficial role in the central nervous system (CNS), not only as immunological sentinels to fend off potentially dangerous infections, but also as constitutively neuroprotective glia that help sustain neuronal function in the normal and especially in the injured CNS when microglia become activated. In recent years, we have reported on the presence of degenerating microglial cells, which are prominent in the brains of aged humans and humans with neurodegenerative diseases, and this has led us to propose a hypothesis stating that loss of microglia and microglial neuroprotective functions could, at least in part, account for aging-related neurodegeneration. In the current review, we sum up the many aspects that characterize microglial activation and compare them to those that characterize microglial senescence and degeneration. We also consider the possible role of oxidative stress as a cause of microglial degeneration. We finish up by discussing the role microglial cells play in terms of amyloid clearance and degradation with the underlying idea that removal of amyloid constitutes a microglial neuroprotective function, which may become compromised during aging.

2. INTRODUCTION

The notion of microglia as neuroprotective cells has been slow to become accepted. For the past two decades, microglial cells have often been portrayed as dangerous immune effector cells thought to be capable of endangering the well-being of neurons upon activation, and this has led to the prevalent notion that microglial activation (glial neuroinflammation) is involved not only in normal aging-related neurodegeneration, but also in age-related neurodegenerative diseases, most archetypically Alzheimer's disease (AD). Contrasting with this perception of microglia as neurotoxic cells, we and others have previously communicated and explained our view of microglia as neuroprotective glia that we consider to be essential for neuronal survival (1, 2). We have also retraced the historical evolution of the neuroinflammation hypothesis of aging-related neurodegeneration and neurodegenerative disease and pointed out a number of caveats, among them, perhaps most importantly, a sweeping overinterpretation of cell culture findings to ascribe detrimental activity to activated microglia (3). Currently, it appears that a paradigm shift is underway in that most authors acknowledge that microglia can have both neuroprotective and neurotoxic functions (4). Our own

Table 1. Comparison of microglial changes associated with activation vs. those reported in the aging brain

Microglial Property	Change with activation (reference)	Change with aging (reference)
Morphology	Hypertrophy and retraction & thickening of processes (19)	Deramification, shortening & twisting of processes; cytoplasmic fragmentation (8)
Mitosis	Proliferative burst peaking 3 days post-facial nerve axotomy that declines by 4 days post-injury (12)	Mitotic dysregulation: proliferative burst at 3 days post-axotomy that remains elevated by 4 days post-injury (52)
Cytokine production	Rapid upregulation of IL-1β, IL-6 and TNF-α mRNAs following traumatic spinal cord damage; prolonged IL-6 upregulation post-facial nerve axotomy (61)	Elevated basal levels of pro- and anti-inflammatory cytokines with equivalent fold-elevation post-injury (64,17)
Immunophenotype	Upregulation of CR3, IgG, thrombospondin, intercellular adhesion molecule 1 and MHC I and II (24, 31, 32, 33)	Upregulation in MHC II, ED1, LCA and CD4 in the absence of overt pathology (16, 39, 40, 41, 42)

perspective remains focused largely on a beneficial role of microglia, and this view has been strongly reinforced by recent observations from our laboratory which demonstrate the presence of degenerating microglial cells in the aged human brain, as well as in the brains of humans and animals with neurodegenerative disease (5-7). The discovery of degenerating (or dystrophic) microglia in the aged human brain (8) has given rise to the microglial dysfunction hypothesis (9), which opens up new possibilities for understanding why the incidence of most neurodegenerative conditions increases with advancing age. The hypothesis states that senescence of microglia produces dysfunctional cells, which are losing their neuroprotective abilities, and that this loss of microglial neuroprotection is in large part responsible for aging-related neurodegeneration and onset of neurodegenerative disease. Thus, neurons and particularly old neurons that may require greater neuroprotection die because they are increasingly neglected by weaker microglial cells.

In the paragraphs that follow, we shall first summarize the changes that occur in microglia during activation, because not only are these complex and involve a number of variables, they also can be difficult to distinguish morphologically from senescent changes. In past studies, senescent microglia have undoubtedly been mistaken for activated ones - and perhaps *vice versa*. We go on to describe the morphological changes that have been used to identify degenerating microglial cells, and to highlight the fact that microglia, as mitotic cells of the CNS, have limited life spans and are subject to telomere shortening and to replicative senescence. We will point out one possible mechanism, i.e. iron-mediated oxidative stress, to account for microglial degeneration. The final portion of this manuscript examines the relationship between microglia and amyloid because amyloid has long been implicated in contributing to neurodegeneration, and clearance of this material by microglia is viewed as an important neuroprotective function that may be declining with age.

3. MICROGLIAL ACTIVATION

Once thought to be immune-privileged, it is now known that the central nervous system (CNS) contains immunocompetent microglial cells. Highly adaptable in structure and function, microglia respond to changes in the microenvironment to help maintain brain homeostasis. Following acute CNS injury, microglia become activated and undergo phenotypical changes that include hypertrophy, an enhanced expression of immunologically relevant cell surface molecules, mitosis, and changes in cytokine and growth factor production. While microglial activation appears to be stereotyped and has been studied extensively, controversy remains concerning the effects of microglial activation on the CNS following brain injury. It also remains unclear what role microglial activation may play in the pathogenesis of neurodegenerative diseases. This area of inquiry has been complicated by interpretations of *in vitro* studies demonstrating highly reactive microglial cells that produce neurotoxic substances, which has led to the idea that activated microglia may be responsible for causing neurodegeneration (10, 11). On the other hand, experiments utilizing *in vivo* models such as the facial nerve axotomy (7, 12) and transient middle cerebral artery occlusion (MCAO) (13), among others (14), have shown that microglial activation is basically a neuroprotective process. When considering reports of microglial activation in aged and diseased brains (Table 1) (15-17), it is important to differentiate between true activation and abnormal cells that may be senescent and dysfunctional (8). A clear understanding of microglial activation is essential in order to properly study microglial function during aging or in the diseased and injured brain. It may also prove valuable in predicting and modulating microglial function for therapeutic purposes.

Cellular morphology is perhaps the most telling aspect of the state of microglial activation. While immunological cell surface markers alone may prove insufficient (18), microglial morphology studied in combination with immunohistochemical markers serves as a reliable assessment of activation states. Following brain injury, resting, ramified microglia undergo a stereotypical, graduated response commensurate with the severity of brain damage incurred. Prior to becoming fully activated, or in the event of a mild perturbation, microglia may take on a hyper-ramified form (19). Fully activated microglia retract their processes and develop an enlarged cell body. Cellular processes exhibit increased thickness and deramification occurs at distal branches (7). Ultimately, activated microglia responding to injury that does not involve frank neuronal degeneration will decrease in number and return to a resting state (20). However, when brain damage leads to neuronal degeneration, microglia further transform into phagocytes (21). Microglia with a macrophage appearance can be detected as early as one to four hours after injury (22, 23). Microglial-derived macrophages take on a rounded, amoeboid shape similar to that of peripheral macrophages, and they may revert to a resting phenotype within a few days to weeks.

Microglial immunophenotype is highly dynamic and fluctuates with changing states of activation. Resting microglia constitutively express several surface molecules including, but not limited to, type three complement receptors (24), Fc, macrophage-specific antigen (25), CD4 (26) and leukocyte common antigen (LCA) (27). In addition, the CD200 receptor (CD200R) has been shown to be expressed on resting microglia (28) and is thought to help maintain the cells in a quiescent state through interaction with its neuronally derived ligand, CD200 (29, 30). Upon microglial activation, there are changes in surface marker expression indicative of changes in cellular function. Within 24 hours of activation, microglia express many molecules important for interactions between lymphocytes and antigen-presenting cells. Specifically, they exhibit an upregulation of CR3 (OX-42) expression (24) accompanied by an increase in IgG-immunoreactivity, thrombospondin, and intercellular adhesion molecule 1 (31-33). Expression of integrin subunits alpha5 and alpha6 peaks at day four post-injury and the alphaM-subunit at day 1 and again at days 14-42. Furthermore, within three days of CNS injury, proliferating microglia have been shown to express the stem cell antigen CD34 (34). Changes have also been shown to occur in the expression of P2 purinoreceptors following LPS-induced activation, suggesting an important role for these receptors in the microglial response to extracellular ATP (35). Consistent with a role as antigen-presenting cells, reactive microglia show enhanced major histocompatibility complex type I and II (MHC I and II) expression during the first week after injury (36, 37). Upregulation of MHC I can be detected on all activated microglia, while MHC II expression is much more restricted affecting fewer cells (36). However, MHC II expression can be widespread on activated microglia in white matter tracts degenerating after injury (37, 38). Notably, there have been numerous reports of an age-related increase in the number of microglia expressing the MHC II antigen (16, 39, 40). While it has been concluded that this increase in MHC II immunoreactivity correlates to an increase in activated microglia, it is important to consider that this expression is often seen in the absence of any overt pathology. Other investigators have also reported age-related increases in the expression of such molecules as ED1, LCA and CD4 (41, 42). Taken together, these results highlight the possibility that microglia undergo age-related changes in cellular function, or alternatively experience secondary effects of an altered microenvironment.

In addition to changes in cellular structure and immunophenotype, mitosis is another prominent and consistent feature of the microglial response to injury. Microglial proliferation has been studied extensively in the facial nerve axotomy model (21, 43-45). This well established injury paradigm is advantageous in the study of microglial activation primarily because there is no direct trauma to the CNS and the blood brain barrier remains intact, providing an opportunity to study purely endogenous glial responses. An additional advantage is that the injury is well tolerated and highly reproducible from animal to animal. Insights gained from studies employing the facial nerve axotomy and other regenerating nerve models, as well as from acute and chronic neural injury models reveal that microglial proliferation begins as early as 12 hours post-lesion (46), peaks at approximately three to four days after insult (21, 34, 47, 48) and declines thereafter. Following population expansion, there is evidence to show that population control is primarily implemented by apoptosis. In models of facial (49), as well as perforant pathway (50) lesions, hypoglossal and sciatic nerve injuries (51), apoptosis of microglia was found to occur beginning three to six days after injury and continue for up to 28 days. Similar to morphology and immunophenotype, changes have also been detected in the proliferative response of microglia in the aged rodent brain. Conde and Streit (52) found that proliferation of microglia in aged rats remains significantly higher than in young rats 4 days after injury, suggesting that adjustments in the regulation of microglial activation occur with aging.

Cytokine production by reactive microglia is context-specific and varies temporally and quantitatively following different types of insult. Some cytokines expressed by microglia act in an autocrine fashion, for example, transforming growth factor-beta (TGF-beta) (53, 54), a pleiotropic growth factor. TGF-beta has been shown to exert inhibitory effects on microglial phagocytosis (55) and proliferation (56), as well as prevent the induction of microglial genes involved in chemotaxis and cell migration, among others (57). Importantly, many microglial-derived cytokines exert both positive and negative effects on the CNS and it is the degree of microglial activation, or severity of neuronal damage, that determines the ensuing cytokine expression patterns and whether the effect will be neurotoxic or neurotrophic (58). For example, microglia rapidly upregulate IL-1beta, IL-6 and TNF-alpha mRNAs following traumatic spinal cord damage (59-62), whereas in the regenerating facial nerve injury paradigm mRNAs of TNF-alpha and IL-1beta, both prototypic proinflammatory cytokines, are only minimally elevated and there is no change in M-CSF mRNA (33, 61). Interleukin-6, which shows prolonged expression after facial nerve axotomy, is rapidly downregulated after initially spiking in spinal cord injury (61). Additionally, Sawada *et al.*, (63) demonstrate that microglial age also impacts cytokine expression patterns and neuronal survival. In their study, neonatal mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) an lipopolysaccharide (LPS), to stimulate microglial activation, showed significant neuronal recovery following initial MPTP-induced cell loss. This was accompanied by microglial activation and an upregulation of IL-1beta and IL-6. Alternatively, while aged mice treated with MPTP also showed significant neuronal loss, this effect was exacerbated by LPS treatment. This result is in concordance with another study showing that microglia derived from aged mice express increased basal levels of pro- and anti-inflammatory cytokines (64). Finally, Sheng and colleagues (17) also report that microglia exhibit increased IL-1beta immunoreactivity with age. The potential causes of these age-related changes are unknown and warrant further research. While most evidence of microglial cytokine expression has come from animal studies, recent work using human primary microglial cell cultures and immortalized human microglial cell lines demonstrate

Table 2. Age-related changes in microglial metabolic activity

Changes in proteolytic activity	Reference
- Impaired protein turnover	195
- Decline in proteasomal function	195, 196
- Decreased protein synthesis	195
- Decreased degradation of proteins from apoptotic vesicles	197
- Accumulation of lipofuscin granules	64
- Increase of inclusions, vacuoles and granularity	64, 198
CHANGES IN CELL SURFACE MOLECULE EXPRESSION	
- Increase in MHC class II antigen expression	199, 200
- Increase in CD40, CD45, and CD86 expression	195
CHANGES IN CYTOKINE PRODUCTION	
- Increased production of IL-6, TNF-α, and IFN-γ	197, 201
- Increased TNF-α, IL-1β, and IL-6 mRNA expression	64
- Decreased production of IL-12	197
- Increased IL-10 and TGFβ1 mRNA expression	64, 202
CHANGES IN REACTIVE OXYGEN SPECIES PRODUCTION	
- Higher basal nitric oxide release	195
- Higher production of ROS	121, 197
- Diminished capacity to evoke oxidative burst	195, 197
CHANGES IN ACTIVATION DYNAMICS	
- Continuous activation	203-206
- Increased release of inflammatory mediators upon activation	64, 207, 208
- Insufficient de-activation by astrocytes	209

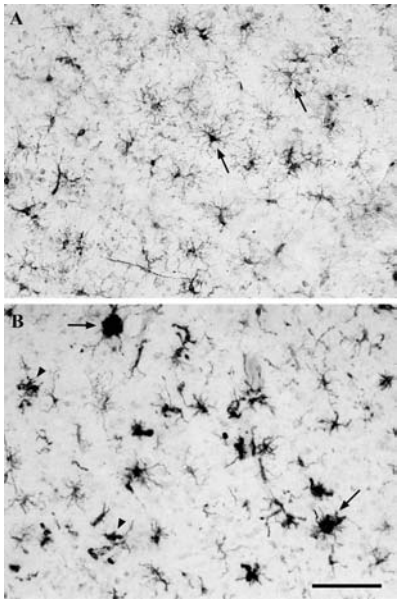

Figure 1. Comparison of microglial morphology using anti-HLA-DR and anti-ferritin immunostaining. **A**, microglial cells immunoreactive for HLA-DR antigens reveal a mostly normal and ramified morphology (arrows). **B**, microglial cells stained for ferritin are mostly dystrophic exhibiting abnormally twisted and deramified processes (arrowheads), and formation of multicellular clusters (arrows). The higher incidence of microglial dystrophy in L-ferritin-positive microglia implicates iron-mediated oxidative stress in microglial degeneration. Temporal cortical gray matter from an 83-year-old non-demented individual (A) and from an 84-year-old AD subject (B). Scale bar: 100-μm.

similar cytokine expression patterns as those previously identified in animal models (65). Human microglial cells stimulated in culture by LPS or amyloid-beta fragments were shown to upregulate mRNA levels of IL-8, IL-10, IL-12, MIP-1beta, MCP-1, MIP-1alpha and TNF-alpha. Lastly, microglial activation resulting from infection, such as viral meningitis or bacteria-induced encephalitis, leads to production of not only those cytokines listed above, but also interferon-gamma (IFN-gamma) (66, 67). IFN-gamma acts to promote upregulation of surface molecules like major histocompatibility complex (MHC) class I and II molecules, complement receptors, Fc receptors and CD14, as well as induce the release of cytokines, complement and nitric oxide (NO) (68). In addition, IFN-gamma acting synergistically with beta-amyloid peptide has been shown experimentally to induce microglial production of the chemokine monocyte chemotactic protein (MCP-1) (69).

4. MICROGLIAL SENESCENCE

Neurons, oligodendrocytes and astrocytes originate from the neuroectoderm. In contrast, microglia arise from myeloid progenitors derived from the hemangioblastic mesoderm (70). Reminiscent of their monocytic lineage is the ability of microglia to proliferate and undergo self-renewal. This mitotic capacity is particularly relevant to studying microglia in the aging brain because it underscores their senescence potential - microglia as mitotic cells have a finite lifespan and are subject to replicative senescence via telomere shortening. Analyses of telomere length have revealed that microglia become senescent with mitotic stimuli *in vitro* (71) and with normal aging *in vivo* (72). The primary consequence of this replicative senescence is the cessation of cell division through the cell's arrest in the G1-S boundary of the cell cycle (73).

Cells that have entered the senescent state remain metabolic active, although there are gradual changes in cellular structure and function. For instance, senescent cells accumulate lipofuscin granules in their cytosol (74-76). Lipofuscin is a mixture of autofluorescent lysosomal lipo-pigments and proteins that cannot be excreted or degraded (77, 78). The amount of lipofuscin in mitotic cells is dependent on both its rate of formation and its rate of dilution by cell division (75). Microglia have been shown to progressively accumulate lipofuscin-containing dense bodies in their cytoplasm, thus providing further evidence for their replicative senescence potential with advancing age. Other age-related changes in microglial metabolic functioning include a decrease in proteolytic activity, an increased production of pro-inflammatory mediators, and an apparent 'primed' state of activation. These results are summarized in Table 2. Senescent microglia undergo profound morphological changes. In aged brain tissues, subpopulations of microglia present signs of cytoplasmic deterioration, including formation of spheroid-like structures along major processes, deramification, atrophy, thinning, twisting and fragmentation of processes (Figure 1). The morphology of these cells is quite different from the ramified appearance of resting microglia or the bushy morphology of hypertrophic (activated) microglia (7, 8),

and cells displaying this kind of degenerative cytoplasmic structure were therefore named dystrophic microglia . The incidence of dystrophic microglia increases with aging, and thus the morphological aberrations are seen as a reflection of cellular senescence. Moreover, dystrophic microglia are found scattered randomly in the brain parenchyma, often alongside normal-appearing, ramified microglial cells suggesting that they form a subset of senescent microglia. In other words, there is heterogeneity of microglia and not all of the cells are of the same age or in the same functional state (79).

Microglial cells exhibiting dystrophic characteristics have been identified in the normal aging brain (5, 8, 80), as well as in several neurological disorders, including Alzheimer's disease (AD) (1, 8, 81), Creutzfeldt-Jakob disease (82), Huntington's disease (HD) (83), and schizophrenia (84). Degenerative changes have also been reported in rat primary microglial cultures exposed to AD-associated amyloid-beta deposits (85), and in murine disease models of HD (83, 86) and amyotrophic lateral sclerosis (6). Assuming that microglial functional activity correlates with their morphology (87), it is likely that dystrophic morphological alterations serve as an impediment to the normal surveillance function of these cells (88, 89), which could have deleterious consequences for the CNS as a whole.

A common feature of both aging and several of the neurological diseases in which dystrophic microglia are present is iron dyshomeostasis. Iron is an essential cofactor required for normal brain functioning, as it plays critical roles in electron transport and cellular respiration, regulation of gene expression, myelin formation, and neurotransmitter synthesis (90). Iron levels must be tightly regulated to prevent iron-mediated toxicity. Too little iron impedes the normal functioning of cells, whereas too much iron promotes iron-induced oxidative stress. Iron metabolism is dysregulated with advancing age because iron accumulates in the aged brain (91-94), especially in brain regions affected by age-dependent neurodegenerative diseases, including AD, PD, and HD (95-97). This increase in brain iron levels places a large toll on microglia, since prolonged storage of excess iron is performed primarily by microglial cells (93, 98). The combined effects of microglial senescence, in which cellular functions are at suboptimal conditions, and increased intracerebral iron levels may predispose microglial cells to degeneration in the aging brain. The following section addresses the potential link between microglial degeneration and iron dysregulation.

5. IRON ACCUMULATES IN MICROGLIAL CELLS WITH AGING

Iron is a highly versatile molecule for biological reactions because it can readily accept or donate an electron as it possesses two valence states: ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}). When iron levels are higher than the needed amount to carry out iron-mediated reactions, excess iron is stored in the intracellular iron storage protein ferritin. Ferritin is composed of a total of 24 functionally distinct heavy (H)-chain and light (L)-chain subunits that come together in varying ratios to form a hollow protein shell (99). Within the ferritin cage approximately 4500 iron atoms can be stored as a soluble inorganic complex (99).

Iron enters the ferritin molecule as Fe^{2+} via the H-subunit, which possesses a ferroxidase center that catalyzes the oxidation and detoxification of Fe^{2+} to its less reactive form Fe^{3+} (100). The L-subunit possesses glutamate residues in its interior that facilitate Fe^{3+} mineralization for long-term storage (101). The subunit composition of ferritin proteins (H:L ratio) is cell- and tissue-specific. In the CNS, microglia contain mostly L-rich ferritins, neurons contain mostly H-rich ferritins, and oligodendrocytes contain equal amounts of both isoforms (98, 102, 103). Iron sequestration within the shell of the ferritin molecule serves antioxidative functions by preventing labile Fe^{2+} from catalyzing the formation of highly reactive hydroxyl ($^{\bullet}OH$) radicals via the Fenton reaction with H_2O_2. The resulting $^{\bullet}OH$ radical is extremely reactive, which indicates that it does not diffuse far from its site of formation. There are no antioxidative mechanisms to neutralize $^{\bullet}OH$ radicals, thus indiscriminate oxidative damage to lipids, proteins, and nucleic acids inexorably occurs upon its generation (104).

Because microglia contain L-rich ferritins, a large portion of the iron accumulation that occurs in aging and neurodegenerative disorders is stored in microglial cells. This iron accumulation is thought to be a consequence of normal aging, since brain iron uptake is maintained at a constant level in adult individuals, while very little iron exits the system (105). Elevated iron levels increase the chances that labile iron will be available to induce oxidative stress reactions. The reason why prolonged iron storage is performed largely by microglial cells may lie in their self-renewal capacity. In contrast to post-mitotic neurons and oligodendrocytes, microglia are relatively expendable cells, as they are subject to gradual replenishment via mitosis (106) and through recruitment of bone marrow-derived progenitor cells (107, 108). Because of this replaceability of microglia one might speculate that they can "afford" to perform the dangerous function of long-term iron storage.

5.1. Microglia and iron-induced oxidative stress

Iron storage is a hazardous cellular activity because Fe^{2+} can be released from ferritin stores and the resultant free Fe^{2+} may catalyze oxidative stress reactions. This process is dependent primarily on the reduction of iron within the ferritin core, since iron accumulates in ferritin in the less reactive Fe^{3+} form, but iron enters and leaves the ferritin shell as Fe^{2+} (109, 110). Superoxide, which is produced and released by activated microglial cells (111, 112), acts as a reducing agent and can therefore release ferrous iron from ferritin (113, 114). Another product of microglial activation, hydrogen peroxide, decreases the microglial ferritin pool by (i) reducing the half-life of ferritin proteins due to oxidation and subsequent removal by the proteasomal system (115, 116), and (ii) reducing ferritin de novo synthesis (115). Hence, microglial activation results in increased intracellular non-ferritin

bound iron levels. Accidental iron leakage can also occur during ferritin turnover, and since the resulting labile iron is not needed for metabolic reactions, it increases the risk of cells to iron-mediated oxidative stress.

To minimize and restrict iron-dependent oxidative damage, only a subset of microglial cells participate in long-term iron storage by expressing ferritin proteins rich in L-chain subunits (Figure 1). In addition, microglia possess a rich antioxidative repertoire for self-protection against oxidative damage, including substantial activities of the antioxidative enzymes catalase and superoxide dismutase (117). Immunocytochemical studies of the antioxidative capacity of microglial cells revealed that, among other neural cells, microglia are strongly immunoreactive for glutathione and glutathione peroxidase (GPx) (118, 119). GPx is an enzyme that uses glutathione to reduce H_2O_2 to water. The predominance of this antioxidant system in microglia pinpoints the potentially deleterious effects of iron-mediated oxidative damage, since iron toxicity is H_2O_2-dependent.

Mitochondrial reactions are the main cellular sites of H_2O_2 production and dystrophic microglia have been shown to contain degenerated mitochondria (84), suggesting that H_2O_2 production might be altered in these cells. In activated microglia, H_2O_2 is also produced as a by-product of NADPH oxidase-induced formation of superoxide (117, 120). As a non-polar molecule, H_2O_2 is able to diffuse across membranes. Moreover, by yet unidentified mechanisms, aging has been shown to increase the basal level of microglial H_2O_2 production (117). Altogether, it appears that with chronic microglial activation as well as advancing age, microglia may be subjected to increased H_2O_2 exposure intracellularly. Thus, in order to minimize iron-induced oxidative stress it is important that levels of iron and H_2O_2 be well controlled.

Oxidative stress takes place when there is an inadequate balance between production and neutralization of reactive oxygen species (ROS). It is not yet known whether microglial antioxidative capacity is maintained at sufficiently high levels in the aging brain to counteract the potentially hazardous effects of increased intracellular iron stores. There are reasons to think it may not: first, microglial production of ROS increases with age (121), and superoxide-mediated ferritin iron leakage have been shown to provoke membrane lipid peroxidation (122-125). Second, oxidative stress markers have been identified in microglia. Specifically, the reactive carbonyl crotonaldehyde, which is generated during lipid peroxidation, localizes in microglial cells in the AD brain (126). Third, lipofuscin is formed by cross-linked proteins that result from iron-catalyzed oxidation of protein and lipid residues (74, 75), and microglia accumulate lipofuscin granules with aging (64). Fourth, our own immunohistochemical analyses of aged human brain tissues revealed that most L-ferritin-positive microglia exhibit dystrophic changes, an indication that senescent microglia are likely undergoing iron-induced oxidative stress. Because aging is characterized by a progressive impairment of cellular function (127), it is possible that senescent microglia gradually lose their ability to maintain viable ferritin-bound iron stores and/or to fight extracellular and intracellular stressors, all of which could result in their greater vulnerability to degeneration and death. The mode of cell death under these conditions of oxidative stress is likely to be cytorrhexis, i.e. complete fragmentation of the cells' cytoplasm (6, 7).

6. THE SELF-RENEWAL CAPACITY OF MICROGLIA IN THE AGED BRAIN

Iron is indispensable for DNA synthesis and the regulation of cell proliferation. As a cofactor of the enzyme ribonucleotide reductase, iron mediates the production of deoxyribonucleotides from the corresponding ribonucleotides, the rate-limiting step in the synthesis of DNA precursors and key control point in DNA synthesis (128). Numerous studies have shown that iron restriction arrests cell proliferation (129). In the aged brain, microglia proliferate more vigorously than in younger brain tissues (52). A possible explanation for this increased proliferation rate is the age-related increase in microglial intracellular iron levels. Thus increased microglial proliferation rate likely reflects an imbalance in microglial self-regulatory mechanisms. Inevitably, higher levels of microglial proliferation in the aged brain would result in further propagation of microglial senescence in the affected tissue.

An interesting question is how microglia can reach replicative senescence in the normal aging brain that is devoid of obvious inflammatory/proliferative stimuli such as infection, neurological trauma or extracellular proteinaceous aggregates. Although the underlying mechanisms of microglial proliferation are partly understood in specific injury models (e.g., facial nerve axotomy) and in cell culture systems, little is known about how normal aging may promote microglial replicative senescence. It is possible, that in addition to stimuli inherent to the CNS, alterations in systemic functioning also play a role in microglial senescence. One such mechanism may be linked to the body's response to stressful events. Microglia express receptors for stress-related hormones (130) and respond to acute physical/emotional stress and associated hormones with proliferation (131, 132) and morphological and functional activation (133). Similarly, microglial activation also occurs in response to closed head trauma (134), systemic infection (135, 136), and even diet (e.g., cholesterol-rich diet) (137, 138). The accumulation of these processes during a lifetime, in addition to age-related changes in iron homeostasis, is likely to lead to senescence of at least a subpopulation of microglial cells.

Microglia are a self-renewable cell population and besides mitosis, microglial replenishment can occur by recruitment of bone-marrow-derived cells, which migrate into the neural parenchyma and differentiate into microglia (139). However, the recruitment of microglial myeloid progenitors across the BBB may decline with aging, and thus this mechanism of microglial replenishment may be insufficient to sustain a nervous system compromised by old age. As a result, senescent microglia would accumulate

in the aged CNS. Higher incidences of senescent microglia may contribute to aging-related neurodegenerative diseases through loss of a number of neuroprotective functions, many of which remain to be elucidated. However, there are some possibilities: perhaps due to increased oxidative stress senescent microglia may begin to oversecrete substances that are harmful to neurons (as well as to themselves and possibly other cell types); senescent microglia may produce insufficient amounts of neurotrophic factors or other substances that help sustain normal neuronal functioning; senescent microglia may be less able to perform efficient phagocytosis leaving insoluble material, such as amyloid, largely unremoved.

7. MOUSE MODELS OF ALZHEIMER'S DISEASE

Given the limitations associated with post-mortem studies on human brains, since the mid-nineties several transgenic mouse lines have been created to model AD. These mouse models emulate with great success the deposition of beta-amyloid in the neocortex and the hippocampus, but not necessarily neurodegenerative changes, such as neurofibrillary tangles. Transgenic mice typically overexpress the amyloid precursor protein (APP) with familial AD mutations under the control of various promoters, and although there are more than a dozen strains available, most studies are done on the PDAPP, Tg2576, APP23 and most recently, the mo/hu APPswe/PS1dE9 mice (140-143). The mutations carried in the hAPP transgene introduced into these mice originated from genetic studies done on families with early onset AD. In general, the transcription of these genes causes dense beta-amyloid plaque to be deposited in the neocortex and the hippocampus usually between six and twelve months of age. The hAPP transgene in the PDAPP model has the V717F mutation (Indiana family origin) (141). The dual point mutations in Tg2576, APP23 mice are at K670N/M671L (Swedish family origin). In addition to the Swedish mutation, mo/hu APPswe/PS1dE9 co-expresses Presenilin 1 with a familial AD mutation (accelerates Aß42 deposition) together with the humanized form of mouse APP. Eponymous PDAPP mice have hAPP under the control of platelet derived growth factor promoter (PD). In the Tg2576 and mo/hu APPswe/PS1dE9 models, the APP gene is driven by the prion protein promoter, while the APP23 model has hAPP driven by the neuron specific Thy-1 promoter. These animals constitutively over express the APP transgene. Recently, a inducible Tet-off mo/huAPPswe/ind transgenic mouse model was generated using a tetracycline responsive promoter (144).

The various mouse models have demonstrable impairment in learning and memory that typically manifest at around the same time plaques deposits become prevalent in the limbic structures of these animals (142, 145, 146). Mice exhibit loss of dendritic spines and loss of synapses in subcortical cholinergic projections, plaque associated gliosis, and cerebrovascular abnormalities (147-151). APP23 mice exhibit neurodegeneration (143) while the plaques in Tet-off/APPswe/ind mice persist for the lifetime the mouse even when APP transgene production is halted early on (144). However, the lifespan of the Tet-off/APPswe/ind mouse is not significantly shortened by the presence of these plaque deposits. This highlights an inherent shortcoming of modeling AD in animals whose normal lifespan is less than 5% that of a normal human. Mouse models also do not have hyperphosphorylated tau neurofibrillar tangles or the same level of complement system activation found in AD (145, 152, 153). Rat models of AD have not gained widespread popularity because rat brains are more resistant to the formation of dense Aß plaques and AD-like learning & memory deficits (154).

The transgenic mice listed above have been mated to other mice deficient or containing mutant proteins that are informative for AD studies. For instance, hybrid mice were created by mating Tg2576 mice to mice deficient in expression of the immune cell chemotaxis receptor CCR2 (155). As described in the following, these mice proved instrumental in further understanding microglial contribution to the CNS challenged with amyloidosis.

7.1. Extracellular trafficking and internalization of beta-amyloid

Though not a complete replicate of Alzheimer's disease, transgenic mouse models are nonetheless useful in understanding the genetics and biochemical cascades that lead to learning and memory deficits found in humans. As mentioned, the amyloid plaques in these models attract glia (150). As the brain's endogenous immunocompetent cells microglia are among the first cells recruited to the plaques (150, 155, 156). The beta-amyloid-protein from these plaques has been found inside microglial lysosomes indicating that these cells actively phagocytose portions of beta-amyloid plaques (156, 157). The process of internalizing beta-amyloid is mediated by several cells surface receptors. In the brain parenchyma, macrophage scavenger receptor Type A (MSR-A) is only expressed by microglia. Studies by Chung and coworkers, and others have shown that MSR-A is responsible for uptake of up to 60% of internalized fibrillar beta-amyloid (non-opsonized) in the brain (158, 159). Interestingly, MSR-A knock-out animals expressing hAPP with Indiana and Swedish mutations under PD control have similar amounts of plaque burden when compared to their littermates with normal MSR-A expression (160). Other receptors, such as MSR-B, and receptor for advanced glycation end products (RAGE) are capable of internalizing beta-amyloid, and it is likely that these compensate for the loss of MSR-A (160-162).

Another way in which beta-amyloid can be internalized is as a non-covalent conjugate to complement factors or antibodies (opsonization). Microglia have an assortment of receptors such as CD11b and Fc gamma receptors which can mediate the phagocytosis of opsonized beta-amyloid (158, 163). A recent study showed that APP is transported to cholesterol rich lipid rafts in neurons by low density lipoprotein receptors like protein (LRP) (164), however, its beta-amyloid cleavage product can be carried by high density lipoprotein like protein (HDL) in the extracellular space and then internalized by microglial LRP (165). Apolipoproteins E (ApoE) and J (ApoJ) in complex

with HDL-beta-amyloid reduce the eventual degradation of beta-amyloid in microglia (157).

Allelic differences in ApoE, along with mutations in APP and PS1/2 are among the most well defined genetic risk factors for familial AD, and it is interesting that microglia & astrocytes are the major contributors of extracellular ApoE in the brain (166). Release of ApoE into the extracellular space is dependent on protein prenylation and is sensitive to statin treatment (167). Micromolar concentrations of beta-amyloid can induce the secretion of ApoE from microglia *in vitro* (168). Conversely, the fibrillization of beta-amyloid is thought to be promoted by ApoE since the ApoE can bind to beta-amyloid (166, 169) and mice with the Indiana or Swedish mutations that have ApoE knocked out no longer have dense plaques or have delayed deposition of plaques, respectively (170, 171).

7.2. Lysosomal and non-lysosomal degradation of beta-amyloid

Frautschy *et al.* quantified up to five-fold increases in microglial density surrounding plaques in mice with the Swedish mutation under the prion promoter (156). El Khoury *et al.* demonstrated that elimination of CCR2 dependent microglial chemotaxis results in earlier appearance of amyloidosis, twice as much beta-amyloid42 and ~36% greater mortality in mice co-expressing the prion promoter Swedish mutation (155). A plethora of reports have provided evidence of mouse and human microglial degradation of beta-amyloid via the endosomal-lysosomal pathway (156, 161, 172). However the rate and quantity of this degradation is a subject of great concern as the kinetics of degradation (in relation to beta-amyloid deposition) has direct physiological relevance to Alzheimer's disease progression. Several articles from Maxfield's group have shown that microglial cells from neonatal mice degrade beta-amyloid at much slower rates compared to blood macrophages (173, 174). In an elegant series of experiments, they showed that in the course of three days, neonatal microglia *in vitro* degrade only 20% of the fibrillar beta-amyloid they are exposed to while peritoneal macrophages degrade close to 80%. While microglia and macrophages are able to make similar cleavages at the N-terminus of the fibrillar beta-amyloid, the macrophages were able to make far more thorough cuts along the beta-amyloid molecule. Neither cell type was able to cut the C-terminal portion of the molecule. Perhaps the localization of beta-amyloid's highly stable twisted beta pleated sheet at the C-terminus confers this resistance to degradation. This pleated sheet is the fundamental secondary structural element underlying multimers of beta-amyloid (175). The authors proposed that microglia are hindered in their capacity to degrade beta-amyloid, relative to their macrophage counterparts, due to an incomplete set of lysosomal enzymes (173). This is indirectly supported by the observation that microglial degradation of beta-amyloid *in vitro* is enhanced when global endocytosis of lysosomal enzymes is enhanced (173). It is worth mentioning that a recent study focusing on macrophages from the blood of AD patients concluded that these cells are impaired in their ability to phagocytose beta-amyloid when compared to non-diseased subjects (176).

TGF-beta1 is a cytokine that attracts and activates microglia. Bigenic mice overexpressing hAPP with Swedish and Indiana mutations under PD promoter in addition to TGF-beta1 (cSJL x B6D2 background) have a 50% reduction in brain parenchymal plaque burden compared to their non- TGF-beta1 overexpressing littermates (177). Since microglia exposed to TGF-beta1 *in vitro* display enhanced degradation of beta-amyloid, it is postulated that the marked *in vivo* reduction of beta-amyloid plaque burden in these TGF-beta1 transgenic mice is due to microglial action. TGF-beta1 and similar factors that stimulate microglia most likely cause degradation of beta-amyloid through either intracellular lysosomal degradation at the N-terminus, as already described, or through extracellular degradation via cell surface enzymes.

Neprilysin, a zinc dependant endopeptidase and insulin degrading enzyme (IDE), a zinc dependant metalloproteinase, are two well defined brain proteases. It is thought that microglia express these proteases on the cell surface and also release them into the extracellular space (172, 178). A series of reports have a built a compelling case for neprilysin being the major soluble beta-amyloid catabolic enzyme in AD animal models, in AD patients and in non-diseased humans (179, 180). Of note are reports quantifying 50% reductions in neprilysin mRNA in hippocampal regions classically susceptible to amyloidosis (181) and ~48% less mouse brain neprilysin mRNA in Tg2576 mice deficient in microglial recruitment to beta-amyloid plaques due to CCR2 knockout (155). As mentioned previously, these CCR2 knockout mice have twice as much beta-amyloid$_{42}$. Even though neprilysin is found in neurons, its major degradative function in the brain parenchyma appears to be microglial based. On the other hand, recent studies show that neprilysin cannot degrade the fibrillar beta-amyloid commonly found in dense plaques (182) or especially neurotoxic oligomeric beta-amyloid (183). Thus, neprilysin activity can modulate normal beta-amyloid catabolism *in vivo* (184) and prevent the onset or progression of AD, however, its specificity for less multimeric forms of beta-amyloid will likely prevent its use as a therapy for reversing the course of symptomatic AD.

The CCR2 knockout mice mentioned (155) have normal IDE mRNA levels. This suggests that neurons and astrocytes are able to supply basal levels of IDE when microglial function is perturbed (155). IDE is present in the cytosol where it degrades the cytoplasmic portion of APP (185), however cell surface IDE and secreted IDE are more likely the species of this protein responsible for extracellular beta-amyloid clearance (172). In 3-month-old mice lacking IDE (and not expressing APP transgenes), a 64% increase in cerebral endogenous beta-amyloid40 has been found (186). However in 16-month-old mice with the Swedish mutation under the prion promoter, astrocytes proximal to plaques display a two-fold increase in IDE immunoreactivity at the same time point diffuse beta-amyloid condenses into plaques (187). Surprisingly, there

was no reported reduction in plaque deposition (187). In these studies, microglia surrounding the plaques do not produce IDE at levels detectable by immunohistochemistry.

IDE is one of the leading drug candidates for AD therapy. A recent study suggested that chemical modifications in its active site for the purpose of keeping the enzyme in a constitutively open state should be pursued (188). This could result in a forty fold increase in catalytic activity and hence a therapeutic increase in Aß degradation. One must be prudent however as insulin is a major substrate for IDE degradation. Unlike other tissues, the brain does not maintain energy reserves so perturbations in sugar homeostasis caused by constitutively active IDE could result in severe side effects. This is less of an issue with neprilysin as a therapy since its other proteolytic substrates lie mostly outside the CNS compartment

8. CONSEQUENCES OF BETA-AMYLOID IMMUNIZATION AND INFLAMMATION

The field of AD research has experienced a significant disappointment following the termination of the beta-amyloid42 immunization (AN-1792) clinical trial due to life threatening inflammatory side effects (189). In contemplating the cause of meningoencephalitis which afflicted 6% of the study's subjects, one must consider the role of microglial interaction with beta-amyloid as a possible activating agent (190, 191). This is thought to be mediated via the binding of complement factor C1 conjugated with beta-amyloid to complement receptors CR3 & CR4 on the microglial cell surface (192) inducing a highly cytotoxic complement cascade. It is worth noting that 20% of the subjects in the trial had the desired antibody response to beta-amyloid immunization. However, in this subgroup, 22% suffered from meningoencephalitis. This suggests that the pathogenic inflammation that halted the study likely involved antibody-induced inflammation. Perhaps the antibody response changed the beta-amyloid to a species that is more inflammatory?

In vitro studies have shown that beta-amyloid can induce synthesis of inflammatory cytokines via a NfκB dependant pathway (192). Floden and colleagues reported that there are age related differences in the ability of different forms of beta-amyloid to induce inflammatory responses (193). Microglia isolated from neonatal and adult mice (C57BL/6 non-transgenics) are able to induce the secretion of TNFalpha when exposed to oligomeric beta-amyloid while fibrillar beta-amyloid can only induce TNFalpha production when exposed to neonatal microglia. If this finding holds true in humans, it could perhaps lend greater understanding to the AN-1792 trial - in the gray and white matter of AD patients with an antibody response to the beta-amyloid42 immunization, post-mortem analyses intriguingly found dramatic increases in soluble beta-amyloid as a result of antibody-dependent plaque disassembly. In fact, the quantity of soluble beta-amyloid increased fifteen-fold in one subject. This antibody-dependent disassembly of fibrillar beta-amyloid into soluble beta-amyloid caused an unintended increase in oligomeric species of beta-amyloid. The investigators found plaque-derived dimers, trimers, tetramers and higher order oligomeric structures of up to 30kDa in the brains of these patients. As described by Floden et al., oligomeric but not fibrillar species of beta-amyloid can selectively induce adult microglial production of proinflammatory TNFalpha (193) and directly cause neuronal death (190, 191).

In closing, it is clear that much remains to be learned about amyloid biochemistry and the immune response to its various forms. However, what is still lacking after many years of amyloid research is a plausible mechanism of how amyloid causes the specific kind of neurofibrillary neurodegeneration characteristically seen in AD brain, if in fact it does cause it. We tend to favor a mechanism whereby plaques and tangles develop independently from each other – plaques occur because of amyloid overproduction and impaired clearance by senescent microglial cells; tangles occur because senescent microglia provide insufficient trophic support to aged neurons. Thus, a common denominator can be found in microglial senescence and the microglial dysfunction hypothesis of AD (9).

9. SUMMARY AND PERSPECTIVES

For too long microglia have been misread as 'enemies within the CNS' (194), cells that, once activated, can endanger adult neurons and neurogenesis, and exacerbate primary CNS injuries caused by ischemia and trauma. Fortunately, the tide appears to be shifting and increasingly neuroscientists realize the neuroprotective roles microglia play, especially in the acutely injured CNS when they get activated. Anthropomorphically speaking, the cells can be likened to a SWAT team (7) in that their normal functions often involve dangerous tasks, as illustrated here by the example of long term iron storage. Thus, in the course of a human life microglia may be exposed to many hazards brought on by various environmental factors and these eventually take their toll on the microglial population as the person ages. It is no wonder that histopathological examination of the aged human brain reveals an abundance of degenerating microglia. Although the microglial dysfunction hypothesis remains an unproven theory, the evidence for it at this juncture is intriguing and certainly warrants additional studies into mechanisms that regulate the life and death of microglia. Once we understand the scenarios that lead to microglial degeneration, we will be able to manipulate them and hopefully devise ways of preventing microglial cell loss in the aging brain and therefore loss of neurons and cognition.

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