Abstract

Amyotrophic lateral sclerosis is a neurodegenerative disorder that results in the selective death of motor neurons in the central nervous system. This progressive motor neuron degeneration leads to death of the patient on average three to five years after onset of the disease. To date, no therapy is available. Many hypotheses have been formulated to explain the selective degeneration of motor neurons. One of the most studied hypotheses is the putative role of the inflammatory response that accompanies motor neuron death. The proliferation of microglia and astrocytes has been considered to be a secondary phenomenon, but recently, evidence is accumulating in favour of a contributory role of the non-neuronal cell populations to the pathogenesis of the disease.

In this review, we will introduce the characteristics of microglial cells in the central nervous system. We will summarize the evidence of the expansion and the activation of the microglial cell population that accompanies motor neuron degeneration. Finally, an overview will be given of the different therapeutic strategies that targeted the inflammatory process in amyotrophic lateral sclerosis.

Introduction

Amyotrophic lateral sclerosis (ALS) is, if not the most common adult onset neurodegenerative disorder by far the most devastating one. Affecting patients in their late fifties to sixties, the progressive and selective death of motor neurons leads to the death of the patient on average three to five years after the onset of the first symptoms due to respiratory insufficiency. Most patients with ALS have no familial history and are considered to have sporadic ALS. In a minority of 10% of patients, the disease is familial with most commonly an autosomal dominant pattern of inheritance, although autosomal recessive and X-linked patterns of inheritance have been described. In approximately 20% of familial cases (which represent only 1 to 2% of all cases) mutations in the superoxide dismutase-1 (SOD1) gene on chromosome 21q can be identified (Rosen et al., 1993). More recently, other motor neuron disease-causing mutations were discovered, such as in the genes encoding vesicle-associated membrane protein (VABP) (Nishimura et al., 2004), alsin (Hadano et al., 2001; Yang et al., 2001) and senataxin (Chen et al., 2004). However, the phenotype of these patients is different from classic ALS, although intrafamilial variability is present. Several other loci for classical ALS have been found, but additional disease-causing mutations still await further identification (Andersen, 2001; Majoor-Krakauer et al., 2003; Robberecht, 2000; Van Den Bosch and Timmerman, 2006). The etiology of sporadic ALS remains obscure, but recent evidence also stresses the important role of SOD1 (Gruzman et al., 2007).

To date, it remains enigmatic why motor neurons in ALS selectively degenerate, while other neuronal structures largely remain intact. The generation of a transgenic mouse model overexpressing mutant human SOD1 (Gurney et al., 1994) resulted in several hypotheses regarding the pathogenic cause (Brown and Robberecht, 2001; Brujin et al., 2004; Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006; Van Damme et al., 2005). These include oxidative damage, intracellular aggregation of mutant protein with secondary entrapment of other vital proteins, axonal strangulation by neurofilamentous accumulation, excitotoxic damage due to excessive glutamate stimulation and subsequent Ca²⁺-influx, mitochondrial dysfunction, decreased availability of growth factors (e.g. vascular endothelial growth factor (VEGF)) and inflammation (Fig. 1).

In this review, we will focus on the role of the microglial cells in the pathogenesis of ALS. A remarkable feature occurring in most neurodegenerative disorders is the marked expansion of both microglia and astroglia preceding and accompanying neuronal death (Eikelenboom et al., 2002; Garden, 2002; Rogers et al., 2002). Initially considered to be a secondary phenomenon due to motor neuron degeneration, evidence is accumulation in favour of an active contribution of microglia expressing mutant SOD1 to motor neuron degeneration (Beers et al., 2006; Boillee et al., 2006b; Clement et al., 2003).
Microglia

Microglia are enigmatic central nervous system (CNS) cells. They represent about 10% on average of the adult CNS cell population (Alliot et al., 1999). The origin of microglial cells in the brain is controversial, but there is evidence that they originate from circulating monocytes that early in development migrate from the yolk sac and invade the immature nervous system (Cuadros and Navascues, 1998). Throughout development, microglia are immunologically active and capable of responding to events associated with CNS organization and formation of the neuronal-glial environment (Bessis et al., 2006; Cuadros and Navascues, 1998; Moore and Thanos, 1996). Microglia, being the resident macrophages of the CNS, are part of the innate immune response. Innate immunity is naturally present and is not stimulated by antigens or mediated by antibodies. It is non-specific and is executed by the complement system and a variety of phagocytic cells including circulating neutrophils, monocytes and macrophages residing in tissues. In the healthy adult brain, microglia exist in a resting state as small cells with multiple small processes that extend towards all directions. Two recent studies showed that resting microglia are not inactive, but rather are highly mobile cells that monitor their environment with continuously moving processes (Davalos et al., 2005; Nimmerjahn et al., 2005). A consequence of this monitoring function is that microglia are well placed to respond to cues from surrounding cells.

Resting microglia become readily activated by both endogenous and exogenous factors (Nakamura, 2002; Raivich et al., 1998). Among endogenous factors are components of the complement system, cytokines, and chemokines, including interferon γ (IFNγ), tumor necrosis factor α (TNFα), macrophage-colony stimulating factor (M-CSF), granulocyte/macrophage (GM)-CSF, interleukin (IL)-1 and IL-6. A potent exogenous activator of microglial cells is the bacterial cell wall component lipopolysaccharide (LPS). Upon activation, microglia can produce a broad spectrum of factors that modulate the functions of surrounding immune cells (chemokines, immunogens, and proinflammatory factors), factors that are toxic to neurons (reactive oxygen species and pro-inflammatory factors) or even beneficial (neurotrophic factors) (Block and Hong, 2005) (Fig. 2). A large body of evidence supports the neurotoxic role of microglia in different neurodegenerative diseases (Boilée et al., 2006a; Garden, 2002; Liu and Hong, 2003; Streit, 2004). In an animal model of Parkinson’s disease, acute injection of LPS into three different regions of rat brain resulted in the largest neuronal death in the substantia nigra (SN), whereas chronic infusion of LPS also resulted in the selective loss of dopaminergic (DA) neurons. The observed difference in neuronal survival was suggested to reflect both the number of microglia present, which was highest in the SN and the higher vulnerability of DA neurons (Gao et al., 2002; Kim et al., 2000). In an in vitro model for Alzheimer’s disease, microglia were activated by β-amyloid (Aβ) resulting in neuronal death due to secretion of TNF-α, IL-1β and nitric oxide (NO) (Giulian et al., 1996). In contrast, in animal models...
of spinal cord injury, activated microglia were observed to enhance axonal regeneration (Prewitt et al., 1997; Rapalino et al., 1998), suggesting that microglia can also exert neuroprotective effects. Hence, the role of microglia is complex and the final outcome of their activation is likely to depend on the cause of microglial activation, the type of neuronal damage, the release of cytokines and the interplay with surrounding cells (Minghetti and Levi, 1998).

**Microglial proliferation in ALS**

Pathological studies already extensively described the increased number of activated microglia and astrocytes in ALS patients compared to controls (Henkel et al., 2004; Kawamata et al., 1992). Similar observations were done in the mouse model of ALS in which extensive microglial and astrogial proliferation was reported to accompany the motor neuron loss, already at early stages of the disease (Alexianu et al., 2001; Almer et al., 1999; Hall et al., 1998). The discovery of the benzodiazepine binding site for the isoquinoline PK11195 that is selectively expressed on activated microglia, but not on astrocytes, made in vivo imaging of activated microglia possible (Banati et al., 1997). Using positron emission tomography with the C11PK11195 ligand, Turner et al. recently documented the presence of activated microglia in the motor cortex, prefrontal cortex, thalamus and pons of ALS patients, whereas only a limited number of microglia was observed in control patients (Turner et al., 2004). Recently, the astrocytic proliferation in ALS patients was documented using deuterium-substituted C11(L)-deprenyl PET, a marker that binds to mono-amino-oxydase (MAO)-B, which in the CNS primarily is located in astrocytes (Johansson et al., 2007).

The increased number of non-neuronal cells in CNS of ALS patients and in the rodent model has long been considered to be a secondary response to motor neuron degeneration. Over recent years, strong evidence accumulated indicating that non-neuronal cells could be involved in the initiation and propagation of motor neuronal loss in ALS. Overexpression of mutant human SOD1 in motor neurons alone failed to cause a significant motor neuron loss in transgenic mice (Lino et al., 2002; Pramatarova et al., 2001). However, in a recent report by Jaarsma (Jaarsma, 2006) homozygous expression of motor neuron specific Thy1.2-SOD1G93A appeared to cause motor neuron death. Furthermore, transgenic mice expressing the mutant SOD1G93A under control of the GFAP promoter resulted in an astrocyte specific expression of the mutant protein, but again, in spite of astrogliosis, failed to result in a clinical phenotype or motor neuron death (Gong et al., 2000). In a very elegant, but methodological challenging study, Clement et al. injected wild type embryonic stem cells constitutively expressing the yellow fluorescent protein (YFP), into human mutant SOD1G93A transgenic blastocysts (Clement et al., 2003). This approach resulted in chimeric mice in which only part of the motor neurons and non-neuronal cells expressed the mutant protein.Interestingly, non-neuronal cells which were free of the mutant SOD1, delayed degeneration and significantly prolonged survival of mutant SOD1 expressing motor neurons. This study strengthened the role of both microglia and astrocytes expressing mutant SOD1 in the pathogenesis of ALS. Additionally, selective knock down of mutant SOD1 from microglial cells in the mutant SOD1 mouse model, significantly increased the life span of the animals. No effect was seen on the absolute number of microglial cells, suggesting that it is the presence of mutant SOD1 that renders the microglial cells neurotoxic (Boillee et al., 2006b). A similar conclusion was drawn from a study using a mouse model deficient of myeloid cells. When these myeloid deficient mice were crossed with mutant SOD1 mice, it was found that transplantation of wild type bone marrow attenuated disease progression compared to transplantation with mutant bone marrow (Beers et al., 2006).

**Microglia are activated in ALS**

In spite of the increasing amount of evidence in favour of the contributory role of microglia to motor neuron degeneration, it remains to be elucidated through which mechanism microglial cell exert their neurotoxic functions in ALS. One attractive hypothesis is that microglial cells become activated in response to the mutant SOD1 in their cytoplasm, resulting in the increased activation of intracellular pathways and the secretion of neurotoxic cytokines. This hypothesis was supported by the observation that the selective knock down of mutant SOD1 from microglial cells resulted in a significant increase in the survival of mutant SOD1 mice (Boillee et al., 2006b). The deleterious effect of mutant SOD1 was further supported by the observation that the mutant protein can be secreted and hence can influence the activation state of microglial cells (Urushitani et al., 2006). In vitro evidence indicated that mutant SOD1 containing microglial cells were more easily activated and produced higher levels of NO, compared to wild type SOD1 expressing microglial cells (Weydt et al., 2004). Chronic administration of LPS to mutant SOD1 mice resulted in increased microglial activation. This sustained activation of innate immunity exacerbated disease progression by 3 weeks and was accompanied by increased motor axon degeneration and microglial activation (Nguyen et al., 2004), underscoring the neurotoxic role of microglial activation. A large body of evidence
exists showing the presence in ALS patients and transgenic mice of factors that may activate microglia and of factors produced by activated microglia including TNF-α, IFN-γ, MCP-1, IL-1α, IL-6 and matrix metalloproteinase-9 (MMP-9) (Beuche et al., 2000; Elliott, 2001; Ferri et al., 2004; Henkel et al., 2004; Hensley et al., 2003; Poloni et al., 2000). Recently, studies using cDNA microarray and RNA protection studies showed that at the age of 80 days numerous inflammation-related genes, including inducible NOS (iNOS), cyclo-oxygenase-2 (COX-2), MMP-9, IL-1, IL-6 and TNF-α were upregulated in the mutant spinal cord, mostly attributed to microglial activation (Hensley et al., 2002; Yoshihara et al., 2002). Moreover, upregulation of the pro-apoptotic p38 MAPK (mitogen activated protein kinase) pathway that mediates microglial activation (Koistinaho and Koistinaho, 2002; Tikka et al., 2001) and down-regulation of the anti-apoptotic Akt pathway (Dewil et al., 2007b), have been described in ALS. We and others reported that microglial cells already at the age of 80 days, when no motor neuron loss had occurred yet express increased levels of the p38 MAPK (Dewil et al., 2007a; Tortarolo et al., 2003), indicating that they were activated. Expression levels remained significantly elevated in the mutant spinal cord compared to the expression levels in the spinal cord of wild type SOD1 mice. A sustained phosphorylation of p38 MAPK also occurred in astrocytes and to a lesser extent in motor neurons (Dewil et al., 2007a; Tortarolo et al., 2003).

When microglial cells become activated, their morphology changes from highly ramified cells towards plump cells with shrunken processes. As they are immune cells the surface receptor profile reflects their activation state (Fig. 2). We recently observed that microglial cells acquire characteristics of dendritic cells at the age of 80 days, as they express CD11c and CD86 (Dewil et al., 2007c). These results were in line with a previous report by Henkel et al. (Henkel et al., 2004), who provided evidence of increased dendritic cell receptor transcripts in the spinal cord of human ALS patients. The number of dendritic cells present in the affected tissue correlated with the rate of disease progression. Moreover, evidence suggests that immature myeloid cells are recruited to the spinal cord and brain as disease progresses to take part in the immune response (Dewil et al., 2007c). This recruitment may be facilitated by the recently described disturbance of the blood brain barrier early in the disease (Garbuzova-Davis et al., 2007).

**Activated microglial cells as therapeutic target**

Since microglial activation and neuro-inflammatory appear to be involved in ALS, different therapeutic strategies have been designed to suppress the inflammatory response. Our research group and others showed that treatment of mutant mice with the semi-synthetic tetracycline, minocycline, not only improved the motor performance of the mutant SOD1 mice, but also delayed onset of the disease and significantly increased survival of the animals, compared to placebo-treated littermates (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002). The precise mechanism of action of minocycline still remains to be fully elucidated, but evidence exists in favour of inhibition of microglial activation by inhibiting the phosphorylation of p38 MAPK (Tikka et al., 2001; Tikka and Koistinaho, 2001; Zhu et al., 2002). However, as pharmacokinetics are different between rodents and humans, extrapolation of these results to humans should be preceded by further research. To study the effect of selective inhibition of p38 MAPK activation, we recently treated mutant SOD1 mice with semapimod, a compound under development as a treatment for a variety of inflammatory diseases characterized by p38 MAPK upregulation (Akerlund et al., 1999; Denham et al., 2000; Hommes et al., 2002). The inhibition of p38 MAPK phosphorylation was paralleled by a significant attenuation of motor neuron and axonal loss in the ventral horn and the ventral root, respectively. The obvious effect of semapimod on motor neuron survival was contrasted with its moderate effect on survival of the animal (Dewil et al., 2007a). This observation suggests that the anti-apoptotic effect of inhibition of p38 MAPK activation most likely rescues the motor neuron perikaryon and to a lesser degree the proximal axon, but that distal denervation progresses, underscoring the notion that dysfunction of the distal neuromuscular compartment is the major determinant of progression and severity in ALS (Fischer et al., 2004; Sagot et al., 1995).

Inhibition of the pro-inflammatory enzyme COX-2 with nimesulide and celecoxib yielded also a delay of the onset of the disease (Drachman et al., 2002; Pompl et al., 2003), whereas a combination trial with creatine and celecoxib extended survival of the mutant mice even more significantly (Klivenyi et al., 2004). Surprisingly, the absence of interleukin-1β had no effect on the life span nor on the extent of motor axon degeneration of mutant SOD1 mice, when crossbred with interleukin-1β knock out mice (Nguyen et al., 2001). Administration of thalidomide, a potent anti-inflammatory and immune modulatory drug, whose effects include inhibition of TNF-α synthesis, delayed death in mutant SOD1 mice (Kiaei et al., 2006). However, it was recently reported that the selective disruption of TNF-α did not influence disease progression, survival and glial proliferation in the mutant SOD1 mouse, suggesting that TNF-α alone is not a key factor in motor neuron degeneration caused by SOD1 mutations (Gowing
Similarly, treatment of mutant mice with an inhibitor of MMP-9, a matrix metalloproteinase thought to play a role in the pathogenesis of several neurological disorders, such as multiple sclerosis, stroke, Alzheimer’s disease, Parkinson’s disease and ALS (Asahi et al., 2000; Lorenzl et al., 2003; Lukes et al., 1999; Yong et al., 2001), yielded a protective effect on survival (Lorenzl et al., 2006). However, the effect of the pharmacological inhibitor was not selective and partial. Therefore, we crossbred MMP-9 knock out mice with mutant SOD1 mice and observed that the absence of MMP-9 enhanced mutant SOD1-induced motor neuron disease rather than delaying it (Dewil et al., 2005).

In another transgenic model, the absence of MMP-9 appeared to be beneficial rather than deleterious (Kiaei et al., 2007), underscoring the complexity of interference with inflammatory pathways.

### Conclusion

Evidence is accumulation indicating that motor neuron death in ALS is a non-cell autonomous process. Initially considered to be a secondary phenomenon, an increasing number of studies supports the contributory role of non-neuronal cells in general and of microglial cells, in particular, to selective motor neuron degeneration. Observational studies reported the early increase of microglial cells in the spinal cord of mice and man. The microglial activation is accompanied by the increased expression of a pleiad of pro-inflammatory molecules that can either induce or enhance progressive and selective motor neuron degeneration. As a consequence, the non-neuronal cell population and the potentially neurotoxic factors released by these cells are a very attractive target for therapeutic strategies. However, as demonstrated by the variable and sometimes contradictory effects of modulation of the microglial activation and the downstream factors they produce, the inflammatory response in ALS is a very complex phenomenon.

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