1 h was up-regulated to become 11.2-fold higher than that in the control cells (Fig. 7a). However, the incubation of the cells with 1 μM NE alone did not affect IκBα mRNA expression at all (Fig. 7a). One-hour incubation of the cells with a cocktail of 0.1 μg/mL LPS and 1 μM NE suppressed the enhanced IκBα mRNA expression caused by the incubation with 0.1 μg/mL LPS alone to 80% of its value (Fig. 7a). The responses of IκBβ mRNA in the cells to LPS and/or NE were almost the same as those of IκBα mRNA, except for the fact that the incubation of the cells with the combination of LPS and NE did not suppress the enhanced IκBβ mRNA expression caused by LPS alone (Fig. 7b). The NF-κB mRNA expression levels in the cells were not affected at all by the 1-h incubation with LPS and/or NE (Fig. 7c).

Discussion

As the first step of a series of experiments, the expression levels of pro-inflammatory cytokines in the LC samples were examined. Under normal conditions, the expression levels of IL-1β and TNF-α mRNAs in the LC were very low (Fig. 1). An i.p. injection of LPS dramatically increased their respective expression levels to values more than 10-fold higher and 4-fold higher than those obtained with the vehicle i.p. injection at 4 h post-injection (Fig. 1). Kaneko *et al.* (2001) previously reported that NE turnover in the LC was enhanced to a statistically significant level within 2–4 h after an i.p. injection of LPS and reached its maximum level at 4 h after the injection. It should be noted that the time profile of NE production in the LC in that study was synchronous with those profiles of IL-1β and TNF-α mRNA expression at the site in our present study. We thought that this increased NE might act to down-regulate the mRNA expression of IL-1β and TNF-α.

Microglia comprise up to 20% of the total glial cell population in the brain, and these cells can be readily transformed to an activated state in response to a wide range of stimuli (Nakamura *et al.* 1999). It is well known that microglia are activated prior to astrocytes when an infection occurs in the brain (Kreutzberg 1998). Because the increases in expression of IL-1β and TNF-α mRNAs in the LC happened very quickly after the LPS injection (Fig. 1), we suspected that microglia were activated in the LC. Therefore, microglia from mouse neonate brains were prepared in order to elucidate whether there is a close correlation between the increase in NE turnover and the up-regulation of pro-inflammatory cytokines. The mRNA and protein levels of IL-1β and TNF-α in microglia were vigorously elevated by the incubation with LPS (Figs 3–6).

NF-κB is one of the transcription factors involved in the transcription of the *IL-1β* gene (Cogswell *et al.* 1994) and *TNF-α* gene (Collart *et al.* 1990). The activation of NF-κB can be estimated by the mRNA induction level of IκB, which functions as an inhibitor of NF-κB in living cells, because the IκB mRNA expression level itself is regulated by NF-κB (Sen and Baltimore 1986; Sun *et al.* 1993). As shown in Fig. 2(a) and Fig. 7(a), IκBα mRNA expression levels were enhanced by LPS stimulation both *in vivo* and *in vitro*. Hence, our results suggest that the enhanced level of IκBα mRNA reflects NF-κB activation, and that the enhanced mRNA expression of IL-1β and TNF-α in microglia incubated with LPS was caused by NF-κB activation.

As shown in Figs 3–6, the effects of NE on the upward expression of mRNA and protein of IL-1β and TNF-α in microglia caused by the incubation with LPS were different between these two pro-inflammatory cytokines. NE enhanced the expression of IL-1β synergistically with LPS, whereas it suppressed the expression of TNF-α that was enhanced by the incubation with LPS. The regulation mechanisms for IL-1β and TNF-α must be explained by different scenarios.

The synergistic effect of NE and LPS on IL-1β expression may be explained by the scenarios in which the binding of NE to β adrenergic receptors on microglia is followed by an enhanced activation of the MAP kinase via cAMP-protein kinase A-dependent pathway (Tanaka *et al.* 2002; Woo *et al.* 2003). Because transcription factor NF-κB regulates both consensus and non-consensus cAMP response element sites in the promoter region of the *IL-1β* gene, NF-κB plays multiple roles in the induction of IL-1β transcription (Cogswell *et al.* 1994).

It is also known that cAMP-elevating agents such as prostaglandin E_2 suppress TNF-α expression in microglia (Aloisi *et al.* 1999; Petrova *et al.* 1999; Kim *et al.* 2000; Facchinetti *et al.* 2003). However, it is reported that protein kinase A-independent pathways may have the major role in the regulation of TNF-α expression in microglia (Woo *et al.* 2003). According to Woo *et al.* (2003), dibutyryl cAMP suppressed NF-κB-mediated transcription of the *TNF-α* gene in microglia. As shown in Fig. 7(a), the addition of NE suppressed the LPS-induced augmentation of IκBα mRNA expression in cultured microglia. Taken together, these data support the view that NE exerts a protective effect against the insults to nerve cells by inhibiting NF-κB activation in microglia.

Because mature IL-1β protein was not released into culture media by LPS treatment, we investigated the expression level and the activity of caspase-1 in primary cultured microglia under LPS and/or NE treatment. The expression level of caspase-1 mRNA in microglia was not affected by LPS and/or NE treatment (data not shown). On the immunoblot, the expression level of 45 kDa caspase-1 precursor protein was not affected by LPS and/or NE treatment. Furthermore, 45 kDa caspase-1 precursor protein in microglia under LPS treatment was not cleaved to produce 10 and 20 kDa subunits (data not shown). In addition, the enzymatic activity of caspase-1 in microglia could not be detected. These data suggest that the activation of caspase-1

in microglia was not to a level that allows the precursor of IL-1β protein to be converted into the secreted form. These data coincided well with the observation that, in spite of the upward expression of IL-1β protein in microglia stimulated by LPS and/or NE, IL-1β protein was not secreted into culture supernatant. The release of processed IL-1β from isolated macrophages was also relatively inefficient, although endotoxin induced the formation of large intracellular stores of pro-IL-1β (Wewers *et al.* 1984). Extracellular ATP has emerged as an inducer of rapid processing and massive release of endotoxin-induced IL-1β in macrophages and microglia (Hogquist *et al.* 1991; Walz *et al.* 1993; Perregaux and Gabel 1994; Griffiths *et al.* 1995; Andrei *et al.* 2004; Kahlenberg and Dubyak 2004; Suzuki *et al.* 2004). Therefore, a sufficient amount of ATP might have to be supplied to the purinergic receptors on microglia that gather in the vicinity of injured nerve cells.

As already described briefly in the Introduction, the noradrenergic regulation of IκBα expression and inflammatory gene expression is relevant to some clinical aspects of Alzheimer's disease, such as the damage to or loss of NE neurons in the LC (Mann *et al.* 1983), to the protection of animal models from experimental autoimmune encephalomyelitis by treatment with a β adrenergic receptor agonist (Chelmicka-Schorr *et al.* 1989; Wiegmann *et al.* 1995), to the decreased levels of β2 adrenergic receptors in astrocytes in multiple sclerosis patients (Zeinstra *et al.* 2000). In this study, we characterized the plausible mechanisms for the regulation of pro-inflammatory cytokines at the LC. Our data suggest that NE exerts neuroprotective and anti-inflammatory actions, mainly by way of the inhibition of TNF-α production in microglia. These findings will be useful for constructing new tactics to treat neurodegenerative disorders in which microglial activation and inflammatory responses are directly linked to their pathological phenomena.

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Inflammatory Process in Parkinson's Disease: Role for Cytokines

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Abstract: Parkinson's disease (PD) is a movement disorder caused by degeneration of the nigrostriatal dopamine (DA) neurons in the substantia nigra pars compacta and the resultant deficiency in the neurotransmitter DA at the nerve terminals in the striatum. We and other investigators found increased levels of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, and IL-6, and decreased levels of neurotrophins such as brain-derived neurotrophic factor (BDNF) in the nigrostriatal region of postmortem brains and/or in the ventricular or lumbar cerebrospinal fluid (CSF) from patients with sporadic PD, and in animal models, such as 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)- and 6-hydroxydopamine (6-OHDA)-induced PD. These changes in cytokine and neurotrophin levels may be initiated by activated microglia, which may then promote apoptotic cell death and subsequent phagocytosis of DA neurons. Cytokines as pleiotropic factors, promote signals that either lead to cell death or exert neuroprotective effects. The discovery of toxic changes in trophic microglia by M. Sawada and co-workers is important to this point. Ultimately, microglial cells may regulate cellular changes that cause either harm or benefit by producing cytokines or neurotrophins depending upon the primary cause and the circumstances during the inflammatory process of PD.

Key Words: Parkinson's disease, inflammation, microglia, astroglia, cytokines, neurotrophins, apoptosis

1. INTRODUCTION

Parkinson's disease (PD), named after the English physician, James Parkinson, who defined it in 1817, is characterized by specific degeneration of the dopamine (DA) neurons in the substantia nigra pars compacta and the resulting loss of the nerve terminals, which is accompanied by a deficiency in the neurotransmitter DA, in the striatum. This DA deficiency is responsible for most of the movement disturbances called parkinsonism, i.e., muscle rigidity, akinesia, and resting tremor. Familial PD (PARK1-PARK10, NR4A2), for which the causative genes and their chromosomal locations have been identified, constitutes a small percentage of PD cases (approximately 5~10%); and most PD is sporadic without hereditary history. Sporadic PD is also called "idiopathic, " because its pathogenesis remains unknown. PD is the second most common aging-related neurodegenerative disease after Alzheimer's disease (AD). Sporadic PD is characterized by the presence of intracellular eosinophilic inclusion bodies called Lewy bodies, which were named after the German physician who described them in 1913.The pathogenesis of sporadic PD is still enigmatic; and many factors are speculated to operate in the mechanism of cell death of the nigrostriatal DA neurons in PD, e.g., oxidative stress and cytotoxicity of reactive oxygen species (ROS), disturbance of intracellular calcium homeostasis regulated by the excitatory neurotransmitter glutamate *via* NMDA receptors, and endogenous or exogenous neurotoxins [1-3].

In the case of familial PD, possible causative factors have recently been defined: *alpha-synuclein* in autosomal-dominant PARK 1 [4]; *parkin*, which encodes a ubiquitin ligase E3, in autosomal-recessive juvenile (early-onset) PARK 2 [5, 6]; *UCH-L1* (ubiquitin C-terminal hydrolase) in autosomal-recessive PARK 5 [7]; *PINK1* in autosomal-recessive PARK 6 [8]; DJ-1 in autosomal-recessive PARK 7 [9]; and *Nurr1* (nuclear receptor-related 1) in autosomal-dominant NR4A2 [10]. The main component of PD-associated intracellular inclusions, Lewy bodies, is alpha-synuclein. The intracellular accumulation of abnormal "unfolded" or "misfolded" proteins consequential to dysfunction of the ubiquitin proteasome system in familial PD produces endoplasmic reticulum (ER) stress, which might subsequently lead to oxidative stress and finally presumed apoptotic cell death (programmed cell death). These findings from studies on familial PD may also give important clues for elucidating the signaling pathway, initiated by possible initiation factors such as oxidative stress, which triggers presumed apoptotic neuronal death in sporadic PD.

However, most cases of sporadic PD start in the elderly in their sixties or seventies and slowly progress over a long period of 10 to 20 years. This characteristic suggests that some continuous pathological process may exist in PD. The presence of activated glial cells, especially reactive microglia, in the substantia nigra in PD, suggests that inflammation *via* reactive glial cells may be an important process that promotes the progressive neurodegeneration of the DA neurons. The inflammatory response in PD, called "neuroinflammation," is different from classical inflammation, which is the defense reaction of living tissues to injury. "Neuroinflammation" is assumed not to be accompanied by dilatation of blood vessels and emigration of leukocytes from the blood to the brain, which is characteristic of the classical inflammation. In the peripheray, the typical initial reaction of classical inflammation consists primarily of changes in the blood vessels, the escape of cells and fluid from the blood

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into the tissues, and subsequent changes in the tissue; but thereafter, chronic inflammation ensues, characterized by exudation of lymphocytes and macrophages with increasing fibrosis [11]. In PD, the blood vessels are assumed to be intact with normal blood-brain barrier functions, although this is a controversial issue. Also controversial, is the exact mode of cell death in PD. At least in certain cases the DA neuronal death in PD appears to be related to the process of apoptosis (programmed cell death). Apoptosis affects isolated cells scattered in normal or diseased tissues and at first elicits no classical inflammatory reaction.

The concept of neuroinflammation and the elucidation of the changes in the glia-neuron network and interaction of cytokines and neural growth factors, i.e., neurotrophins, in PD gained much attention during the 1990s [16], and neuroinflammation is now considered to be fundamental to at least the progression if not the pathogenesis of PD. Thus, elucidation of the mechanisms underlying neuroinflammation in PD may contribute to new and effective therapies [18, 19]. As the first features of inflammation in PD, McGeer and the collaborators reported an increased numbers of major histocompatibility complex (MHC) class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive microglial cells in the substantia nigra [12, 13]. In agreement with this finding, we also found that the level of beta 2-microglobulin, the light chain of MHC class I molecules, was higher in the striatum of PD patients than in that of control subjects [14]. High expression of mRNAs of MHC class I heavy chain and beta2-microglobulin were observed in nigral DA neurons and brainstem motoneurons in adult rats, and these neurons also displayed interferon (IFN)-gamma receptor mRNA [15]. The MHC initiates and propagates the immune response. MHC class I molecules present antigens to CD8 cytotoxic T cells (CTL), whereas MHC class II do so to CD4 positive helper T cells (Th1 and Th2). Not only microglia, but also astrocytes supposedly play a role in the loss of DA neurons, although a lesser one than microglia [16, 17].

Recent findings support the hypothesis that the process of DA cell death either in sporadic PD or in familial PD might be programmed cell death, i.e., apoptosis [20-23]; although this is still a controversial issue [24, 25]. We and other researchers have found changes in the levels of pro-inflammatory cytokines, anti-apoptotic neurotrophins and factors related to programmed cell death, specifically, in the nigrostriatal region of postmortem brain and/or in the ventricular or lumbar cerebrospinal fluid (CSF) from patients with sporadic PD or from animal models of PD such as mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD or rats with 6-hydroxy-DA (6-OHDA)-induced PD.

The findings concerning familial PD (effects of causative genes) and sporadic PD (activated glial cells that may produce pro-inflammatory cytokines, anti-apoptotic neurotrophins and apoptosis-related factors) may be mutually related, and consequently may provide a clue as to the effect of neuroinflammatory responses on neuronal death not only in PD, but also in other neurodegenerative diseases such as Alzheimer's disease (AD). Both of these diseases are speculated to be "protein conformational diseases" caused by the accumulation of "unfolded or misfolded proteins" with abnormal protein conformations, possibly as the result of a compromised ubiquitin proteasome system.

2. IMMUNE RESPONSE AND CYTOKINE CHANGES IN CEREBROSPINAL FLUID IN PARKINSON'S DISEASE

The brain is generally considered to be an "immune privileged" site, i.e., one free from immune reactions, since it is protected behind the blood-brain-barrier. However, accumulating findings have revealed that immune responses may occur in the brain, especially due to activation of the microglia that are known to produce pro-inflammatory cytokines. If a significant immune response and changes in cytokine levels can be detected in the CSF during the course of PD, cytokines would become valuable clinical markers. However, since the immune response-mediated inflammatory changes, if present, may be localized mainly in the nigrostriatal region of the brain in PD, representative detection of cytokines in the CSF would be difficult.

Wachter's group first reported that neopterin(N) is released from macrophages during immune responses and is a sensitive index of immune reactions [26]. Activated microglia in the brain also produce this molecule. Neopterin is a metabolite formed *via* D-erythro-6, 7-dihydroneopterin (NH2) from D-erythro-6, 7-dihydroneopterin triphosphate (NH2P3), which is synthesized as the main pteridine in microglia. It is also an intermediate in the biosynthesis of (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4), the cofactor of pteridine-dependent monooxygenases (phenylalanine hydroxylase [PAH] for phenylalanine degradation in the liver [27], tyrosine hydroxylase [TH] for catechloamine biosynthesis [28], and tryptophan hydroxylase [TPH] for indoleamine biosynthesis [29]. Likewise, neopterin is also an intermediate of nitric oxide synthase (NOS) for NO biosynthesis [30, 31]) in neurons having catecholamines [DA, noradrenaline/norepinephrine, adrenaline/epinephrine], indoleamines [serotonin (5-HT), melatonin] and nitric oxide (NO) as neurotransmitters. The reaction mechanism of TH, TPH, and PAH coupled with BH4 as a cofactor is essentially similar, but that of NOS seems to be more complex (Fig. **1**). More precise reactions of TH coupled with BH4 are shown in Fig. (**2**). BH4 is synthesized from guanosine triphosphate (GTP) *via* 3 enzymes, GTP cyclohydrolase I (GCH1), 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. Catecholamines (DA, noradrenaline/norepinephrine, adrenaline/epinephrine) are synthesized from tyrosine. TH is the key enzyme for DA biosynthesis. TH in nonprimate animals is generally a single protein encoded by a single TH gene consisting of 13 exons. Only the human TH gene has an extra exon 2, giving it 14 exons; and this TH produces 4 isoform proteins (human TH type 1 [hTH1] to type 4 [hTH4]) [32, 33]. In contrast, the monkey TH gene, lacking the extra exon 2 of humans, has 2 isoform proteins (TH1 and TH2)[34], arising by alternative mRNA splicing. An interesting question is whether the multiple isoforms of TH in humans and monkeys bear any relation with the fact that humans and monkeys are highly sensitive to the PD-producing neurotoxin MPTP. BH4 formed in catecholamine neurons is metabolized *via* quinonoid BH4 (formed in the TH reaction) and 7, 8-dihydrobiopterin (BH2) to biopterin

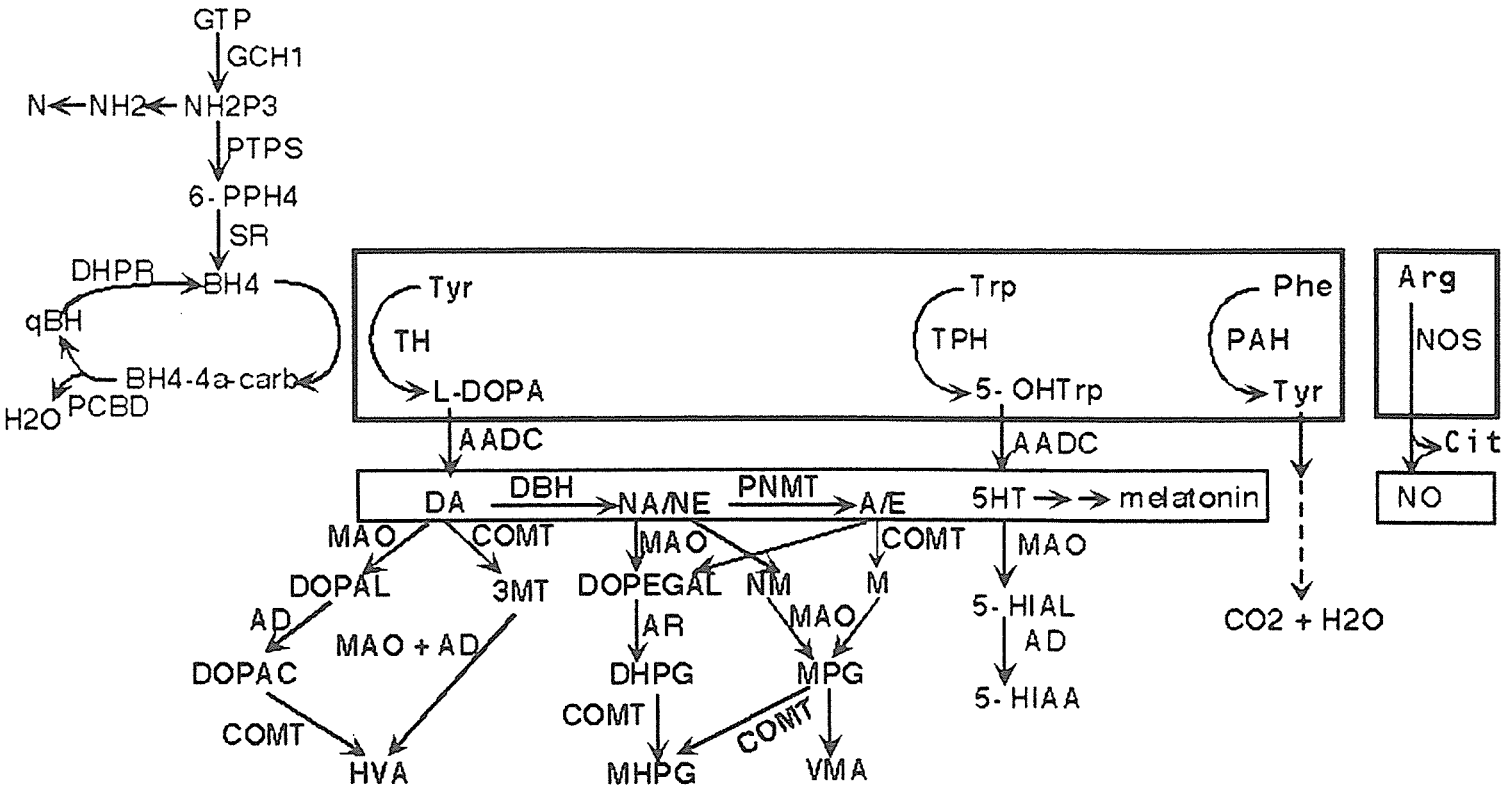

Fig. (1). Metabolism of catecholamines (dopamine, noradrenaline, adrenaline), indoleamines (serotonin, melatonin), phenylalanine, and nitric oxide in relation to tetrahydrobiopterin metabolism by pteridine-dependent monooxygenases (tyrosine hydroxylase, tryptophan hydroxylase, phenylalanine hydroxylase, and nitric oxide synthase).

A/E: adrenaline/epinephrine, AADC: aromatic L-amino acid decarboxylase, ALD: aldehyde dehydrogenase, ALR: aldehyde reductase, BH4: tetrahydrobiopterin, BH4-4a-carb: tetrahydrobiopterin-4a-carbinolamine, qBH2: quinonoid dihydrobioptein, Cit: citrulline, COMT: catechol O-methyltransferase, DA: dopamine, DHPG: 3, 4-dihydroxyphenylglycol, DHPR: dihydropteridine reductase, DOPA: 3, 4-dihydroxyphenylalanine, DOPAC: 3, 4-dihydroxyphenylacetic acid, DOPAL: 3, 4-dihydroxyphenylacetaldehyde, DOPGAL: 3, 4-dihydroxyphenylglycolaldehyde, E: epinephrine, GCH1: GTP cyclohydrolase I, GTP: guanosine triphosphate, 5HIAA: 5-hydroxyindoleacetic acid, 5HIAL: 5-hydroxyindoleacetaldehyde, 5HT: 5-hydroxytryptamine, serotonin, HVA: homovanillic acid, M: metanephrine, MAO: monoamine oxidase , MHPG: 3-methoxy-4-hydroxyphenylglycol, MOPGAL: 3-methoxy-4-hydroxyphenylglycolaldehyde, 3MT: 3-methoxytyramine, NA/NE: noradrenaline/norepinephrine, NM: normetanephrine, PAH: phenylalanine hydroxylase, PCBD: pterin-4a-carbinolamine dehydratase, Phe: phenylalanine, 6-PPH4: 6-pyruvoyltetrahydropterin, PTPS: 6-pyruvoyltetrahydropterin synthase, SR: sepiapterin reductase, TH: tyrosine hydroxylase, TPH: tryptophan hydroxylase, Trp: tryptophan, Tyr: tyrosine, VMA: vanillylmandelic acid.

(B) (Fig. **2**) [35]. The high contents of TH, BH4, GCH1, and sepiapterin reductase (SPR, the last enzyme acting in BH4 synthesis) in the nigrostriatum of the human brain suggest that the nigrostriatal DA neurons produce the largest amounts of BH4 and thus biopterin in the brain [36-38].

In our earlier studies, we found that, during the progression of PD, the ratio of total neopterin (NH2P3+NH2+N) to total biopterin (BH4+BH2+B) in the lumbar CSF gradually increased, even though the absolute amounts of total biopterin and total neopterin were significantly decreased as compared with those of control subjects. During the progression of PD the contents of total biopterin and total neopterin seen in CSF may be decreased due to the loss of nigrostriatal DA neurons. However, activated microglia, if present, may release neopterin, resulting in the increased ratio of total neopterin to total biopterin seen in the CSF [39]. Activation and production of neopterin from microglia in the PD brain are thought to occur in the nigrostriatal region. We found that the activity of GCH1, the first and rate-limiting enzyme for BH4 biosynthesis, in DA neurons was decreased specifically in that region [40]. Since GCH1 activity is high in the nigrostriatal DA neurons, large amounts of BH4 and the intermediate neopterin are produced there; and their concentrations in the nigrostriatum may be decreased due to the degeneration of the DA neurons. Thus, the increase in the ratio of total neopterin to total biopterin in the CSF during the progression of PD may reflect neuroinflammation accompanied by microglial activation and neopterin release in the nigrostriatal region. This concept agrees with the fact that, in patients with autosomal-dominant GCH1 deficiency/DOPA responsive dystonia (DRD) without DA cell death, both biopterin and neopterin concentrations in the CSF are decreased in parallel without a change in the neopterin / biopterin ratio [41]. This condition is also called "Segawa's disease, " which was first described by Segawa in 1971 as "hereditary progressive dystonia with marked diurnal fluctuation (HPD)" [42, 43].

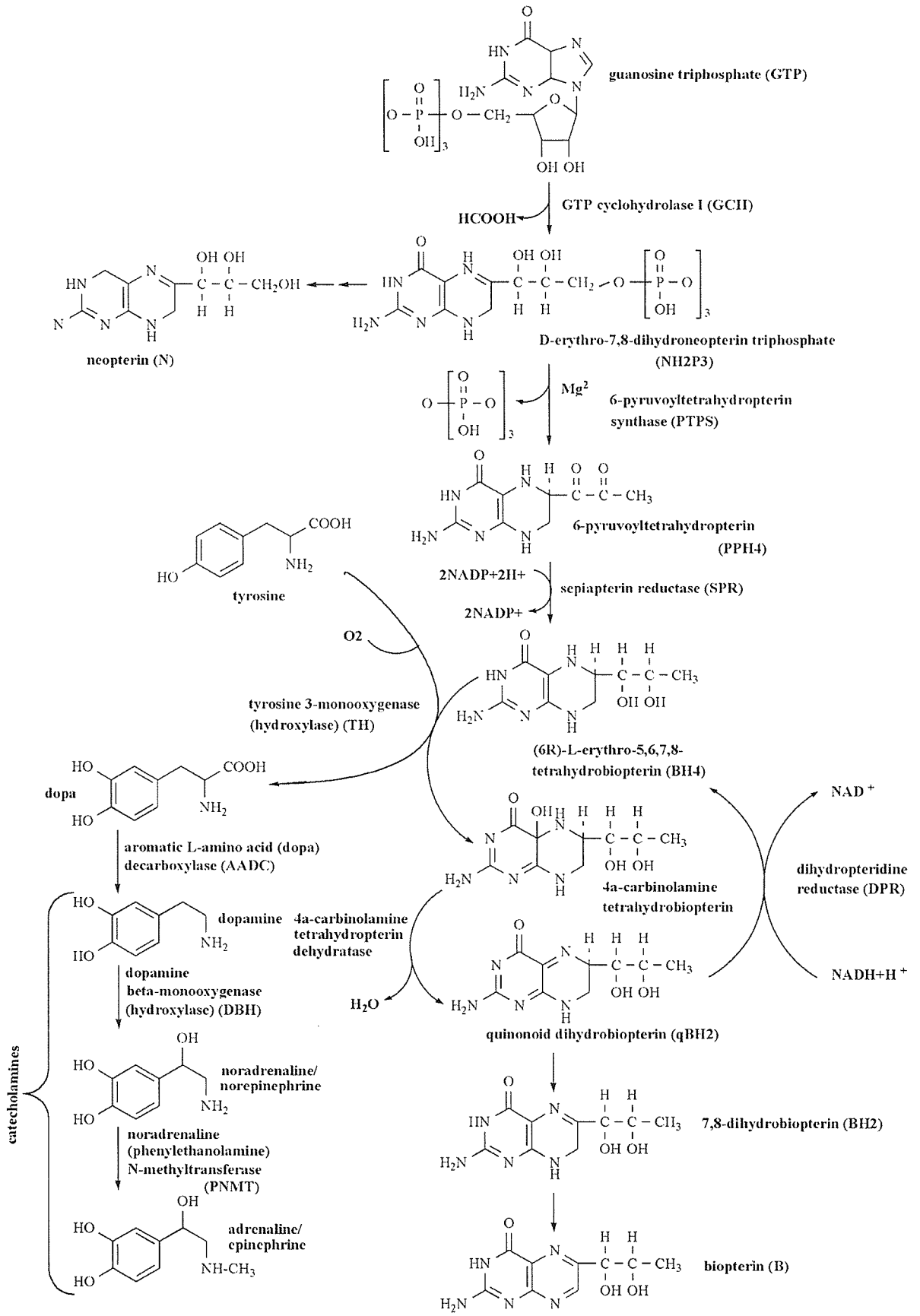

Fig. (2). Biosynthesis of catecholamines (dopamine, noradrenaline, adrenaline) in relation to biosynthesis of tetrahydrobiopterin, the cofactor of tyrosine hydroxylase.

Ichinose *et al.* [44, 45] cloned the human GCH1 gene, and discovered mutations in the GCH1 gene that caused a partial deficiency (2-20% of the normal value) in GCH1 activity and BH4 levels. Decreased GCH1 activity not only decreases BH4 levels but also decreased TH protein, as indicated by a study on BH4-deficient mice [46] as well as by one on the postmortem striatum of patients with autosomal-dominant GCH1 deficiency [47]. In this deficiency only 1 allele of the GCH1 gene is mutated and there is a lack of degeneration of nigrostriatal DA neurons. However, DA levels are decreased specifically in the nigrostriatal DA neurons; and the dystonia is controllable for many years by DA supplementation with L-DOPA without the development of dyskinesia (i.e., the "on-off" effect of L-DOPA, as frequently observed during L-DOPA therapy in PD patients) [42, 43]. In contrast, in autosomal-recessive GCH1 deficiency, which was first described by Niederwieser *et al.* in 1984 [48], both alleles of the GCH1 gene are mutated, resulting in GCH1 activity that is less than 2% of the normal value and in decreased levels of all of the neurotransmitters (DA, noradrenaline, adrenaline, serotonin, NO) produced by pteridine-dependent enzymes. The consequences are clinical phenotypes that are completely different and more severe than the phenotype of autosomal-dominant GCH1 deficiency: parkinsonism, hyperphenylalaninemia, muscle hypotonia, fever episodes, and epilepsy [44, 48]. Neither autosomal-dominant GCH1 deficiency nor autosomal-recessive sepiapterin reductase deficiency, a newly recognized BH4 deficiency disease recently reported by Blau *et al.* [49], show hyperphenylalaninemia, thus suggesting adequate PAH activity in the liver.

Autosomal-recessive TH deficiency was first reported in Germany in 1995 as autosomal-recessive DOPA-responsive dystonia or autosomal-recessive Segawa's syndrome [50, 51]. Several mutations were found in the human TH gene in this disorder; and the clinical symptoms depended upon the degree of decrease in TH activity ranging from mild DOPA-responsive dystonia to severe DOPA-nonresponsive parkinsonism [52]. Complete loss of TH would be lethal, for TH(-/-) mice were found to die around birth [53, 54] and Kobayashi *et al.* [53] found this neonatal death to be caused by cardiac dysfunction owing to noradrenaline deficiency in the sympathetic noradrenaline neurons innervating the heart. In the case of autosomal-dominant GCH1 deficiency, DA neuronal death does not occur; DA transporter levels are normal, as determined by single-photon emission computed tomography [57]. However, there is an indication of degeneration in patients with autosomal-recessive DOPA-nonresponsive TH deficiency [52] and this deficiency was similar to that reported for autosomal-recessive juvenile familial PD (PARK2) caused by mutations of the *parkin* gene [5]. However, it should be underscored that differential diagnosis among autosomal-dominant GCH1 deficiency, autosomal-recessive TH deficiency, and autosomal-recessive juvenile PARK2 are sometimes difficult [55, 56].

GCH1, which is the first enzyme involved in the biosynthesis of BH4, is colocalized with TH in the nigrostriatal DA neurons [36]. Choi *et al.* [58] proposed a hypothesis that BH4 itself together with DA quinones may induce cell death. As described below, we propose that the selective neurotoxicity in the nigrostriatal DA neurons among the catecholamine neurons may be explained by the higher biosynthesis rate of both BH4 and DA in the residual nigrostriatal DA neurons in PD. The hypothesis proposed by Choi and co-workers is consistent with our proposition and helps explain why neuronal death is not produced in autosomal-dominant GCH1 deficiency, in which both BH4 and DA levels are decreased.

Although there is a lack of evidence concerning inflammatory responses in GCH1 deficient patients, microglia activation may occur in autosomal-recessive juvenile PD (PARK2) or sporadic PD. Furthermore, since BH4 is a cofactor of NOS, the induction of GCH1 with consequent increase in the BH4 levels may stimulate the formation of inducible NOS (iNOS) in microglia, thus causing the synthesis and release of more NO from the activated glial cells. It would be interesting to examine whether the neopterin/biopterin ratio in CSF increases in the severe forms of autosomal-recessive TH deficiency, in which microglial activation might be expected.

Since the elevated ratio of total neopterin/total biopterin in the CSF in PD patients indirectly suggests microglia activation in the brain, Mogi and co-workers in our group further examined, by using a highly sensitive enzyme-linked immunosorbent assay (ELISA), changes in the contents of pro-inflammatory cytokines in the CSF that may reflect glia activation and inflammation in the brain. Cytokines are unstable proteins, and so we examined the levels in both lumbar CSF and ventricular CSF. As shown in Table **1**, we found that the level of tumor necrosis factor-alpha (TNF-alpha) was significantly increased in the lumbar CSF and in the nigrostriatal region of the brain in PD [59]. As the concentrations of other cytokines were generally low in the lumbar CSF, we measured them in the ventricular one. We found elevated levels of the following cytokines: interleukin-1beta (IL-1beta), IL-2, IL-4, IL-6, transforming growth factor-alpha (TGF-alpha) [60], and TGF-beta1 [61]. In agreement with our data on increased cytokines in the CSF from patients with PD, Blum-Degan *et al.* [62] also reported increased levels of IL-1beta and IL-6 in the lumbar CSF from patients recently diagnosed with PD or with AD without drug treatment. Interestingly, a significant inverse correlation between severity of PD and IL-6 CSF levels was observed [63]. These results indicate that elevated IL-6 levels in the CSF of untreated PD patients may reflect the inflammatory reaction in the brain during the course of the disease. This inverse correlation may suggest a neuroprotective compensatory reaction of IL-6 in neuroinflammation. Increased levels of TGF-beta1 and TGF-beta2 in the ventricular CSF of PD patients were also reported [64]. These changes in the levels of cytokines as well as the relative increase in the neopterin in lumbar or ventricular CSF may be considered to reflect the changes in the nigrostriatal regions of the brain in PD, where glial activation and inflammatory responses might occur in a tissue-specific manner.

3. PRO-INFLAMMATORY CYTOKINES AND NEUROTROPHIC GROWTH FACTORS (NEUROTROPHINS) IN THE BRAIN IN PARKINSON' DISEASE

An increased neopterin/biopterin ratio and increased levels of pro-inflammatory cytokines, such as TNF-alpha,

IL-1beta, and IL-6 in lumbar and ventricular CSF from patients with sporadic PD may reflect increased production from activated glial cells, especially microglia caused by the immune reactions at nigrostriatal brain regions. Therefore, Mogi and colleagues in our group assessed pro-inflammatory cytokines and anti-apoptotic neurotrophin expression in the postmortem brain from patients with idiopathic (sporadic) PD. Many precautions regarding postmortem changes in cytokines or neurotrophins are necessary when interpreting the results, since these proteins are easily degraded by proteases in the brain. We examined postmortem samples from the brain bank at Würzburg University, Germany, in collaboration with Dr. Peter Riederer, and those from the Japanese brain banks of Dr. Yoshikuni Mizuno (Juntendo University, Tokyo, Japan) and Dr. Sadako Kuno (Utano Hospital, Kyoto, Japan). The results are summarized in Table **1**.

In agreement with the CSF data described in the aforementioned section, we found increased levels of the following cytokines within nigrostriatal regions: TNF-alpha [59], IL-1beta [65], IL-2 [66], IL-6 [65], TGF-alpha [65], and TGF-beta1 [61]. We also found that levels of the TNF-alpha receptor R1 (TNFR1, p53) were elevated in the substantia nigra in PD in comparison with that of controls [67]. In agreement with our ELISA results, Boka *et al.* [68] found TNF-alpha immunoreactive glial cells in the substantia nigra of PD patients. They also reported immunoreactivity for TNF-alpha receptors in cell bodies and processes of most DA neurons, suggesting that TNF may participate in the degeneration process occurring in PD, at least after a primary insult capable of inducing reactive gliosis. Interestingly, expression of Fc epsilonRII/CD23 (low-affinity IgE/Fc epsilon receptor), whose engagement results in the production of nitric oxide and TNF-alpha, was found in glial cells of PD patients [69]. These immunohistochemical data suggest that proinflammatory cytokines are produced around DA neurons, probably in activated glial cells.

The presence of interferon-gamma (INF-gamma)-synthesizing cells in the brain has been a controversial issue. Hunot and Hirsch [70] found IFN-gamma-positive cells in the parkinsonian brain with morphology suggestive of a lymphocytic phenotype, indicating the possibility of infiltration of peripheral lymphocytes. Thus, these IFN-gamma

Table 1. Changes in Cytokines, Neurotrophins, and Apoptosis-related Proteins in the Brain (Nigrostriatal Regions), Ventricular Cerebrospinal Fluid (VCSF), and Lumbar CSF (LCSF) in Parkinson's Disease

	Brain	CSF	
	(striatum/substantia nigra)	VCSF	LCSF
Neopterin/Biopterin ratio			↑↑
TNF-alpha	↑↑		↑↑
IL-1beta	↑	↑	ND
IL-2	↑	↑	ND
IL-4		↑	ND
IL-6	↑	↑	↑
EGF	↑		
TGF-alpha	↑	↑	ND
TGF-beta1	↑	↑	ND
bFGF	→		
NGF	↓↓		
BDNF	↓↓		
GDNF	→		↑
sFAS	↑	ND	ND
TNF-alpha Receptor 1 (p55)	↑		
beta2-MG (MHC I)	↑	↓	↓
Bcl-2	↑	ND	ND
Caspase 1	↑		
Caspase 3	↑		

ND, not detectable; ↑↑ : markedly increased; ↑ : increased; → : no change; ↓ : decreased

positive cells were clearly distinguishable from TNF-alpha- and IL-1beta-positive cells, which appear to be of a neuronal or glial origin. Although T cell infiltration is typically not observed in the PD brain, there are a few cases where the presence of CD8-positive T cells were detected within the substantia nigra of patients with PD [71]. There is also a report indicating changes in blood vessels occur in the mesencephalon during PD [72]. Thus, the IFN-gamma-positive cells in the parkinsonian brain may be T cells that arrived there due to a malfunctioning blood-brain-barrier.

Neurotrophic growth factors, i.e., neurotrophins (nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], neurotrophin-3, and neurotrophin-4/5) are proteins produced by glial cells and neurons that support the differentiation and survival of neurons, and also appear to act as anti-apoptotic factors. In contrast to the increased cytokine levels, nigrostriatal BDNF and NGF concentrations (on the order of ng/mg and pg/mg protein, respectively) were significantly lower in PD patients than controls [73]. The neurotrophins, BDNF and NGF, have neuroprotective consequences for DA neurons and their depletion triggers apoptotic processes. Thus, the lack of neurotrophins together with the increased levels of pro-inflammatory cytokines may be fundamental in the pathogenesis of PD by accelerating progressive apoptotic death of nigrostriatal DA neurons. Glial cell line-derived neurotrophic factor (GDNF) is also a neurotrophin that has attracted considerable interest in relation to PD, largely owing to its particularly potent effects on DA neurons [74-77]. We showed earlier that GDNF levels in the human brain were significantly higher in the nigrostriatal DA regions (substantia nigra, caudate nucleus, putamen) than in the cerebellum and frontal cortex from either control or PD brains; however, nigrostriatal GDNF did not significantly differ between PD and control patients [78]. This is in contrast to the markedly reduced levels of BDNF or NGF found specifically in the nigrostriatal region in PD. The unchanged levels of GDNF in PD could be due to compensatory production by glial cells, which does not occur for either BDNF or NGF [73]. Interestingly, the concentration of another DA neuron-protective growth factor, basic fibroblast growth factor (bFGF), which was also shown to be abundant in the nigrostriatal region (on the order of ng/mg protein), was unchanged between control and PD striatum [66], again suggesting the possibility of compensatory production by glial cells.

4. APOPTOSIS-RELATED FACTORS DOWNSTREAM OF CYTOKINE OR NEUROTROPHIN SIGNALING PATHWAYS IN THE NIGROSTRIATAL REGION IN PARKINSON'S DISEASE

The presence of pro-inflammatory cytokines such as TNF-alpha, coupled with the deprivation of neurotrophins together may be a potent apoptotic signal [79]. Consequently, we examined changes in the levels of apoptosis-related factors in PD brains [Table **1**]. Belonging to the TNF-alpha/NGF receptor family, the Fas antigen/APO-1/CD95 is a cell-surface receptor protein known to trigger apoptosis upon binding to the Fas ligand (FasL). Fas antigen and the two TNF receptors, p55 and p75, have been implicated in triggering cell death upon stimulation by their natural ligands, i.e., TNF-alpha and FasL [80, 81]. Activated Fas/CD95 was reported to induce pro-inflammatory cytokine responses by human monocytes and monocyte-related macrophages [82]. Thus, in addition to its classical intracellular apoptotic neuronal pathway, Fas may also induce pro-inflammatory responses by activated microglia.

Molecular cloning and nucleotide sequence analysis revealed a human Fas mRNA variant capable of encoding a soluble Fas (sFas) molecule lacking the transmembrane domain. Moreover, sFas is known to protect against Fas-mediated apoptosis. We found that the concentration of sFas in nigrostriatal DA regions was significantly higher in PD patients than controls [83], suggesting attenuation of the Fas-signaling pathway in PD. However, the content of Fas-FasL as well as that of Fas-associated death domain (FADD) was reported to be reduced in DA neurons of PD patients [84]. This finding suggests that a reduction in Fas signaling may be a defense mechanism of these neurons for PD-related pathology. We also found that the concentration of anti-apoptotic bcl-2 protein, which is localized in several cell components such as inner and outer mitochondrial membranes [85] in cells in the nigrostriatal DA region, was significantly higher in PD patients than in those of controls [86]. Marshall *et al.* [87] also reported upregulation of bcl-2 in the basal ganglia in PD. Importantly, the anti-apoptotic activity of bcl-2 was linked to reduced generation of ROS [88, 89]. One would expect the upregulation of sFas and bcl-2 and downregulation of Fas-FasL in the nigrostriatum to prevent the apoptosis that is presumably occurring in PD. These results are thus against the hypothesis that apoptosis is indeed responsible for the neurodegeneration. However, the possibility should be entertained that these changes in the apoptosis-related factors in PD may reflect responses of yet unknown compensatory mechanisms operative during ongoing Fas-mediated apoptosis.

Members of a novel family of aspartate-specific cysteine proteases, which include caspase-1 (IL-1beta-converting enzyme) and caspase-3, have been implicated as mediators of apoptotic cell death [90, 91]. Indeed, both caspase-1- and caspase-3-like proteases are involved in TNF- and Fas-receptor-mediated apoptosis. The activities of caspase-1 and -3 were significantly higher in the substantia nigra from PD patients relative to control patients [67]. Activated caspase-3 was also detected immunohistochemically and was proposed to be the final effector in the apoptotic cell death of DA neurons in PD [92]. Since both caspase-1 and -3 together with TNFR1 may be fundamental for apoptotic cell death through the TNF-alpha-induced signal pathway, the presence of a pro-apoptotic environment in the nigrostriatal region of the PD brain suggests vulnerability of neurons and glial cells towards a variety of noxious factors. In support for this contention, ribozyme-mediated inhibition of caspase-3 activity reduced apoptosis induced by 6-hydroxydopamine (6-OHDA) in PC12 cells [93]. In another human study, a significantly higher percentage of DA neurons than of other cells in the substantia nigra displayed caspase-8 activation, as indicated by their immunoreactivity [94]. As caspase-8 is known to cause the release of cytochrome c from mitochondria to trigger caspase-3 activation, it might be involved in the apoptotic cell death of DA neurons.

5. CYTOKINES AND NEUROTROPHINS IN THE BRAIN IN ANIMAL MODELS OF PARKINSON'S DISEASE

In several animal models of PD, we observed changes in cytokines and neurotrophins similar to those observed in postmortem PD brain. The dopaminergic neurotoxin, MPTP, which causes PD in humans [96], also produces apoptotic cell death of the nigrostriatal DA neurons in monkeys and rodents [95, 97]. MPTP is a synthetic N-methylated amine product that easily enters the brain from circulation and is oxidized to 1-methyl-4-phenylpyridinium ion (MPP^+) by monoamine oxidase type B (MAO-B) in glial cells. Subsequently, MPP^+ is transported into DA neurons *via* the DA transporter, where it causes neuronal death (possibly through apoptosis) by inhibiting complex I of mitochondria and producing ROS. MAO-B inhibitors such as deprenyl (selegiline) completely prevent PD induction by MPTP, and deprenyl is clinically effective for the treatment of PD.

It was also reported that MPTP had multiple immune/inflammatory effects in mice, including upregulation of MHC expression in the nigrostriatum, microglial activation and infiltration of CD4- and CD8-T cells into the substantia nigra [98]. We observed that repeated intraperitoneal injection of MPTP increased IL-1beta concentration 23-fold and decreased NGF concentration by approximately 50% within the striatum [99]. These results agree with data from postmortem PD brains and hint at a possible induction of apoptosis within DA neurons. As well, in support for cytokine-mediated apoptotic and inflammatory pathway in PD, mice lacking in caspase 11 (an apical caspase mediating the activation of caspase 1) were somewhat resistant to MPTP toxicity [100]. Gene expression analysis in MPTP treated mice by cDNA microarray also revealed neuroinflammation to be an important event in the process of DA neuronal cell death [101]. Moreover, the anti-inflammatory drug, aspirin or salicylate, was found to protect against MPTP-induced dopamine depletion in mice [102], through inhibition of cyclooxygenase type 2 (COX2) [103], which is essential for synthesis of inflammatory prostaglandins. Correspondingly, increased expression of COX2 as well as the inducible form of NOS (iNOS) was evident in glial cells of the substantia nigra in patients with PD [104, 105]. Again, these data support a role for inflammatory pathways in PD, specifically involving COX2 and the oxidative stressor, iNOS, which may also be related to apoptotic factors (e.g. caspases).

Further support for a link between inflammation and apoptotic processes in PD comes from a recent study by Hayley *et al.* [106] that revelaed that MPTP increased nigral Fas expression and that genetically deficient Fas mice displayed attenuated DA neuron loss. As well, MPTP increased nigrostriatal microglia immunoreactivity within wild type but of the Fas null mice, however, it remains to be determined if these glial variations stemmed from direct actions of Fas or a secondary to variations of neuronal survival. Interestingly, nigrostriatal expression of a dominant-negative c-Jun adenovirus blocked the induction of Fas and also protected DA neurons from MPTP-induced damage. These data suggest the critical nature of the immediate early gene, c-Jun, and the Fas signaling pathway in MPTP induced degeneration. This group of investigators also reported that Fas-deficient mice displayed a pre-existing reduction in striatal DA levels and locomotor behavior, but were resistant to further MPTP induced decreases of DA levels. However, MPTP increased the metabolites, DOPAC and HVA in Fas mice, raising the possibility again that compensatory responses may have been engendered to maintain stable DA stores and behavioral responses.

Changes in cytokine levels were also observed in rats with 6-OHDA-induced PD. We found that in hemiparkinsonian rats, produced by injecting 6-OHDA unilaterally into the ventrotegmental bundle without or with L-DOPA treatment, levels of TNF-alpha were significantly increased only in the substantia nigra and striatum on the injected side. L-DOPA administration did not produce any significant change in TNF-alpha concentrations in the 6-OHDA-untreated control side of any of the brains [107]. These results agree with previous findings demonstrating increased TNF-alpha levels in the striatum and lumbar CSF of PD patients, and also suggest that the increased TNF-alpha levels in PD patients may not be due to the secondary effects of L-DOPA therapy. In a subsequent study, we showed that the increased TNF-alpha levels in the 6-OHDA-lesioned striatum was suppressed by the immunophilin ligand FK506 [108]. This finding is in keeping with the proposition that the immunosuppressant effects of FK506 may prevent 6-OHDA-mediated activation of microglial cells in the nigrostriatal DA region of rats. All these reports indicate that changes in cytokines and neurotrophins in the CSF and in the nigrostriatum of postmortem brains of idiopathic PD patients are consistent with data obtained from animal models using MPTP and 6-OHDA [109-111].

Although MPTP [96] is the only neurotoxin proved to produce PD in humans, the effects of neurotoxins similar to MPTP have also been examined in animals. Several MPTP-like neurotoxins were identified in the human brain [3, 109-117], but these compounds were found to produce PD only by direct injection into the striatum of animals. Among the various MPTP-like neurotoxins found endogenously in the PD brain, such as isoquinolines and beta-carbolines [3, 109-117], N-methyl(R)salsolinol (R-N-methyl-6, 7-dihydroxy-1, 2, 3, 4-tetrahydroisioquinoline) may be synthesized from DA within nigral neurons. Unilateral injection of N-methyl (R) salsolinol into the striatum of rats produced hemiparkinsonism, much like that associated with 6-OHDA [118, 119], and caused activation of caspase-3, suggesting possible apoptotic death of DA neurons [120]. This endogenous neurotoxin was reported to inhibit complex I in mitochondria, thereby producing ROS and ultimately provoked apoptotic cell death, as evidenced by DNA fragmentation [119].

Beta-calbolinium cations, which are produced from beta-carbolines by MAO-B, exist in the CSF from patients with PD at higher concentrations than those in controls [115]. These beta-calbolinium neurotoxins were also found to destroy the nigrostriatal DA neurons after their direct injection into the striatum of rats [113, 114]. Endogenous isoquinolines and beta-carbolines in the brain, like MPTP, are assumed either to be first N-methylated by N-methyltransferase and then oxidized by MAO-B to the corresponding isoquinolinium ions or beta-carbolinium ions, or to be

directly oxidized to more toxic metabolites by MAO-B [3, 109-117]. It is suggested that the mechanisms of the DA neuronal death induced by PD symptom-producing neurotoxins, i.e., MPTP/MPP^+, isoquinoline/isoquinolinium, or beta-carboline/beta-carbolinium, may be primarily apoptotic in nature. However, it remains to be determined whether isoquinoline/isoquinolinium or beta-carboline/beta-carbolinium produce changes in cytokine or neurotrophin levels along with inflammatory factors (e.g. COX2) in the nigrostriatum similar to those changes effected by MPTP/MPP^+.

Epidemiological studies have suggested that insecticide exposure is associated with an increased risk of developing PD. Rotenone is a naturally occurring, lipophilic compound from the roots of certain plants (*Derris* species), and is the main component of many insecticides. Rotenone is not structurally related to MPTP, but, just like MPP^+, its repeated peripheral injection selectively and partially inhibits mitochondrial complex I to produce a PD-like syndrome in rodents. Rotenone, as a highly lipophilic compound, may be able to affect both DA neurons and glial cells in the nigrostriatal region. Chronic systemic injection of rotenone into rats caused highly selective degeneration of nigrostriatal DA neurons and symptoms of muscle rigidity. An important morphological finding in rotenone-treated rats was that the nigrostriatal DA neurons accumulated fibrillar cytoplasmic inclusions, containing ubiquitin and alpha-synuclein, similar to the Lewy bodies seen in idiopathic PD [121]. This finding stands in contrast to the effects of MPTP in animals and humans, in which cytoplasmic inclusions or Lewy bodies are not observed. Lewy body formation is common in PD and is often taken to indicate failure of the ubiquitin-proteasome system; thus, rotenone may induce morphological changes closely aligned with idiopathic and familial PD involving PARK1 (alpha-synuclein mutation) [122].

The effects of other endogenous factors in the nigrostriatal DA neurons related to PD remain to be elucidated. For example, the formation of neurotoxic DA orthosemiquinones [123] or DA quinones associated with high levels of neuromelanin and iron [124, 125]. As well, low levels of the normally protective factors calbindin (a calcium-binding protein), and glutathione (GSH) may render neurons vulnerable to excitotoxic and free radical damage.

An important question regarding the changes in the levels of cytokines, neurotrophins, and apoptosis-related factors in the nigrostriatum of the PD-animal models is whether these changes might be either the cause of neuronal cell death or secondary responses, and possibly neuroprotective reactions in glial cells, that occur in reaction to the degenerating DA neurons. Specifically, glial cells that are activated around degenerative DA neurons release cytokines that may either promote or inhibit neuronal survival. Thus, we asked the question of whether the microglia activation in the MPTP-mouse model is neuroprotective or neurotoxic by using histochemical methods. We compared the effect of single and repeated (7 doses) MPTP administration on microglia activation in the nigrostriatum. After a single dose of MPTP, no remarkable microglia activation was observed. However, after repeated administration, significant microglia activation was detected simultaneously with an approximately 50% decrease in the number of TH-positive cells. However, the numbers of TH-positive cells gradually recovered after the last dose of MPTP. We speculate from these histochemical findings that the activated microglia may not cause direct damage to TH-positive neurons but rather that a secondary neuroprotective reaction by them contributes to the recovery of TH-positive neurons in mice, since the glial cells were strongly associated with TH-positive cells [Sawada *et al.*, to be published].

There are also some reports indicating that the cytokines induced by the initial insults could be neuroprotective at least at the early stages of pathology in mice. It will be recalled; TNF-alpha is secreted from activated glial cells and acts *via* two different receptors, TNF-R1 and TNFR2. Rousselet *et al.* [126] reported that MPTP reduced motor activity in TNFR1 and TNFR2 double knock out mice but not in mice lacking only one of these receptors. The striatal DA level was slightly decreased in double TNF knockout mice and reduced even more in these mice after MPTP injection. From these results, the authors concluded that TNF-alpha may not directly participate in the death of DA neurons in PD, but may slightly alter DA metabolism, affecting the survival of DA terminals by a mechanism involving both TNFR1 and TNFR2 receptor subtypes. In another report by Suzuki *et al.* [127], microglia activated *via* the $P2X_7$ receptor through ATP, was found to release TNF to protect neurons against glutamate toxicity. These data suggest that TNF released from activated microglia may actually serve a neuroprotective role, at least in the initial stage of neuronal injury. However, several reports indicated that activated microglia might initiate or promote progression of DA neuronal death.

Intranigral or intrastriatal injection of the potent bacterial immunostimulant, lipopolysaccharide (LPS), produced PD-like pathology in rodents. Specifically, LPS induced microglia activation and damaged nigrostriatal neurons resulting in decreased levels of TH-positive neurons [128]. In another report [129], LPS-induced loss of DA neurons in the rat substantia nigra was accompanied by iNOS induction in fully activated microglia (with amoeboid morphology), suggesting that NO and/or its metabolites may play a crucial role in inflammation-mediated degeneration of DA neurons. The impact of glial activation provoked by IFN-gamma plus LPS was investigated in rat midbrain slices by Shibata *et al.* [130]. Application of IFN-gamma followed by LPS elicited an induction of iNOS and COX2 together with increased NO production, and resulted in DA neuronal death [130]. Aminoguanidine, an inhibitor of iNOS, or SB203580, an inhibitor of p38 mitogen-activated protein kinase (MAPK), prevented the IFN + LPS induced DA cell loss as well as NO production; whereas, selective COX-2 inhibitors such as NS-398 and nimesulide did not protect DA neurons. These results indicate that iNOS-derived NO in activated glial cells plays a crucial role in IFN-gamma /LPS-induced DA cell death.

MPTP-induced PD mice recover spontaneously after a few months. However, such recovery does not occur in humans and monkeys and this effect may be attributable to the progressive consequences of chronically enhanced glial activation. McGeer *et al.* [131] reported the presence of HLA-DR-positive reactive microglia, the accumulation of extracellular melanin, and the extensive loss of DA neurons

in the substantia nigra of monkeys administered MPTP 5 to 14 years before death. The monkeys had been drug free for at least 3 years before death, indicating that a brief exposure to MPTP had instituted an ongoing process. Highly reactive microglia appeared to surround DA neurons and a strong relationship was suggested between damage to DA neurons and the intensity of the neuroinflammatory response. Interestingly, such chronic neuroinflammation years after MPTP exposure is similar to that observed in humans with MPTP-induced PD [132]. Based on these changes at the chronic stage of MPTP-induced PD in monkeys, McGeer *et al.* [131] proposed that PD itself involves exposure to one of a variety of agents that disappear after instituting long-lasting inflammatory changes.

6. FAMILIAL PARKINSON'S DISEASE AND POSSIBLE IMMUNE REACTION

In idiopathic PD as well as in familial PD involving PARK1 (alpha-synuclein mutation), Lewy bodies consisting of mainly alpha-synuclein and spherical filamentous masses are common characteristic features. The question remains unanswered as to whether Lewy bodies are causative (neurotoxic) or compensatory (neurorpotective) in PD. Intraneuronal filamentous deposits similar to PD Lewy bodies are generally observed in Alzheimer's disease (AD), Huntington's disease, and other neurodegenerative conditions. Misfolded or unfolded proteins in cells are normally degraded by the ubiquitin-proteasome system thereby preventing their aggregation as intracellular inclusion bodies. Thus, dysfunction of the ubiquitin-proteasome system causes accumulation of these aggregates. The above neurodegenerative diseases in general are speculated to be "protein-conformational diseases or protein-misfolding diseases" due to a faulty ubiquitin-proteasome system [133]. However, in autosomal-recessive early-onset familial PD, PARK2, which is caused by the mutated *parkin* gene encoding inactive ubiquitin ligase E3 (loss of function), Lewy bodies are not observed. It is unclear why Lewy bodies are not formed in the presence of a dysfunctional ubiquitin-proteasome system in the case of PARK2 mutation. However, these results suggest that Lewy bodies may not be essential for the provocation of PD.

The Pael receptor (parkin-associated endothelin receptor-like receptor) is a substrate of parkin and is abundantly distributed in the nigrostriatal region. It would be expected that in PARK2 (autosomal-recessive juvenile PD), owing to the loss of functional parkin, Pael receptors may accumulate in the endoplasmic reticulum (ER) to cause ER stress [134], which in turn, may cause mitochondrial dysfunction and apoptotic death. The fact that TH-immunoreactive cells in the substantia nigra pars compacta are largely Pael receptor-positive may help explain the sensitivity of these DA neurons in PD [134]. A remaining question is the role of the Pael receptor in the sporadic PD. If sporadic PD with Lewy bodies might also be a "protein-conformational disease, " then oxidative stress of environmental factors coupled with the PD susceptibility genes might cause the unfolded protein response leading to ER stress [135]. ER stress arises from the failure of the ubiquitin proteasome system to remove misfolded or unfolded proteins. This build-up of abnormal proteins might finally induce the programmed cell death (apoptosis). However, the mechanism and the signal transduction pathway causing unfolded proteins to initiate apoptosis and the interaction between ER stress and oxidative stress, which is supposed to be the main trigger for sporadic PD, have not been made clear yet [136]. Since the parkin protein is a component of Lewy bodies, the formation of such bodies might contribute to neuronal death by the sequestration of functional parkin, ubiquitin ligase E3, which would prevent the removal of toxic proteins [137].

Alpha-synuclein (mutated protein in PARK1) is the main component of Lewy bodies and is speculated to act to presynaptically regulate DA release, synthesis or storage as well as regulation of synaptic plasticity. Curiously, in MPTP treated mice and in PD linked to PARK2 (parkin mutations) Lewy bodies are not produced. Thus, the contribution of alpha-synuclein to PD pathology is not clear. Thus, MPTP or rotenone was administered to alpha-synuclein(-/-) mice to assess the relation between ROS and alpha-synuclein. In these mice, DA neurons seem to be resistant to MPTP, but not to rotenone. This is curious given that both MPP^+ and rotenone inhibit complex I to produce PD in rodents. However, as mentioned before, the toxicity of MPP^+ formed from MPTP depends on the uptake of MPP^+ into DA neurons by the DA transporter, whereas rotenone is highly lipophilic and does not depend on this transporter. Therefore, one may speculate that alpha-synuclein is required for MPP^+ transport into DA neurons and that its deficiency would protect DA neurons. Accordingly, alpha-synuclein may be necessary for MPTP toxicity in mice.

Reactive oxygen species (ROS) may accelerate the process of alpha-synuclein aggregation and lead to the formation of more ROS through toxic alpha-synuclein fibrils, therein generating a repetitive cycle of death for the DA neurons. It is also important to note that DA itself, when oxidized, may stabilize the protofibril form of alpha-synuclein, thereby increasing the concentration of toxic protofibrils and of ROS in DA neurons [138]. Other reports indicated that the accumulation of normal alpha-synuclein in cultured human DA neurons resulted in ROS mediated apoptosis that also required endogenous DA production [139]. In contrast to alpha-synuclein toxicity in DA neurons, alpha-synuclein is not toxic in non-DA human cortical neurons, but rather exhibits neuroprotective activity, confirming the DA-dependent toxicity of alpha-synuclein. DA-dependent alpha-synuclein neurotoxicity is mediated by 5483-kDa soluble protein complexes (containing alpha-synuclein and 14-3-3 protein), which are elevated selectively in the substantia nigra in PD. Thus, the accumulation of soluble alpha-synuclein protein complexes can render endogenous DA toxic, suggesting a potential mechanism for the selectivity of neuronal loss in PD [139].

In accordance with *in vitro* data concerning DA specific toxicity of alpha-synuclein, nigrostriatal alpha-synucleopathy was induced *in vivo* by adeno-associated viral vectors overexpressing either wild-type or mutant human alpha-synuclein in the substantia nigra of adult marmosets. Indeed, the vectors promoted the loss of TH-positive neurons, formation of alpha-synuclein-positive inclusions and dystrophic neurites, as well as motor impairment, indicating a new primate model of PD [140].

A new gene implicated in familial PD, DJ-1 is beginning to receive attention. The crystal structure of DJ-1, which is the causative protein of another form of autosomal-recessive juvenile PD, PARK7 [9], was recently resolved [141, 142]; and studies revealed a highly conserved cysteine residue and an exsquisite sensitivity to oxidative stress. This cysteinyl structure suggests the possible involvement of DJ-1 protein in the cellular oxidative stress response and in the general etiology of sporadic PD [142].

All results concerning the causative mutated proteins of familial PD, i.e., alpha-synuclein in PARK1, parkin in PARK2, UCH-L1 in PARK5, and DJ-1 in PARK7, suggest the induction of apoptotic cell death of DA neurons due to the production of misfolded or unfolded proteins. Since the postmortem brain has not yet been adequately examined for the presence of cytokines or inflammatory response in familial PD, the relation between misfolded proteins and presumed neuroinflammation in the substantia nigra in familial PD is not clear yet. However, it is possibile that damaged DA neurons, either through misfolding or ER stress in familial PD, or by various insults in sporadic PD, may send unknown signal molecules to microglia to activate them [143]. Activated microglia would then produce pro-inflammatory cytokines, ROS, NO, etc. and accelerate the apoptotic pathway in the damaged DA neurons, which cells might finally be removed by phagocytosis by the activated microglia. Along these lines, caspase-12, a caspase that induces cytokine maturation, has been proposed as a mediator of apoptosis induced by ER stress and might contribute to the pathogenesis of AD [144].

It is possible that misfolded proteins associated with familial PD, i.e., PARK1 (alpha-synuclein), PARK2 (parkin), PARK5 (UCH-L1), or PARK7 (DJ-1), may be produced not only in DA neurons but also in glial cells. Accordingly, immune responses may be initiated in microglia which then damage DA neurons. Biochemical and histological data from patients with familial PD may give clues about possible neuroinflammatory reactions in familial PD. In a study using cultured neural cells, inhibition of the ubiquitin-proteasome system was shown to induce a pro-inflammatory response manifested by upregulation of COX2, its accumulation as ubiquitin conjugates and the production of prostaglandin E(2) [145]. Thus, ubiquitin-proteasome system disturbances might be a common mechanism in both familial PD and sporadic PD. Similar neuropathlogical findings that have been reported between familial and sporadic PD, except for the difference in the presence or absence of Lewy bodies, strongly suggest involvement of common molecular mechanisms for neuroinflammation leading to the death of nigrostriatal DA cells.

7. NEUROTOXIC AND NEUROPROTECTIVE EFFECTS OF CYTOKINES IN NEUROINFLAMMATION IN PARKINSON'S DISEASE

7.1. Roles of Microglia in Neuroinflammation

As already indicated, microglia together with astroglia play critical roles in the pathogenesis of various neurological disorders by acting as cytokine-producing and MHC class II antigen-positive immunoregulatory cells. Microglia produce IL-1alpha, IL-1beta, IL-5, IL-6, IL-10, IL-12, TNF-alpha, and TGF-beta, whereas astrocytes produce IL-1alpha, IL-1beta, IL-5, IL-6, IL-8, IL-10, INF-alpha, and -beta, RANTES (regulated on activated normal T cell expressed and secreted), macrophage inflammatory protein-1, TNF-alpha, TGF-beta, G-CSF, GM-CSF, and M-CSF (granulocyte-, granulocyte-macrophage-, and macrophage-colony-stimulating factors). Microglia also express mRNAs of cytokine receptors for IL-2, IL-3, IL-4, IL-6, IL-7, GM-CSF, and M-CSF. These cytokines affect functions of both neuronal and glial cells *via* specific receptors on the cells, and form a unique cytokine network in the brain [146-151].

Changes in levels of pro-inflammatory cytokines and the upregulation of inflammation-associated factors such as iNOS or COX2 in activated microglia in the brain from patients with PD and in animal PD models strongly suggest that the loss of the nigrostriatal DA neurons may be triggered by glial derived pro-inflammatory cytokines. However, owing to their pleiotropic effects, cytokines may have either neurodeleterious or neuroprotective effects depending upon the circumstances at a particular time in the progression of PD. In terms of neurodeleterious effects, pro-inflammatory cytokines might start the cascade of the events leading to apoptotic cell death of the nigrostriatal DA neurons [18, 149-151]. Microglial activation may also occur as a secondary reaction to the various primary insults to the DA neurons. The process of oxidative stress in DA neurons involves decreased complex I activity in mitochondria, a decreased level of GSH, an elevated level of iron, and increased lipid peroxidation, all of which initiate the apoptosis cascade from mitochondria finally leading to DNA damage in the nucleus and subsequent apoptotic cell death. Damage to DA neurons in the nigrostriatum may initiate neuroinflammation and aggravate the disease process by causing glial cell activation, especially, microglia activation, to produce pro-inflammatory cytokines, ROS, and NO [152, 153]. However, a neurotoxin such as rotenone, which is highly lipophilic and penetrates any cells without depending upon DA transporter action, may inhibit complex I of mitochondria in both DA neurons and glial cells. As a result rotenone might also produce direct glial activation to produce cytokines, ROS, and NO.

Viruses have been suspected for many years as a cause of certains instances of PD, ever since the pandemic von Economo's disease in the early 20th century [154]. In fact, a rat model of PD was induced by Japanese encephalitis virus [155]. Interestingly, neither viral antigen nor viral genome could be detected in the substantia nigra. This fact suggests that a virus infection may trigger the chronic process of PD long after disappearing from the brain. As mentioned before, reactive microglias exist in the substantia nigra of monkeys [131] and humans [132] with MPTP-induced PD years after MPTP exposure.

The signal transduction pathway from the initial trigger of neurodegeneration of DA neurons to the final cell death has been extensively examined in various animal models of PD, particularly aspects involving oxidative regulatory factors. For instance, Hunot *et al.* [156] showed that nuclear translocation of transcription factor nuclear factor kappa B (NF-kappa B), which is activated by oxidative stress, is increased in DA neurons of patients with PD. As described

in the article by Barger in this issue, NF-kappa B is also known to be upregulated by TNF-alpha, IL-1beta or IL-6. As well, NF-kappa B induces iNOS expression to increase NO production, which may finally cause apoptotic death of DA neurons. Additionaly, oxidative stressors may be provoked by cytokine induced activation of the low-affinity IgE receptor Fc epsilonRII/CD23 on glial cells. Indeed, cytokines were reported to stimulate Fc epsilonRII/CD23 expression, which resulted in the induction of iNOS (with the subsequent release of NO) and TNF-alpha in substantia nigra glial cells of PD patients [69]. The activation of iNOS, which mediates the synthesis of high levels of NO, is supposed to be the key intermediate to produce apoptosis of DA neurons [105]. Activated microglia produce ROS and NO *via* induction of NADPH oxidase and iNOS and release ROS, NO, and H_2O_2, which may mediate DA cell injury. Accordingly, MPTP treated mice displayed elevated NADPH oxidase activity that was shown to regulate oxidative stress induced injury [157]. Thus, the immune/inflammatory pathology and oxidative stress caused by induction of iNOS and NADPH oxidase may be tightly linked in PD, and activated microglia may play an important deleterious role in propagating and amplifying oxidative neuronal injury and possibly even in initiating such injury [153].

In mesecephalic-microglia co-culture models, both LPS as well as IgGs from patients with PD activated microglia and provoked DA neuronal death [158]. However, PD IgGs only provoked neuronal demise in the presence of DA quinone- or H_2O_2-modified DA cell membranes, suggesting possibly important interactions between the IgGs and altered DA epitopes. Importantly, activated microglia released several proinflammatory cytokines, ROS, H_2O_2 and NO, which appeared to mediate the DA cell injury [158]. It is likely that PD IgGs activate Fc receptors on microglia, thereby promoting glial responding against DA neurons. Initially, the reactive IgGs may arise in response to altered DA epitopes associated with ongoing degeneration in PD.

As already alluded to, PD has been associated with an induction of MHC-I and MHC-II and increased expression of complement components, HLA-DR antigens, and Fc epsilonRII/CD23 in glial cells [12, 69]. We have also demonstrated increased beta2-microglobulin expression, i.e., the light chain of MHC-I, specifically in the striatum in PD patients [14]. Since TNF-alpha levels (which may induce beta2-microglobulin) were also increased in the striatum, it may be the case that a local immune reaction may occur within the nigrostriatal region in PD. Indeed, MHC expression of microglia enable these cells to present antigen to any infiltrating lymphocytes as well as elicit respiratory oxidative activity. Although immune responses are kept to a minimum in the healthy brain by active neuronal inhibition of such processes [159], microglia with MHC-regulated antigen-presenting capacity induced in response to degeneration may further precipitate DA loss. Further, increased proinflammatory cytokine expression would further serve to stimulate microglia MHC expression and phagocytosis, thereby perpetuating a cycle of inflammation and degeneration.

Neurotoxins such as MPTP/MPP^+ can produce apoptotic cell death in the PD animal models and this effect may be mediated by inflammatory glia. Indeed, human microglia express CD95 (Fas) ligand (CD95L) and can induce apoptosis in CD95-expressing target cells *in vitro* [160]. However, the issue of apoptotic cell death of the DA neurons in the PD brain is still controversial. Apoptotic cells in postmortem brain in PD have been demonstrated using histochemistry, however, the numbers of such cells are admittedly few [20, 21, 161]; and in one report, they were undetectable [162]. Furthermore, the intracellular signaling pathway causing the deleterious effects of activated microglia leading to the apoptotic cell death of DA neurons in the substantia nigra in PD is not yet clearly understood. The tumor suppressor protein p53 plays a pivotal function in neuronal apoptosis triggered by oxidative stress. However, oxidative stress induces p53-mediated apoptosis also in glial cells, although the signaling pathway may differ between neural and glial cells. Pifithrin-alpha (PF-alpha), which has been reported to protect neurons from ischemic insult by specifically inhibiting p53 DNA-binding activity, was unable to prevent oxidative stress-induced apoptosis of astrocytes. In astrocytes p53, acting *via* caspase-dependent and transcription-independent pathways, mediated an apoptotic response by directly promoting mitochondrial release of cytochrome c release and nucleosomal fragmentation [163].

Cytokines, such as TNF-α, released from microglia can activate the apoptotic pathway *via* intracytoplasmic death domains on DA neurons. In fact, caspase-3 and caspase-8, which are the effectors of apoptosis, are activated in PD. However, caspase inhibitors or blockade of the TNF-alpha or IL-1beta receptors has not yielded substantial protection of DA neurons against degeneration in experimental models of PD. Perhaps, manipulation of a single signaling pathway may not be sufficient to completely protect DA neurons. Neuroprotection strategies for PD may require a poly-therapy acting on different cell-death pathways to block the degeneration process of the DA neurons, as in the case of poly-therapies used in the clinical treatment of AIDS [163].

It should be realized that the glial response in PD might not only be toxic to the DA neurons, but also neuroprotective in certain cases owing to the secretion of neurotrophic factors that can buffer against ROS and gluatamate toxicity. In fact, delivery of GDNF by neural stem cells induced neuroprotection in a mouse model of PD [74] and GDNF expressed by viral vectors prevented DA neuron cell death in primate models of PD *in vivo* [76] or in mecencephalic cells *in vitro* [77]. Interestingly, Sawada *et al.* [164] discovered that intravenous injection of immobilized microglial cells promoted brain-specific gene expression, which may make these cells useful for the delivery of GDNF into the nigrostriatal region.

In order to address the question as to whether microglial activation is neurotoxic or neurotrophic [165] *in vivo* in PD, we examined activated microglia in the autopsy brain from PD patients by immunohistochemistry using HLA-DR antibody. We found 2 types of activated microglia, one associated with and one without neuronal degeneration; the former was found in the nigrostriatum; and the latter, in the hippocampus and cerebral cortex. We observed the expression of genes related to pro-inflammatory cytokines in the nigrostriatum, but not in the cerebral cortex or hippocampus.

In contrast, increased gene expression of neurotrophic growth factors such as BDNF, GDNF, or TGF-beta was observed in the hippocampus. We also found that in postmortem brains from early stage-PD patients double-stained for microglia and TH, such that the activated microglia were in close contact with TH-positive nerve fibers [166]. We speculate that since TH staining was intense in these nerve fibers, some neuroprotective role may be played by microglia, at least in early stages of disease and in regions outside the nigrostriatal pathway. Along these lines of Sawada and coworkers [166], Hirsch *et al.* [167] also proposed separate popula-tions of microglia. One subpopulation of glial cells may play a neuroprotective role by metabolizing DA and scavenging oxygen free radicals and another that may be deleterious to DA neurons by producing NO and pro-inflammtory cytokines.

Thus, immune responses by activated microglia in the nigrostriatal region in PD patients appear to be either neuro-protective or neurotoxic depending upon the circumstances present at a particular time during the progression of the disease. Activated microglia may be neuroprotective at least at an early stage of PD, but may assume a deleterious role after chronic activation over the course of disease. The fragments of DA neurons produced by apoptosis of DA neurons may be removed by phagocytosis by activated microglia. Phagocytosis of damaged DA neurons by microglia would be an important process in PD pathology.

7.2. Roles of Astrocytes in Neuroinflammation

In addition to microglia, astrocytes may contribute, although to a lesser extent, to the neurodegenerative process in PD [168]. Along these lines, astrocytes can express MHC class II antigens and can produce a wide range of cytokines. The presence of reactive astrocytes often leads to substantial neuronal degeneration in the injured adult brain [169]. Although astrocyes typically release neurotrophins or small antioxidants with free radical-scavenging properties (GSH, ascorbic acid, GDNF, BDNF, NGF, bFGF), in certain disease conditions they may produce toxic products such as NO, pro-inflammatory cytokines [170]. Thus, altered astro-cyte function might also contribute to nigral degeneration in PD [171]. Indeed, neuroprotection of mesencephalic DA neurons elicited by cyclic AMP may require the repression of astrocytes through inhibition of cyclin-dependent kinase 1 (CDK1) [172].

In terms of a protective role for astrocytes, de Bernardo *et al.* [173] reported that mesencephalic astroglia-condi-tioned medium greatly increased the expression of the DA phenotype and protected cells from spontaneous or neuro-toxin-induced death through a complex signaling network involving protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulation kinase (ERK)/ mitogen-activated protein kinase (MAPK), and phos-phatidylinositol-3 kinase (PI-3K). Thus, neuroprotection and differentiation of DA neurons mediated by astroglia may require the activation of PKA, PKC, ERK/MAPK, and PI-3K pathways. Astrocytes also influence the formation and maintenance of the blood-brain-barrier. *In vivo*, astrocyte foot processes are in close apposition to the abluminal surface of the microvascular endothelium of the blood-brain-barrier [174].

8. INTERACTIONS BETWEEN GLIA AND DOPAMINE NEURONS *VIA* CYTOKINES IN INFLAMMATORY PROCESS OF PARKINSON'S DISEASE

Communication between neurons and glia plays a key role in shaping the quiescent and reactive states of microglia. The demonstration of numerous receptors for brain signaling molecules (neurotransmitters, neuropeptides, ATP) and neurotophins on microglial cells has suggested that these cells not only monitor but are also under the strict control of the neurochemical environment. Given the heterogeneity of neuronal populations within distinct neuroanatomical regions, the effects of the neurochemical environment on microglia are site-specific, and this could account for differences in the degree of microglia activation and inflammatory reactions in different brain regions [175, 176]. Therefore, the interaction between the nigrostriatal DA neurons and activated microglia in PD may have complex sequences, resulting in neurotoxicity or neuroprotection. It is important that we obtain a better understanding of the neuron-glia communication that takes place during the development of PD.

Use of anti-inflammatory drugs may prove to be a viable adjunct treatment strategy to augmnent the efficacy of current pharmacological regimens for PD. Indeed, ongoing clinical trials for AD have suggested a tremendous potential for such compounds. Furthermore, animal models have shown the anti-inflammatory drugs, pioglitazone, a PPAR (peroxisome proliferators-activated receptor)-gamma agonist, and minocycline, a tetracycline derivative, provide clinical benefit for neurotoxin treated mice [151]. In summary, microglial cells may thus regulate tissue changes that confer either harm or benefit, depending on the specific circumstances. A hypothetical scheme of neuroinflammation in PD is shown in Fig. (3).

CONCLUSIONS

Recent discoveries of the causative genes and the products of familial PD, such as alpha-synuclein, parkin, UCH-L1, and DJ-1, suggest that the neuronal death in PD as well as in other neurodegenerative diseases such as AD may be "protein conformational diseases" caused by accumula-tion of disease-characteristic misfolded proteins that have adopted some non-native conformation often rich in beta-sheets. However, most (about 95%) cases of PD or AD are sporadic, without any finding of mutated causative genes. Recent findings of increased levels of pro-inflammatory cytokines and reduced neurotrophin levels accompanied by inflammatory responses in the nigrostriatal regions in sporadic PD rasise the possibility that immunogenic pro-cesses are fundamental to PD.

Although sporadic and familial PD are thought to be different with respect to the initial trigger, they might have in common apoptotic neuronal death as the final event, reached by multiple signaling pathways. Inflammatory response accompanied by glial activation and cytokine release may be neuroprotective in the early stages of PD but exacerbate pathology later in the disease. In this respect, Swada and colleagues [165] recently proposed different microglial populations that may act in either a protective or destructive capacity, depending upon the cytokines and other factors

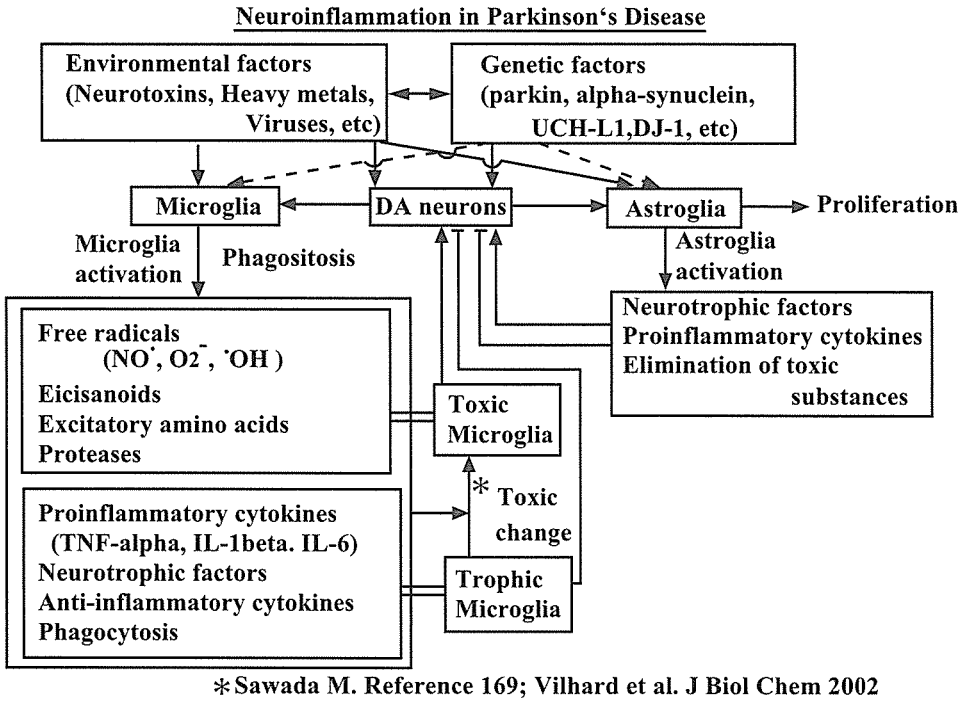

Fig. (3). Hypothetical scheme depicting neuroinflammation and interactions among the triggers, dopamine (DA) neurons, and glial cells in Parkinson's disease (PD).

Lines: possible relations; dotted lines: unsettled relations.

released within the microenvironment. A more comprehensive understanding the neuroinflammatory response and the roles of cytokines in PD should prove to be useful for the designing of new drugs to prevent or protect against the neurodegenerative process that occurs in the disease.

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