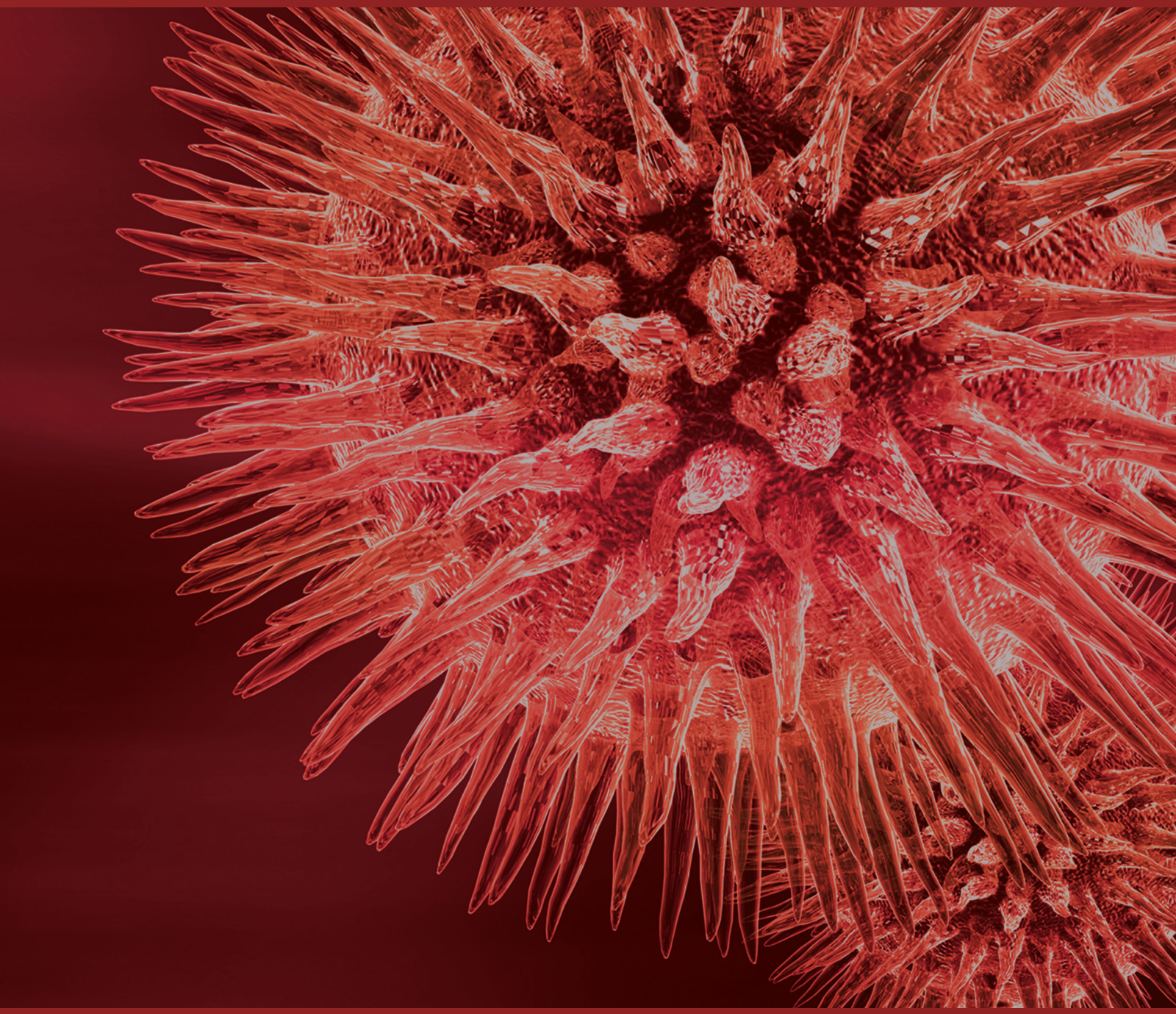
BioMed Research International

Inflammatory Reaction in Neurological Diseases

Guest Editors: Hung-Chen Wang, Cheng-Hsien Lu, Kuang-I Cheng, and Jason Cheng-Hsuan Chiang



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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 408514, 2 pages
http://dx.doi.org/10.1155/2014/408514

Editorial

Inflammatory Reaction in Neurological Diseases

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Received 14 October 2014; Accepted 14 October 2014; Published 31 December 2014

Inflammatory reaction in the central nervous system (CNS) is now recognized to be a feature of all neurological disorders. In neurological degenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), there is prominent infiltration of various leukocyte subsets into the CNS or there is intense activation of microglia with resultant elevation of many inflammatory mediators within the CNS. In acute critical CNS diseases, such as delayed deterioration associated with vasospasm after subarachnoid hemorrhage (SAH), ischemic stroke, spontaneous intracerebral hemorrhage (ICH), and traumatic brain injury (TBI), recent evidences show that inflammation may be a potential target for therapy. Inflammation has become a promising area of research for new treatments. To accelerate the process of translating this information to clinical applications, a number of important issues must be addressed such as their ability to consistently detect characteristic cerebral deficits in individuals with neurological degenerative diseases, the relationships of cerebral injuries to clinical symptoms and genetic characteristics, and the degree to which these injuries respond to different therapies. In this special issue, several research groups report findings that address some of these issues.

In neurological degenerative diseases, the paper by C. Millington et al. reviewed the role of chronic neuroinflammation in the pathogenesis of *Alzheimer's disease* (AD). With the glial fibrillary acidic protein-interleukin 6 (GFAP-IL6) transgenic mice model, the authors found that this animal model, in which chronic neuroinflammation triggered constitutive expression of the cytokine interleukin-6 (IL-6) in astrocytes, could serve as an excellent tool for drug discovery and validation in vivo. The paper by X. Su and H. J. Federoff reviewed the role of inflammation in *Parkinson's disease* (PD) neuropathology. They provide an overview of current knowledge on the temporal profile of central and peripheral immune responses in PD and discuss the potential synergistic effects of the central and peripheral inflammation in disease development. The study by W.-C. Lin et al. used TRODAT-1 SPECT to evaluate leukocyte apoptosis in PD patients and its association with central dopamine neuron loss. The leukocyte apoptosis and striatal dopamine transporter uptake ratios were further associated with increased severity and longer duration of disease. The interaction between brain and systemic inflammation may be responsible for the neurodegenerative disease progression. The paper by K. Lu et al. used the Longitudinal Health Insurance Database 2000 (LHID2000) to investigate and compare the risk of *dementia* between patients clinically diagnosed with *autoimmune rheumatic diseases* (ARD) and non-ARD patients during a 5-year follow-up period. Their findings suggest that patients with and without ARD were found to have similar risks of developing dementia.

In acute critical CNS diseases, the study by K.-W. Wang et al. used *traumatic brain injury* (TBI) model to determine whether simvastatin combined with an antioxidant could attenuate cerebral vascular endothelial inflammatory response after traumatic brain injury in rat. Their findings

support that simvastatin combined with an antioxidant could provide neuroprotection and it may be attributed to a dampening of cerebral vascular endothelial inflammatory response. The study by W. Winardi et al. used a structural equation modeling to evaluate the predictive value of admission Glasgow Coma Scale (GCS) scores, duration of unconsciousness, neurosurgical intervention, and countercoup lesion on the impairment of memory and processing speed functions six months after a TBI. The study demonstrated that admission GCS score is a robust predictor of memory/processing speed dysfunctions after TBI. The study by N.-W. Tsai et al. investigated serum thiobarbituric acid-reactive substances (TBARS) and free thiol levels in different subtypes of *acute ischemic stroke* (AIS) and evaluated their association with clinical outcomes. They found that patients with large-vessel disease have higher oxidative stress but lower antioxidant defense compared to those with small-vessel disease after AIS. Serum TBARS level at the acute phase of stroke is a potential predictor for three-month outcome. And the paper by C.-M. Su et al. aimed to determine whether serum adhesion molecules are associated with *septic encephalopathy* (SE). Their findings demonstrate that SE implies higher mortality in nontraumatic, nonsurgical patients with severe sepsis. Serum vascular cell adhesion molecule-1 (VCAM-1) level on presentation is a more powerful predictor of SE in these patients than lactate concentration and other adhesion molecules on admission.

In the CNS malignancy, the study by D. Winardi et al. investigated the relationship between protein expressions of two autophagy markers, LC3B and Beclin-1, with clinical parameters in *astrocytoma patients*. Their results suggest that astrocytoma cancer stem-like cells together with enhanced autophagy may cause resistance to radiation therapy/chemotherapy and that targeting the cancer stem-like cell in astrocytoma may offer a viable therapeutic approach. And the study by C.-L. Chung et al. investigated DAPK protein expression and promoter hypermethylation in *central neurocytoma and oligodendroglioma*. Their results show that DAPK promoter hypermethylation and repressed expression of DAPK protein were more common in central neurocytoma than in oligodendroglioma. Thus, DAPK promoter hypermethylation could be useful for differential diagnosis between these two types of tumors.

In summary, the papers in this series highlight several important research strategies that are making it increasingly evident that the neuroinflammation is of translational value for different types of neurological diseases. The results from these studies not only help us to understand the pathogenesis of these disorders but also show great potential to provide urgently needed objective biomarkers for clinical diagnosis and evaluation. Knowledge and understanding of these conditions have led to the development of animal models, successful therapies, and novel tools to characterize these clinical conditions and provide better care to patients.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 309129, 10 pages
http://dx.doi.org/10.1155/2014/309129

Review Article

Chronic Neuroinflammation in Alzheimer's Disease: New Perspectives on Animal Models and Promising Candidate Drugs

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Received 28 February 2014; Revised 14 May 2014; Accepted 14 May 2014; Published 16 June 2014

Academic Editor: Cheng-Hsien Lu

Chronic neuroinflammation is now considered one of the major factors in the pathogenesis of Alzheimer's disease (AD). However, the most widely used transgenic AD models (overexpressing mutated forms of amyloid precursor protein, presenilin, and/or tau) do not demonstrate the degree of inflammation, neurodegeneration (particularly of the cholinergic system), and cognitive decline that is comparable with the human disease. Hence a more suitable animal model is needed to more closely mimic the resulting cognitive decline and memory loss in humans in order to investigate the effects of neuroinflammation on neurodegeneration. One of these models is the glial fibrillary acidic protein-interleukin 6 (GFAP-IL6) mouse, in which chronic neuroinflammation triggered constitutive expression of the cytokine interleukin-6 (IL-6) in astrocytes. These transgenic mice show substantial and progressive neurodegeneration as well as a decline in motor skills and cognitive function, starting from 6 months of age. This animal model could serve as an excellent tool for drug discovery and validation *in vivo*. In this review, we have also selected three potential anti-inflammatory drugs, curcumin, apigenin, and tenilsetam, as candidate drugs, which could be tested in this model.

1. Alzheimer's Disease

1.1. Alzheimer's Disease: A Disease without a Neuroprotective Treatment. Dementia is one of the major causes of disability and dependency among older people worldwide. In 2012, 35.6 million people have dementia worldwide and there are 7.7 million new cases reported every year [1]. It has been predicted that the number of people affected by dementia will double every 20 years to 81.1 million by 2040 [1]. Alzheimer's disease (AD) is the most common form of dementia, accounting for up to 70% of all sporadic, late-onset cases of dementia. In an aging population, the number of dementia sufferers increases every year and this poses significant health and social problems for society in the very near future. Despite decades of research dedicated to the development of new pharmaceutical compounds designed to slow down disease progression, no drugs with real disease-modifying properties are available at this point in time. There is a growing consensus in the scientific community that disease-modifying treatments which start before the onset of clinical dementia are needed [2].

1.2. Neuropsychology and Pathophysiology of AD. The first behavioural symptoms of AD include significant memory problems in immediate recall and short-term or long-term memory. Additionally there are significant deficits in at least

one of four areas: expressing or comprehending language; identifying familiar objects through the senses; poor coordination, gait, or muscle function; and the executive functions of planning, ordering, and making judgments. These symptoms appear gradually and become steadily impaired over time [3].

The main pathological hallmarks of AD are two types of protein deposits, amyloid plaques and neurofibrillary tangles and the degeneration of cholinergic neurons in the basal forebrain and the associated loss of cholinergic neurotransmission in the cerebral cortex and other areas. [4]. Amyloid-beta (Aβ) peptide is the main constituent of senile plaques and creates one of the key pathological features of AD. Although the initial cause of sporadic AD is still debated, the "amyloid cascade hypothesis" states that the aberrant production, aggregation, and deposition of Aβ is a causative process in the pathogenesis of AD [5]. Some researchers have found evidence that Aβ fibrils form pores in neurons, leading to calcium influx and the neuronal death associated with AD [6]. Apart from the direct role in cell death, Aβ-mediated glutamate receptor modifications can lead to synaptic dysfunction, resulting in excitotoxic neurodegeneration during the progression of AD [7]. However, the long list of negative clinical trials targeting amyloid production or deposition compels us yet again to reexamine the amyloid cascade hypothesis as only a marginally significant pathogenic mediator of disease and to perhaps revert back to traditional science principle where repeated negative data leads one to consider other ideas [8–10].

Tau is one of the microtubule-associated proteins (MAPs) that stabilize neuronal microtubules for their role in the development of cell processes, establishment of cell polarity, and intracellular transport. Neurofibrillary protein aggregates are one of the major hallmarks of AD. These aggregates, also known as neurofibrillary tangles (NFT), are formed as a result of abnormal hyperphosphorylation of tau protein, aggregation into "paired helical filaments" (PHFs), cross-linked by reactive carbonyl compounds [11, 12], and loss of ability to maintain the microtubule tracks [13]. Despite a lack of understanding of the intermediate steps involved, NFT formation appears to be caused by aberrant signalling that leads to an imbalance of kinases/phosphatases, resulting in hyperphosphorylation of tau and its detachment from microtubules. Microtubule breakdown follows, an aggregation of tau into PHFs, which in turn bundle into NFTs of "neuropil threads" that then leads to the disintegration of intracellular transport and neuronal degeneration [14].

1.3. Current Treatment Strategies for AD. The two major pharmacological treatment options against AD currently available are acetylcholinesterase inhibitors and memantine, a glutamate receptor associated channel blocker. Among those, cholinesterase inhibitors are the most common medications used for the treatment of AD; however these treatments are symptomatic and only temporarily effective [15]. Although antiamyloid drugs were initially hoped to slow down disease progression, all respective clinical trials have failed to yield positive results. As there are no disease-modifying therapies available for the treatment of AD at the moment, alternative targets such as low-grade neuroinflammation are attracting more and more interest in the search of a disease-modifying treatment for AD [16–18].

1.4. The Cholinergic Deficit in AD. Cholinergic neurons are one of the most prominent features of the mammalian basal forebrain that can be described as a collection of aggregated and nonaggregated, large, hyperchromic neurons; many of them contain choline acetyl transferase (the key enzyme in the synthesis of acetylcholine) and project to the cerebral cortex and hippocampus [19]. Cholinergic neurons are widely distributed in the basal forebrain, including the medial septum, the nucleus of the horizontal, vertical, and lateral limbs of the diagonal bands of Broca, and the ventral pallidum, in the basal part of substantia innominata, and in the extension of amygdala. Cholinergic input from the basal forebrain to the cortex and the hippocampus has a key role in mechanisms of cognitive functions, including arousal, attention, sensory processing, and memory [20, 21]. The number and size of the basal forebrain cholinergic neurons as well as cortical acetylcholine axon density have been found to decrease with normal aging and in AD [22]. In AD, in contrast to normal ageing, the progression of brain atrophy caused by neuronal and synaptic loss is rapidly accelerated. Massive cholinergic cell death in the nucleus basalis was originally suggested to be one of the major indicators of AD [23], and the resulting cholinergic deficits in the cortical and hippocampal regions have been correlated with the severity of dementia [24]. Patients with AD have a significant decrease of acetylcholine in the cortex and show pathological changes in cholinergic basal forebrain neurons [25]. Recent evidence suggests that atrophy of the cholinergic basal forebrain in AD can be distinguished from normal age-related degeneration even at predementia stages of the disease [26]. These findings considered collectively suggest that cholinergic neuronal cell death in the basal forebrain together with the decline of the cholinergic synapse numbers in the hippocampus and the neocortex is characteristic pathological manifestation of AD. Therefore, animal models of AD need to be able to demonstrate these features to be suitable for disease modelling and drug discovery.

1.5. Chronic Neuroinflammation Occurs in Early Stages of AD. AD is associated with an inflammatory response as shown by an increased presence of activated microglia and astrocytes, activated complement proteins, cytokines, and reactive oxygen, nitrogen, and carbonyl species [27–31]. These histochemical studies have been confirmed by imaging studies using positron emission tomography and the peripheral benzodiazepine ligand PK11195 as a marker for activated microglia indicates that activation of microglia occurs already in mild and early forms of AD and precedes cerebral atrophy [32, 33]. Genetic studies on AD further confirm the importance of inflammation. For example, genome-wide

association studies have identified three genes that are associated with an increased risk of developing AD, CLU (clusterin), CR1 (complement receptor 1), and TREM2 (triggering receptor expressed on myeloid cells 2) [34]. Furthermore, pharmacological evidence also points to the importance of neuroinflammation for AD pathogenesis. Long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been shown to delay the onset of AD, but randomized trials show no benefit from NSAIDs in AD patients. However, asymptomatic individuals treated with the NSAID, naproxen, experienced reduced AD incidence, after only 2 to 3 years [35]. In summary, these data support the significance of neuroinflammation for AD and gave rise to the hypothesis of the "cycle of self-perpetuating inflammatory neurotoxicity" [36]. (a) Multiple inflammatory triggers can lead to microglial activation. These triggers can be peripheral (such as systemic infections or peripheral chronic inflammation) or central (e.g., caused by beta-amyloid peptide in senile plaques). (b) Activated microglia release neurotoxic factors such as cytotoxic cytokines like TNF-α and reactive oxygen/nitrogen species and thus cause damage to neighbouring neurons; direct neurotoxic insults (such as energy depletion and hypoxia) might further weaken neurons and make them more susceptible to microglial attack. (c) These damaged or dying neurons release microglia activators, for example, damage associated molecular patterns (alarmins), resulting in a self-perpetuating cycle of neurotoxicity. In summary, neuropathological, epidemiological, and genetic findings show clear evidence for the involvement of neuroinflammation in the early stages of sporadic AD.

2. Shortcoming in the Current Amyloid-Based Mouse Models of AD

Transgenic (Tg) mice that overexpress mutant familial Alzheimer's disease (AD) amyloid precursor protein (APP) genes have contributed to an understanding of dementia pathology and support the amyloid cascade hypothesis. Although many sophisticated mice APP models exist, none comprises all features of AD cellular and behavioural pathology. The greater resilience of transgenic mice to substantial Aβ burdens suggests the Aβ levels and forms that are deleterious to human neurons are not as noxious in these animal models [37]. For example, the APP23 mouse model does not demonstrate all features of the human disease, such as cholinergic axon terminal deficits and extensive cholinergic cell loss in relevant areas such as in the cerebral cortex and CA hippocampal area 1 [38, 39]. Furthermore, these mice do not demonstrate the same variety of proinflammatory markers as human AD patients and generally develop a much weaker neuroinflammatory phenotype [40]. For example, when the Tg2576 mice (containing the Swedish double mutation of human APP) were examined for the expression pattern of various cytokines, only a few IL-6-immunoreactive astrocytes were observed, and iNOS immunoreactivity was completely absent [41]. Moreover, in one of our studies investigating the effect of vitamin D depletion and supplementation with vitamin D enriched mushrooms, there was no clear difference between wild-type and amyloid AD transgenic mice (APPswe/PS1dE9) in terms of learning and memory, despite significant deposition of amyloid plaques observed in the AD mice [42].

In summary, amyloid overexpressing transgenic mice were initially thought to provide a useful model to investigate the mechanisms by which cytokines contribute to the progression of AD, including cognitive decline. However, there is decreasing confidence that this is the best animal model for this purpose. Therefore, a novel model of chronic neuroinflammation with resulting neurodegeneration would be helpful; the GFAP-IL-6 mouse model could be ideal for this purpose.

3. The GFAP-IL-6 Transgenic Mouse Model as Novel Model of Chronic Neuroinflammation Relevant for AD

The GFAP IL-6 mouse line was initially generated to investigate cytokine signalling in the CNS. In this model, the murine IL-6 gene (and lacZ) is expressed in astroglia under the transcriptional control of the murine glial fibrillary acidic protein (GFAP) promoter, resulting in brain-specific forced expression of IL-6 [43]. In the wild-type (WT) mouse brain, IL-6 levels are undetectable, while in the GFAP-IL6 transgenic, elevated levels of IL-6 are observed in the cerebellum, the striatum, the hippocampus, the hypothalamus, the neocortex, and the pons, resulting in accelerated age-related structural changes seen within 3–6 months compared to normal aged mice [44]. It has also been shown that the level of transgene-encoded IL-6 expression in the CNS of these transgenic mice is similar to that found in EAE and thus falls within a pathophysiological range [45]. Astrocyte production of IL-6 results in a localised inflammatory state within the CNS with the activation of many acute-phase response genes including α1-antichymotrypsin, complement C3, and metallothionein [45]. Cellular changes include the activation of astrocytes and microglia, proliferative angiopathy with blood-brain barrier (BBB) breakdown [46], reduced hippocampal neurogenesis [44], and neurodegeneration which is accompanied by age-related deficits in learning and memory [47]. In many respects, the GFAP-IL6 mouse replicates the structural and functional neuropathology of human neurodegenerative diseases including Alzheimer's disease and HIV-associated dementia [46]. Thus, this transgenic mouse not only highlights the capacity for endogenously produced IL-6 to induce inflammation and neurodegeneration in the CNS but also provides a powerful tool in which to explore the basic mechanisms that underpin IL-6 actions and associated neurodegeneration in the brain.

While it is arguable, which of the major proinflammatory cytokines might be the best to create a suitable animal model providing both severe neurodegeneration and being of relevance for AD, we believe that IL-6 is a particular good candidate. As it serves as one of the inflammatory triggers for the following reasons; IL-6 can be consistently detected in the brains of AD patients but not in the brains

of nondemented elderly people [48], and increased IL-6 levels in serum have been shown to differentiate dementia from normal ageing [49]. Additionally, connections between genetic variants of IL-6 and the volume of the hippocampus were analyzed using voxel-based morphometry indicating that the IL-6 allele has a significant role in the development of brain atrophy [50]. Based on preliminary data and previous findings, this IL-6 mouse model provides an exceptional opportunity to investigate the detrimental effects of chronic neuroinflammation on the structure and function of the mammalian brain, paying special attention to its effects on the cholinergic system.

4. The IL-6 Overexpressing Mouse as a Drug Validation Model

Animal models like the GFAP-IL-6 mouse can be used to perform preclinical proof-of-concept studies and assess the efficacy, mechanism of action, and safety profile of anti-inflammatory and neuroprotective compounds. In animals treated with these compounds, cognitive, functional, and behavioural tests can be performed and complemented with tissue-based assays, to demonstrate *in vivo* the molecular basis, drug efficacy, and mechanism of action. Based on previous research, we have selected three candidate compounds, curcumin, apigenin, and tenilsetam, to evaluate if anti-inflammatory treatment can slow down the progression of neurodegeneration and the decline in cognitive function in the GFAP IL-6 mouse model, which will be discussed in this review.

5. Potential Anti-Inflammatory Drug Candidates for the Treatment of AD

5.1. Curcumin. Curcumin is a component of the Indian curry spice turmeric (*Curcuma longa Linn*). Curcumin has been shown to have various antioxidant and anti-inflammatory properties. For example, curcumin was shown to decrease the level of inflammatory mediators such as tumour necrosis factor- (TNF-) α and inhibited the production of interleukins (IL) 1, 2, 6, 8, and 12, monocyte chemo attractant protein (MCP), and migration inhibitory protein [51–55]. Curcumin inhibits inflammatory cytokines through a number of mechanisms, with *in vitro* studies indicating that curcumin inhibits the activation of certain transcription factors such as activating protein-1 (AP-1) and nuclear factor kappa B (NF-κB) in stimulated monocytes and macrophages, thereby blocking cytokine gene expression [56, 57]. Furthermore, the pharmacokinetic properties of curcumin are favourable in achieving clinical efficacy. Curcumin crosses the blood-brain barrier and curcumin preparations with enhanced bioavailability (delivered orally) can achieve therapeutic concentrations in the brain [58]. For example, brain levels of the curcuminoids reached concentrations of up to 3 μM for curcumin and up to 6 μM for tetrahydrocurcumin (TC) [59]. These concentrations are indicative of clinical efficacy, since they are in the same range as the tissue concentrations for inhibition of mRNA production of inducible nitric oxide synthase (iNOS) *in vivo*, where the EC_{50} values were determined at 1.2 and 0.701 μM for curcumin and TC, respectively [59].

Lim et al. have tested curcumin for its ability to inhibit the combined inflammatory and oxidative damage in Tg2576 transgenic mice. In this study, Tg2576 mice aged 10 months were fed a curcumin diet (160 ppm) for 6 months. These results indicated that the curcumin diet significantly lowered the levels of oxidised proteins, interleukin- (IL-) 1β, and soluble and insoluble Aβ, in addition to plaque burden [58]. Following on from this work, Yang et al. evaluated the effect of feeding a curcumin diet (500 ppm) to 17-month-old Tg2576 mice for 6 months [60]. When fed to the aged Tg2576 mice with advanced amyloid accumulation, curcumin resulted in reduced soluble amyloid levels and plaque burden [60]. Moreover, the effect of curcumin in the brain was demonstrated by its capability to modulate cholinergic neurotransmission and, consequently, improve cognition deficits and memory impairment in aged rats [61].

So far, large clinical trials with curcumin in AD patients have been lacking. However, one small study conducted in Japan reported some interesting results [62]. In this study, Hishikawa et al. described three patients with AD whose behavioural symptoms improved remarkably as a result of turmeric (the spice containing curcumin as major ingredient) treatment [62]. After 12 weeks of the treatment, the total score of the Neuropsychiatric Inventory-brief questionnaire decreased significantly in both acuity of symptoms and burden on caregivers. In one case, the minimental state examination (MMSE) score was up by five points, from 12/30 to 17/30. In the other two cases, no significant change was seen in the MMSE; however, they came to recognize their family within 1 year of treatment. In all cases that had been taking turmeric for more than 1 year reexacerbation of behavioural and psychological symptoms of dementia (BPSD) was not seen. Though it is a small sample size, the three AD cases treated with turmeric suggest a significant improvement of the cognitive and behavioural symptoms, suggesting a probable benefit in the use of turmeric in individuals with AD for BPSD [62].

The reported *in vitro* and *in vivo* studies indicate that curcumin is a suitable compound to target pathways involved in neuroinflammation. However, preparations with enhanced bioavailability need to be developed in order to realize therapeutic concentrations in the human CNS [63].

5.2. Apigenin. Apigenin is a dietary flavonoid found in a wide variety of plants, fruits, and vegetables. This polyphenolic compound is particularly abundant in the ligulate flowers of the chamomile plant and in other sources such as celery, parsley, and grapefruit [64]. Previous investigations of the biological activity of apigenin have demonstrated potent antimicrobial, anti-inflammatory, antioxidant, and antitumorigenic properties [65–67]. Evidence from several reports suggests a broad and potent anti-inflammatory activity of apigenin [68, 69]. For example, apigenin has been shown to have inhibitory effects on *in vitro* releasing of several proinflammatory mediators in lipopolysaccharide (LPS) and upregulation of proinflammatory markers in murine and

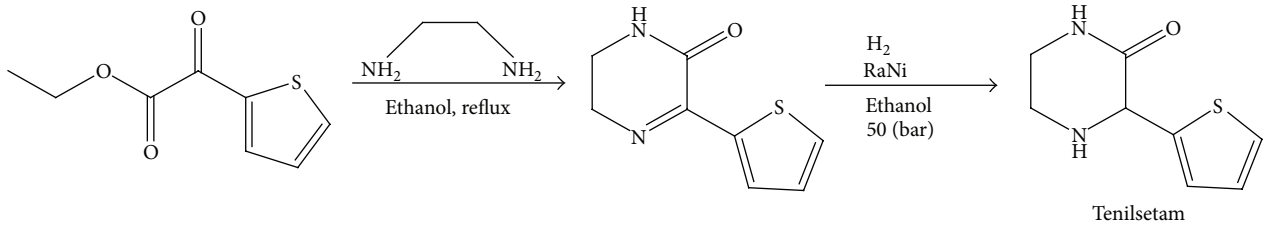

Figure 1: Synthetic route for tenilsetam.

human cell lines. Apigenin strongly decreased levels of IL-6 in LPS activated mouse macrophages [70] and suppressed CD40, TNF-α, and IL-6 production via inhibition of interferon gamma (IFN-γ) induced phosphorylation of STAT1 in murine microglia [71]. Evidence of its anti-inflammatory properties is also demonstrated in studies that show dose-dependent suppression of the inflammatory mediators nitric oxide (NO) and prostaglandin E_2, through inhibition of iNOS and cyclooxygenase-2 (COX-2) expression in both microglial and macrophage mouse cells [67, 72]. In human cell cultures, apigenin has been demonstrated to attenuate the release of inflammatory cytokines by inactivation of NF-κB, mediated by suppression of LPS-induced phosphorylation of the p65 subunit [73]. Other effects reported for apigenin include decreasing expression of adhesion molecules [74] and its well-known defensive properties against oxidative stress, such as free radical scavenging and increasing intracellular glutathione concentrations [75]. Apigenin is reported to exert many of its effects through interactions with the signaling molecules in the 3 major MAPK pathways (ERK, JNK, and p38) in both murine and human cell culture models [76–78].

There are very few studies on apigenin in models of neuroinflammation and/or cognitive decline including AD animal models. One recent study by Zhao et al. tested the neuroprotective effects of apigenin in the APP/PS1 double transgenic AD mouse [79]. Four-month-old mice were treated orally with apigenin (40 mg/kg) for 3 months. Results indicated that apigenin-treated mice displayed improvements in memory and learning deficits and a reduction of fibrillar amyloid deposits with lowered insoluble Aβ concentrations, mediated by a decrease in β-CTF and BACE1. Additionally, the apigenin-treated mice showed restoration of the cortical ERK/CREB/BDNF pathway involved in learning and memory typically affected in AD pathology. Enhanced activities of superoxide dismutase and glutathione peroxidase were also observed and increased superoxide anion scavenging [79, 80]. Similarly, in another study Aβ-$_{25-35}$-induced amnesia mouse models were treated with apigenin (20 mg/kg), resulting in improvements in spatial learning and memory, in addition to neurovascular protective effects [79]. Other *in vivo* studies with non-AD-related animal models report significant reductions in LPS-induced IL-6 and TNF-α production in apigenin pretreated mice [70].

Based on the published literature, only one study in humans had been conducted with apigenin with respect to inflammation or cognitive performance. Forty-two patients with AD (12), PD (17), and MS (13) were included in the study; a formulation containing apigenin was administered to them every 12 hours and they were given appointments every three months for a clinical evaluation and a review of general lab analyses. Subjects were submitted to follow-up evaluations between 3 and 24 months (mean: 8.85 ± 5.99 months). Clinical stabilisation was achieved in all the patients (100%) with MS and the scores on the Expanded Disability Status Scale improved in four patients. Clinical stabilisation was achieved in 17 patients (100%) with PD and improvements in the score on the Unified Parkinson's Disease Rating Scale are in 15 of them. Of the AD patients, 12 attained clinical stabilisation (100%) with an improvement in minimental test in nine cases [81]. In view of the role of oxidative stress and neuroinflammatory processes involved in the pathophysiology of AD, there is evidence to suggest that apigenin is a suitable and promising natural compound to further investigate.

5.3. Tenilsetam. The nootropic drug (±)-3-(2-thienyl)-2-piperazinone (CAS 997, Tenilsetam, Figure 1) has antidementia, antiamnesic, and antihypoxidotic properties. *In vivo*, Tenilsetam has been assayed in human plasma and urine, where recovery in both media was achieved with endogenous background material separated accurately via a HPLC method [82]. Several pharmacokinetic studies were completed and plasma concentrations were observed over 72 h period. With an initial 150 mg dose, the mean plasma concentration reached a maximum value of 3.39 ± 0.64 μg/mL and the mean urine cumulative recovery was 47.3 ± 3.4%. Tenilsetam was absorbed reasonably fast, showing peak plasma concentrations at 2 hours, which were determined to be dose-dependent. The half-life was determined to be between 18 and 22 hours [83].

In human studies, encephalotropic and psychotropic properties were studied in ten aged subjects using quantitative EEG and psychometric analysis. EEG spectral analysis displayed significant CNS activity caused by tenilsetam, where alpha brain activity increased and delta activity decreased. Time-efficacy calculations showed two pharmacodynamic peaks occurring in the 4th and between the 8th and 24th hour. The lag between pharmacokinetic and pharmacodynamic changes is consistent with the hypotheses that tenilsetam needs to penetrate the blood-brain barrier [83].

Several mechanisms of action for tenilsetam have been hypothesized. For example, tenilsetam has been shown to be

a carbonyl scavenger (e.g., of methylglyoxal) or inhibitor of advanced glycation end-product (AGE) formation [84, 85]. It inhibits AGE-derived amino acid and protein crosslinking *in vitro* by covalently scavenging toxic reactive carbonyl compounds such as methylglyoxal [86–88]. In addition, mounting evidence suggests that the interaction of AGEs with their receptor RAGE perpetuates inflammation and participates actively in various vascular and inflammatory diseases [11, 27, 89, 90]. There are various RAGE ligands including AGEs, S100 proteins, and amphoterins [91, 92]. It is proposed that tenilsetam could exert an indirect anti-inflammatory action by minimizing proinflammatory AGE formation [84, 89].

In vitro experiments with human neuroblastoma SH-SY5Y cells, incubated with methylglyoxal which was preincubated with tenilsetam for 2 or 24 hours, tenilsetam completely nullified the toxicity of methylglyoxal at both time points [87]. A few studies have investigated the effect of tenilsetam in humans in regard to memory and cognition. For example, Wesnes et al. [93] examined the influence of tenilsetam on 18 male undergraduates with the hypothesis that tenilsetam would improve cognitive performance in normal conditions and after scopolamine was administered. In an additional study, healthy volunteers ($n = 15$) received randomized tenilsetam doses (150, 300, and 900 mg) to study proof of antihypoxidotic activity. Blood gas, EEG, and psychometric results were obtained under hypoxic (9.8% O_2) and normoxic (21% O_2) conditions. Spectral EEG analysis indicated deterioration in vigilance under the hypoxic conditions, which was attenuated by tenilsetam [94].

Tenilsetam has been used successfully as a treatment in a pilot trial for AD patients. Over a 3-month period at 150 mg/day doses, AD patients showed significant improvements in the favorability judgment task (FJT) and reaction time [95]. The influence of tenilsetam on the P300 response test was also observed with the same dose causing a significant reduction in latency after 4 weeks of treatment [96]. The supporting data surrounding tenilsetam suggest that it would make a suitable compound to test in the GFAP IL-6 mouse to learn more about the drug's mode of action, about its anti-inflammatory and cognitive enhancing properties, and if it attenuates sustained neuroinflammation and prevents cognitive decline.

6. Conclusion

Dementia, in particular Alzheimer's disease (AD), poses a substantial challenge to health, aged care, and social policy. Chronic neuroinflammation, demonstrated by the activation of microglia and astrocytes as well as the release of reactive oxygen species and cytokines, has attracted considerable interest in AD over the past decade, not only for its potential role in contributing to neuronal degeneration, but also as a target site for developing potent therapeutics in the future. To investigate the connection between neuroinflammation and AD and to test potential anti-inflammatory drugs, a more suitable "neuroinflammatory" animal model is needed, showing the level of neuroinflammation comparable to the human conditions. Using such animal models, including the GFAP-IL6 mouse line, will allow researchers to investigate the effects of chronic low-grade neuroinflammation on brain structure and function, as well as the effects of anti-inflammatory agents in prevention and recovery.

In this review, we introduced the GFAP-IL6 transgenic mouse, in which the proinflammatory cytokine interleukin-6 (IL-6) is produced by astrocytes under the control of the GFAP promoter, highlighting this as a suitable animal model to study the role of chronic neuroinflammation in neurodegeneration. Previous studies show that these transgenic mice display progressive neurodegeneration in the hippocampal and cerebellar regions and a decline of both cognitive function and motor skills from 6 months of age. It has not been evaluated if the cholinergic system, the neurotransmitter system most affected in AD, is impaired in this model, and it is important to understand if IL-6 induced inflammation may cause cholinergic system degeneration and contribute to cognitive impairment.

Previous studies suggested that curcumin, apigenin, and tenilsetam are promising compounds due to their pharmacokinetic properties and previous success in animal models of familiar AD or in patients. In addition to these three drugs, we anticipate that this animal model can be used to validate a variety of cytokine suppressive anti-inflammatory drugs (CSAIDs) with potential for the prevention and/or treatment of AD from both synthetic and natural sources in the future. The GFAP-IL6 mouse line as animal model of chronic neuroinflammation might be even useful for the investigation and therapeutic intervention relevant for other neuroinflammation related diseases, such as multiple sclerosis, lupus, bipolar disorder, Rasmussen's encephalitis, and traumatic brain injury, which are all associated with microglial activation and neurodegeneration. Furthermore, we believe that this animal model can serve as a valuable *in vivo* bioassay for screening of anti-inflammatory drugs, including the class of CSAIDs, and a successful treatment can be monitored by amelioration of inflammation, neurodegeneration, and cognitive decline.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Christopher Millington and Sandra Sonego contributed equally to this paper as first authors.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 910260, 6 pages
http://dx.doi.org/10.1155/2014/910260

Research Article

Simvastatin Combined with Antioxidant Attenuates the Cerebral Vascular Endothelial Inflammatory Response in a Rat Traumatic Brain Injury

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Received 26 February 2014; Revised 17 April 2014; Accepted 22 April 2014; Published 11 June 2014

Academic Editor: Cheng-Hsien Lu

Traumatic brain injury (TBI) leads to important and deleterious neuroinflammation, as evidenced by indicators such as edema, cytokine production, induction of nitric oxide synthase, and leukocyte infiltration. After TBI, cerebral vascular endothelial cells play a crucial role in the pathogenesis of inflammation. In our previous study, we proved that simvastatin could attenuate cerebral vascular endothelial inflammatory response in a rat traumatic brain injury. This purpose of this study was to determine whether simvastatin combined with an antioxidant could produce the same effect or greater and to examine affected surrogate biomarkers for the neuroinflammation after traumatic brain injury in rat. In our study, cortical contusions were induced, and the effect of acute and continuous treatment of simvastatin and vitamin C on behavior and inflammation in adult rats following experimental TBI was evaluated. The results demonstrated that simvastatin combined with an antioxidant could provide neuroprotection and it may be attributed to a dampening of cerebral vascular endothelial inflammatory response.

1. Introduction

Traumatic brain injury (TBI) remains one of the leading causes of death and disability in industrialized countries. Despite numerous studies on animal models of TBI that have investigated therapeutic strategies, no effective therapy is currently available [1]. TBI leads to important and deleterious neuroinflammation, as evidenced by edema, cytokine production, induction of nitric oxide synthase, and leukocyte infiltration. Strategies that block inflammatory and oxidative mediators have been shown to induce neuroprotective and anti-inflammatory effects after brain injury [2].

After TBI, cerebral vascular endothelial cells play a crucial role in the pathogenesis of inflammation and it has been comprehensively reviewed [3]. In this study, we chose to assay ICAM-1 and IL-10 as the markers of vascular endothelial cell inflammation according to the result of our previous study [3]. Statins, a class of lipid-lowering drugs, inhibit 3-hydroxy-3-methylglutaryl-CoA reductase, thereby suppressing cholesterol biosynthesis. Apart from their lipid-lowering activities, statins have been shown to mediate pleiotropic effects *in vitro* and *in vivo* by reducing inflammation and oxidative stress [4, 5]. Several studies have shown that the administration of statins induced neuroprotective and anti-inflammatory effects and improved neurological outcomes after experimental TBI [3, 6–9].

Vitamin C in human must be ingested for survival. It is an electron donor, and the property accounts for all its known functions. The antioxidant effects of vitamin C have been demonstrated in many experiments *in vitro*. Human diseases such as atherosclerosis and cancer might occur in part from oxidant damage to tissues. The relationship of this oxidant to human disease conditions is not very clear at this time, but it has been studied [10–12].

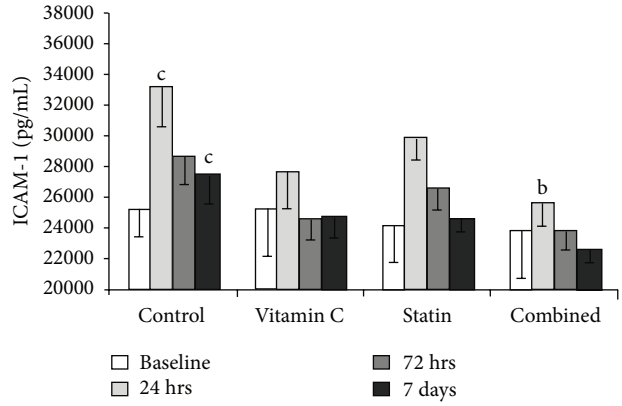

Figure 1: The labels "a," "b," and "c" denote $P \leq 0.05$ for the comparison versus vitamin C group, statin group, and combined group at a specific time-point, respectively. In the time- point of 24 h and day 7 the combination group showed a significant reduction in ICAM-1 as compared to the control group. In the time-point of 24 h the combination group had a significant reduction in ICAM-1 when compared to the statin group. However, there was no difference in each time-point between vitamin C and combination group.

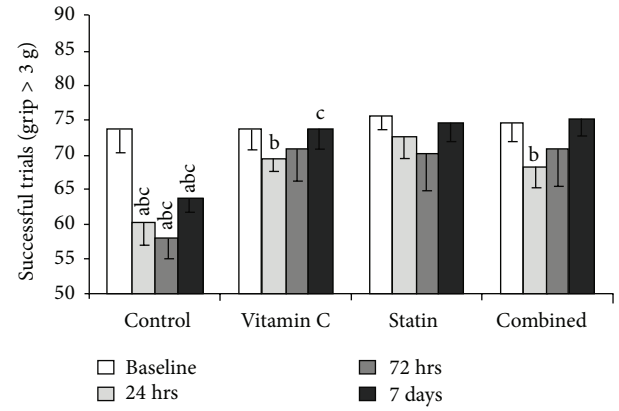

Figure 2: The labels "a," "b," and "c" denote $P < 0.05$ for the comparison versus vitamin C group, statin group, and combined group at a specific time-point, respectively. In the time- point of 24 h the statin group had the best neurological function when compared to the other 3 groups. At the time-point of the 7th day the combination group had better performance in neurological function when compared to the vitamin C group.

To date few published studies have investigated the consequences of a combination therapy following TBI, and that it may decrease the side effects of the single drug. In this study, we investigated the effect of acute and continuous treatment of simvastatin combining with vitamin C on behavior and inflammation in adult rats following experimental TBI.

2. Materials and Methods

2.1. Animals. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of E-DA Hospital and complied with the IACUC Guide for the Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats ($n = 30$; weight: 400–450 g) were group-housed on a 12-12 h light-dark cycle and were provided with standard diet.

2.2. TBI in Rats. Cortical contusions were induced using a device adapted from the impact method described in detail elsewhere [13, 14]. Briefly, rats were anesthetized with halothane, body temperature was maintained at 37°C, and other vital signs were held stable. The scalp was cleaned with Ioprep, and aseptic techniques were used throughout surgery. The scalp was opened, and a craniotomy was performed over the left hemisphere; the center of the footplate was positioned 1.5 mm posterior and 2.5 mm lateral to the bregma [14, 15]. Contusions were made in the "hind paw" area, which consists of overlapping motor and somatosensory fields [16]. This area was selected because it is readily accessible and relatively flat, and if injured, it produces a readily observable deficit. Animals were randomly assigned as unilateral contusion or craniotomy controls.

Following the removal of a small bone flap, a stainless steel circular footplate was placed so that it rested just upon the surface of dura, which remained intact. To prevent contused cortex from herniating into the opening, craniotomies were only slightly larger than the diameter of the footplate. A 40 cm long stainless steel tube kept at a 90° angle was used to guide a falling 20 g brass weight onto the footplate. To prevent air compression in the tube, the tube was perforated at 1 cm intervals.

Following surgery, animals were placed in the prone position on a 10 cm foam block, and the foam block was placed beneath the contusing device. Injury was induced by release of a 20 g brass weight from a height of 40 cm onto the foot plate. The degree of injury was created by repeated controlled cortical impacts, and the injured rats received 10 cortical impacts. After impact, the bone flap was replaced and sealed with bone wax, the scalp sutured closed, and the animals were allowed to recover.

2.3. Experimental Protocol. There were 5 groups utilized for the study: (1) sham group, craniotomy only ($n = 6$); (2) control group, TBI without treatment ($n = 6$); (3) treatment group ($n = 6$), TBI with vitamin C, administration only; (4) treatment group ($n = 6$), TBI with simvastatin only; and (5) treatment group ($n = 6$), TBI with combination therapy. According to our previous study and other studies (Shao et al. [17]), we chose to administer an even higher dose (15 mg/kg) of simvastatin (Merck). The treatment group received 15 mg/kg of simvastatin in 1 mL of distilled water daily and vitamin C (20 mg/kg) (Chi Sheng) for 3 days via an orogastric tube inserted each day [18]. The first dose was given 1 h after experimental TBI. Each animal in the control and sham groups received 1 mL/day of distilled water via the same route [19]. The rats were sacrificed 7 days later and brain specimens were preserved for immunochemistry analysis.

2.4. Neurological Score. Neurological function was assessed with the grip test (grip strength meter, Singa). The grip test

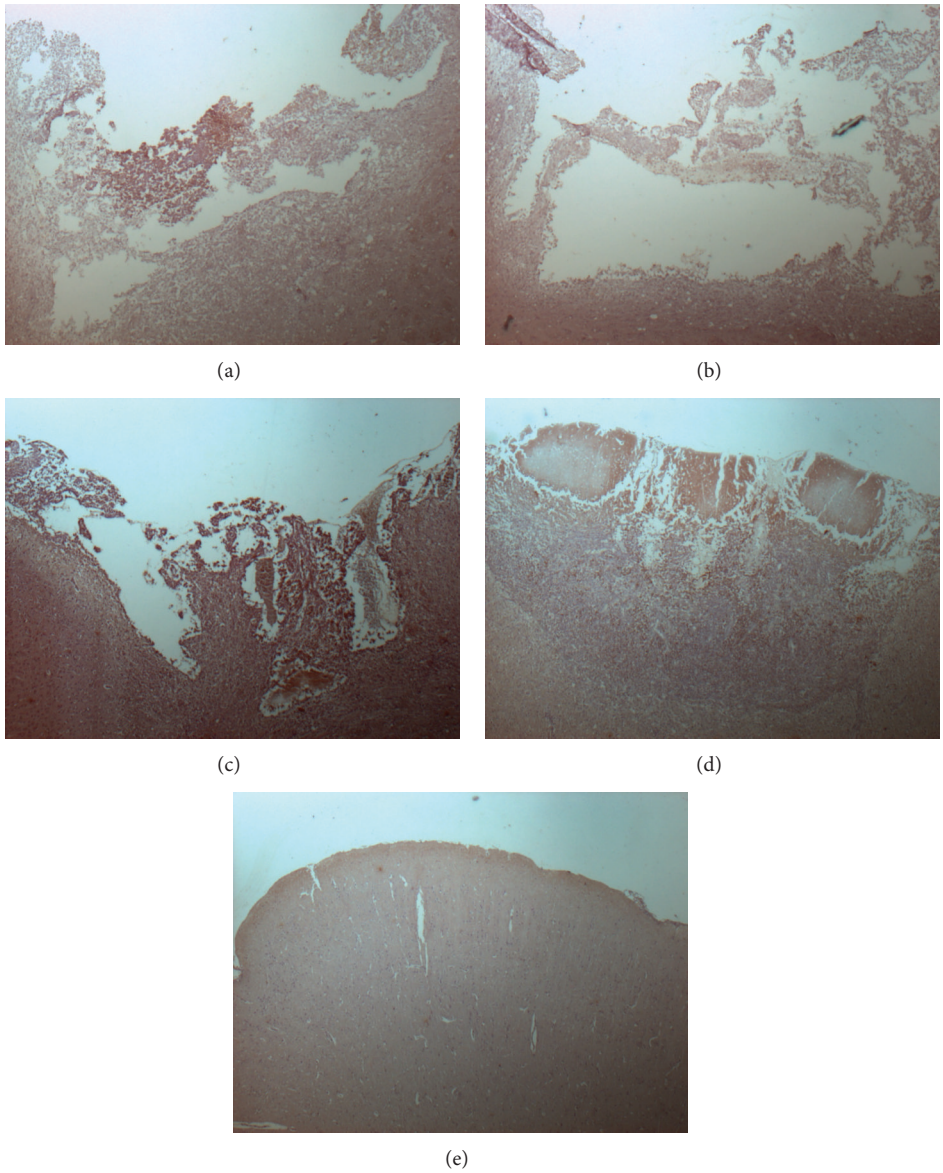

FIGURE 3: (a) The control group showed severe cortex injury and brain tissue loss. (b) The simvastatin treatment group showed some cortex injury and brain tissue loss. (c) The vitamin C treatment group also showed some cortex injury and brain tissue loss. But the area of injury was smaller. (d) The combination treatment group showed mild cortex injury and minimal brain tissue loss. (e) The sham group showed intact cortex.

was performed before TBI induction and then at 24 h, 72 h, and 1 week after TBI. We evaluated the muscle power of all 4 limbs, and a trial was successful if the effective grip power of limbs was more than 3 g. We recorded the frequency of successful trials in 60 seconds [20].

2.5. Determination of ICAM-1 and IL-10 Levels. Blood samples were collected in tubes with potassium acetate before injury and at selected times after injury (24, 48, and 72 h and 7 days). Samples were then centrifuged at 3000 ×g for 5 min, immediately frozen, and stored at −80°C. The ICAM-1 level was measured using commercially available quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D System, USA).

2.6. Statistical Analysis. Data shown in figures are presented as mean ± SEM. Repeated measure ANOVA with robust standard error and exchangeable working correlation matrix (compound symmetry) of generalized estimating equation (GEE) was used to determine the P values for the main effects of time and treatment and interaction effects of time by treatment. When a significant interaction effect of time by treatment was revealed, simple main effects (pairwise comparisons) were calculated with GEE within-model

contrast (LSD method). In the analysis of simple main effects, comparisons among groups at a specific time point were performed. SPSS software package 15.0 for Windows was used for all analyses. $P < 0.05$ was considered to be statistically significant.

3. Results

In Figure 1, we present the results of biomarker findings of inflammation after traumatic brain injury and the treatment groups had a significant reduction in ICAM-1. There was no result in IL-10. In the time-point of 24 h and day 7 the combination group showed a significant reduction in ICAM-1 as compared to the control group. In the time-point of 24 h the combination group had a significant reduction in ICAM-1 when compared to the statin group. However, there was no difference in each time-point between vitamin C and combination group.

In Figure 2, we present the fact that all treatment groups had better grip test than the control group for 24 h, 72 h, and the 7th day, respectively. In the time-point of 24 h the statin group had the best neurological function when compared to the other 3 groups. At the time- point of the 7th day the combination group had better performance in neurological function when compared to the vitamin C group. In contrast, there was no difference in each time-point between the combination group and statin group.

In Figures 3(a), 3(b), 3(c), 3(d), and 3(e), the result of immunochemistry staining showed mild injury and minimal brain tissue loss in combination treatment group when compared with control group and other treatment groups. Smaller area of loss of brain tissue was noted in vitamin C group when compared with simvastatin treatment group.

4. Discussion

In this study, we have shown that treatment with vitamin C, simvastatin, or combination therapy could attenuate the cerebral vascular endothelial inflammatory response in a rat traumatic brain injury and reduce neurological deficit after traumatic brain injury. At the time- point of 24 h the combination group showed a significant reduction in ICAM-1 when compared to the statin group. But there was no difference between the vitamin C and combination group. All treatment groups had better grip test than the control group at 24 h, 72 h, and on the 7th day, respectively. Our findings suggested that vitamin C and combination therapy could play a critical role in the TBI-mediated inflammatory response and reduce the neurological deficit.

The clinical and experimental studies of antioxidant and anti-inflammatory effects of vitamin C have been reported and the antioxidant effects of vitamin C on vascular endothelium have been proposed but not studied in traumatic brain injury. Vitamin C may increase endothelial nitric oxide (NO) by protecting it from oxidation and increasing its synthesis [21, 22]. Vitamin C and the other antioxidant vitamin, vitamin E, appear to have beneficial effects on vascular endothelial function in healthy subjects and in patients with cardiovascular disease [23, 24]. In healthy subjects, vitamin C administration restored endothelium-dependent vasodilation that was impaired by acute hyperglycemia [25]. Thus vitamin C may have favorable effects on vascular dilatation, possibly through its antioxidant effects on NO, but these findings are not consistent [26–28]. In stroke study vitamin C may play a critical role in stroke-mediated inflammatory response and may be associated with neurological changes and cognitive impairment [12]. In different animal models antioxidant depletion was observed after focal cerebral ischemic brain injury. A number of factors may be responsible for this phenomenon, including the physiological need of glial cells, particularly astrocytes, coupled with the removal of increased levels of glutamate after stroke. Another factor may be the formation of free radicals after stroke. In our study we proposed that vitamin C could attenuate the vascular endothelial inflammatory response after traumatic brain injury in rats with the reduction of ICAM-1 level and it was never mentioned before.

ICAM-1 plays a critical role in mediating cell-cell contact between leukocytes and cells of various origins. ICAM-1 expression is upregulated by inflammatory cytokines and appears to play a critical role in the posttrauma inflammatory response [2]. Endothelial activation plays an important role in the pathophysiology of the inflammatory reaction and vascular injury after traumatic injury. After injury ICAM-1 interacts with leukocyte integrins to mediate firm adhesion of activated neutrophils to endothelium and diapedesis [4–7]. ICAM-1 also contributes to brain leukocyte accumulation and leukocyte-mediated tissue injury in experimental models of stroke, meningitis, and systemic trauma [4–7].

IL-10 is a member of the interferon/IL-10 family and it is designated as an anti-inflammatory cytokine. It inhibits proinflammatory cytokine production and stimulates cytotoxic T-cell development and B-cell proliferation [24]; we chose to use ICAM-1 and IL-10 as markers of endothelial activation and inflammation.

Our results demonstrated that all treatment groups could attenuate the expression of ICAM-1, but not IL-10. At these time-points IL-10 expression declined to basal or below basal levels after its maximum increase and after traumatic brain injury [2, 12]. The effect of simvastatin for TBI has been confirmed, but the effects of vitamin C and such combination therapy have not been discussed. Our data showed that these two groups could attenuate the vascular endothelial inflammatory response, but there was no difference between these two groups in some time- points. It may be the effect of dose. We chose a higher dose of simvastatin and a lower dose of vitamin C [12, 18]. From previous studies with vitamin C, a higher dose of vitamin C was used and it could be in the range of 50 mg/100 g [29–31]. As a logical next step we would like to adjust the dose of vitamin C and simvastatin and investigate the effect of vitamin C at a higher dose.

4.1. Limitation. There are some limitations about the study. First this is an experimental study and we can control the timing of treatment. But in clinical condition the acute treatment about traumatic brain injury is started several hours later even more. Second, we treated higher dose of simvastatin

in traumatic brain injury of rat. But we cannot prescribe such higher dose to human patients. However, we confirmed that such treatment could reduce neuroinflammation after traumatic brain injury.

5. Conclusion

This is the first study showing the efficacy of a simvastatin-vitamin C combinational therapeutic approach in achieving molecular, histological, and neurological recovery after TBI. Our results showed that such combinational therapy could attenuate the cerebral vascular endothelial inflammatory response in a rat traumatic brain injury. Another finding was that vitamin C also could attenuate the vascular endothelial inflammatory response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This study was supported by a Research Program of E-DA hospital (EDAHP 101013).

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BioMed Research International
Volume 2014, Article ID 723176, 8 pages
http://dx.doi.org/10.1155/2014/723176

Research Article

Correlation of Altered Expression of the Autophagy Marker LC3B with Poor Prognosis in Astrocytoma

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Received 28 February 2014; Accepted 10 April 2014; Published 12 May 2014

Academic Editor: Hung-Chen Wang

Glioblastoma multiforme is one of the most serious malignant brain tumors and is characterized by resistance to chemotherapy and radiation therapy. Recent studies suggest that autophagy may play an important role not only in the regulation of cancer development and progression but also in determining the response of cancer cells to anticancer therapy. The purpose of the present study was to assess the relationship between protein expressions of two autophagy markers, LC3B and Beclin-1, with clinical parameters in astrocytoma patients. Furthermore, the expression of CD133, a marker of the cancer stem-like cells, in astrocytoma patients was also investigated. A total of 106 thin-section slides were retrospectively collected from astrocytoma patients. LC3B, but not Beclin-1, protein expression was found to significantly correlate with resistance to radiation- or chemotherapy. In addition, high intensity of LC3B staining was predictive of poor prognosis. Furthermore, survival time of patients with high-level expression in both CD133 and LC3B was significantly shorter than those with weak expression in both CD133 and LC3B. These results suggest that astrocytoma cancer stem-like cells together with enhanced autophagy may cause resistance to radiation therapy/chemotherapy and that targeting the cancer stem-like cell in astrocytoma may offer a viable therapeutic approach.

1. Introduction

Astrocytoma is the most frequent brain tumor found in humans. The World Health Organization (WHO) [1] has classified astrocytomas into four grades based on the degree of malignancy. Grade I tumors are benign and slow-growing, as represented by pilocytic astrocytomas. The grade II tumors consist of relatively slow-growing diffuse astrocytomas and pilomyxoid astrocytomas. The grade III and the grade IV tumors are highly malignant and are, respectively, exemplified by anaplastic astrocytoma and glioblastoma multiforme (GBM), which is the most common and most aggressive malignant primary brain tumor in humans. Extensive efforts have been focused on identifying biomarkers that correlate with the severity of astrocytomas in order to facilitate diagnosis as well as to develop therapeutic agents for the treatment of this devastating disorder. In this regard, increased protein and/or gene expression of several biomarkers, such

as cycloxygenase-2 [2], insulin-like growth factor-binding proteins [3], and epidermal growth factor receptor [4], have been shown to correlate with poor survival in astrocytoma patients. By contrast, protein and/or gene expression of *myo*-inositol [5] and N-myc downstream-regulated gene [6] have been reported to be negatively correlated with pathological grading in astrocytoma. However, these markers are rarely related to mechanisms by which the development of astrocytomas is regulated.

The cancer stem cell (CSC) theory postulates that tumors arise from a subpopulation of cells that are characterized by self-renewal, infinite proliferative potential, and multipotency and are able to initiate new tumors in vivo [7]. CSC cells mediated radio- and chemoresistance and these cells with stem-like features have been identified in glioblastoma [8]. A previous study showed that these cells express the transmembrane glycoprotein prominin-1 (CD133) (a cell-surface marker expressed on normal human neuronal stem cell) and have the ability to initiate new tumor in vivo after xenotransplantation in mice. But few data are available on the actual prognosis of CD133 expression in malignant gliomas. Glioblastoma stem cells are highly resistant to conventional chemotherapy and radiotherapy [9, 10] and the chemo-radioresistance of these cells may be responsible for the poor clinical outcome of these patients. The CSCs display strong capability of tumor resistance to TMZ. This resistance is probably contributed by the CD133+ cells with downregulation of autophagy-related proteins [11].

Autophagy constitutes the basic catabolic mechanism that involves degradation of unnecessary or dysfunctional cellular components by lysosomes [12]. Recent studies suggest that autophagy may play an important role not only in the regulation of cancer development and progression but also in determining the response of cancer cells to anticancer therapy [13–15]. Subsequent studies have also identified microtubule-associated protein 1 light chain 3 (LC3) [16–18] and Beclin-1 [19–21] as essential markers for autophagy. There are three isoforms of LC3, namely, LC3A, LC3B, and LC3C. Lines of evidence have shown that LC3B is a prognostic marker in advanced breast cancer after chemotherapy [22].

In the present study, protein expressions of LC3B and Beclin-1 in astrocytoma patients were evaluated and the results were utilized to correlate with clinical parameters. Furthermore, since cancer stem-like cells have been found to be attractive targets for novel anticancer therapies [23, 24], the expression of CD133, a marker of these cancer stem-like cells [25, 26], in astrocytoma patients was also investigated.

2. Material and Method

2.1. Astrocytoma Samples. Based on the operative notes, medical records, pathological reports, and MRI images, 218 thin section slides were retrospectively collected from astrocytoma patients who were diagnosed between 2000 and 2010 at the Neurosurgery Department of Chung-Ho Hospital, Kaohsiung, Taiwan. However samples with poor immunohistochemical staining were excluded, so were those from patients who were diagnosed by biopsies only, had incomplete medical records or no follow-up visits, or showed low quality pathological results. A total of 106 samples were finally selected for the present study. This study was approved by the Kaohsiung Medical University Hospital Review Board (KMUH-IRB-20120238).

2.2. Immunohistochemistry. In order to retrieve antigens for immunohistochemical staining, 3 μm sections from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, and autoclaved at 121°C for 10 min in Target Retrieval solution (Dako, Glostrup, Denmark), pH 9.0. Endogenous peroxidase in the sections was blocked by incubating in 3% hydrogen peroxide at room temperature for 5 min. The sections were then washed in a Tris-buffered solution (Dako, K9001) and incubated with a 1 : 200 dilution of rabbit polyclonal anti-human CD133 (Biorbyt, orb18124, UK), Beclin-1 (abcam, ab16998, USA), and LC3B (SANTACRUZE, sc-16755, Europe) antibodies for 1 hr at room temperature. After washing with Tris-buffered solution, the sections were incubated with secondary antibodies conjugated with horseradish peroxidase for 30 min at room temperature. Subsequently, the slides were incubated in 3,3-diaminobenzidine for 5 min followed by Mayer's hematoxylin counterstaining for 60 sec and mounted with Entellan (product no. HX247305, Merck). The immunohistochemically stained slide sections were evaluated by an investigator blinded to the experimental procedures. The staining score of Beclin-1 and LC3B was as follows: negative: 0 (without or less 10% positive cells of tumor); weakly positive: 1 (10~30% positive cell of tumor); positive: 2 (30~70% positive cell of tumor); strongly positive: 3 (70%~100% positive cell of tumor). The staining score of CD133 was as follows: negative: 0 (if 65% of cells were stained by the antibody); weakly positive: 1 (>5–20%, including 20%, of stained cells); positive: 2 (>20–50%, including 50%, of stained cells); strongly positive: 3 (>50% of stained cells) [27]. For statistical analysis, scores of 0 and 1 were defined as low-expression group and scores of 2 and 3 were defined as high-expression group [28]. The results of protein expressions were correlated with clinical parameters such as age, gender, tumor grade, being accepted for radiation- or chemotherapy, and Karnofsky performance status scale (KPS) [29].

2.3. Data Analyses. Social Sciences for Windows, Version 19.0 (SPSS, Chicago, IL, USA), was used for statistical analysis. Chi-square test was performed to determine whether there was a correlation between Beclin-1 and LC3B protein expressions with a specific clinicopathological parameter. A P value of <0.05 was considered statistically significant. The survival rate was analyzed by the Kaplan-Meier method with log-rank test.

3. Results

3.1. Correlation between LC3B and Beclin-1 Protein Expressions with Clinical Parameters. Figures 1 and 2 show representative immunochemical staining sections for Beclin-1 and LC3B, respectively, with weak, low, moderate, and high intensities.

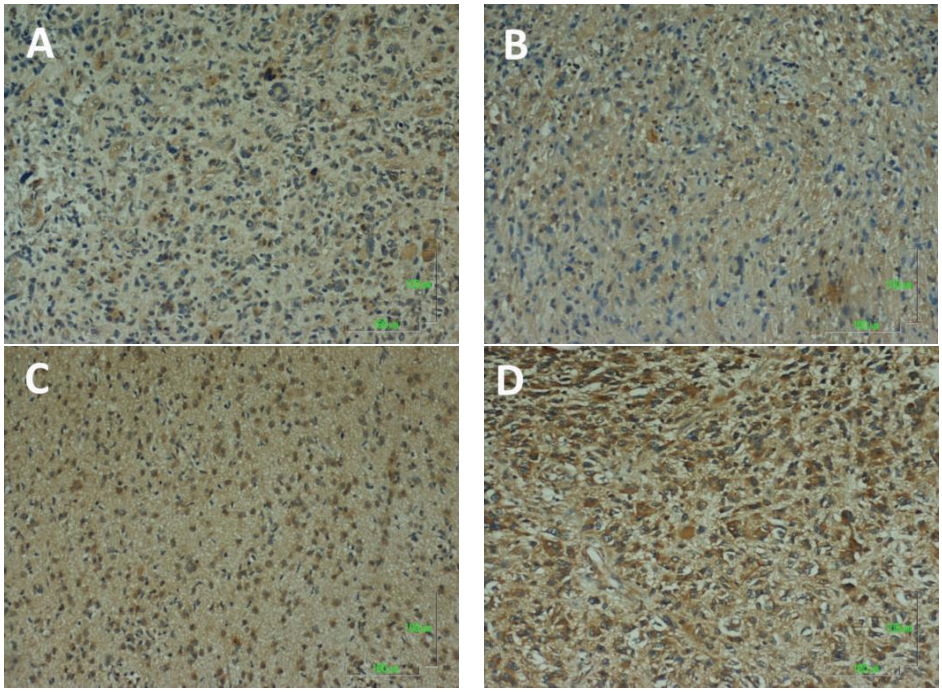

Figure 1: Representative immunohistochemical staining for Beclin-1 protein expression in astrocytoma sections. A: Score 0, negative or weak intensity. B: Score 1, low intensity. C: Score 2, moderate intensity. D: Score 3, high intensity. Magnification, 100x.

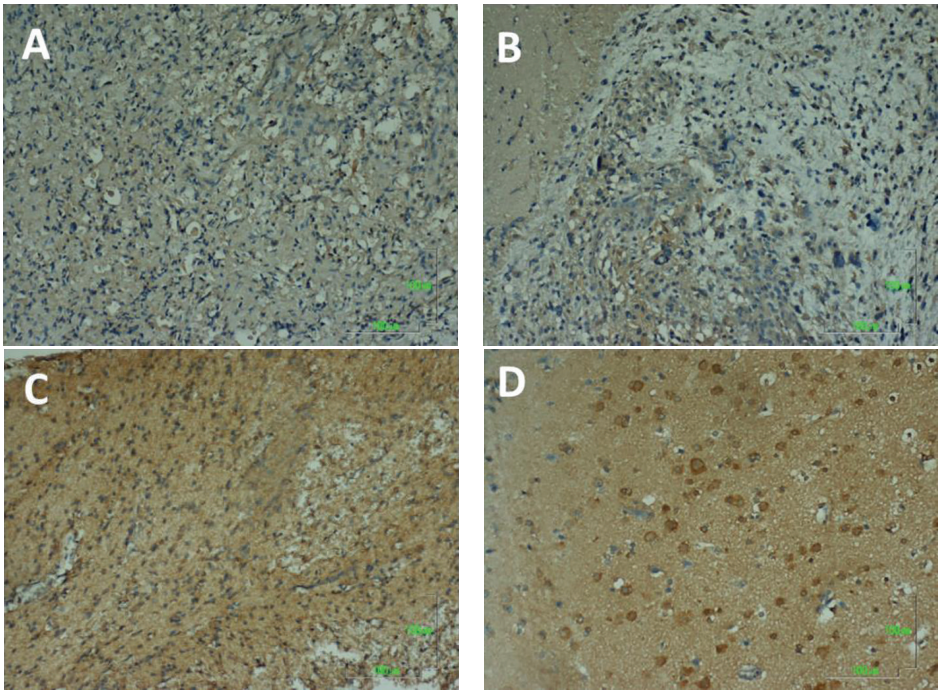

Figure 2: Representative immunohistochemical staining for LC3B protein expression in astrocytoma sections. A: Score 0, negative or weak intensity. B: Score 1, low intensity. C: Score 2, moderate intensity. D: Score 3, high intensity. Magnification, 100x.

Table 1: Correlation between Beclin-1 and LC3B protein expression with clinicopathological parameters. P values were determined by Chi-squares analysis. KPS: Karnofsky performance status scale.

Score	LC3B					Beclin-1				
	0	1	2	3	P	0	1	2	3	P
Age					0.312					0.244
>65	16	19	14	15		9	17	18	20	
<65	12	12	17	1		7	12	13	10	
Gender					0.221					0.453
M	15	17	18	6		4	14	19	19	
F	13	14	13	10		12	15	12	11	
Tumor grade					0.563					0.312
I	2	5	3	0		0	2	4	4	
II	4	8	2	2		1	4	5	6	
III	5	4	6	2		4	5	4	4	
IV	17	14	20	12		11	18	18	16	
Radiation/chemotherapy					0.021*					0.071
Yes	8	10	24	15		8	18	19	21	
No	20	21	7	1		8	11	12	9	
KPS					0.452					0.549
>70	10	15	17	6		10	15	8	15	
<70	18	16	14	10		6	14	23	15	

The results of immunohistochemical staining of Beclin-1 and LC3B were separately analyzed to determine the relationship of protein expression with clinicopathological parameters of astrocytoma patients, such as age, gender, tumor grade, resistance to radiation- or chemotherapy, and KPS scale. None of these parameters were significantly correlated with Beclin-1 protein expression (Table 1). LC3B protein expression was found to significantly correlate with radiation- or chemotherapy ($P < 0.05$). However, none of other clinical parameters were shown to correlate with LC3B protein expression (Table 1). Furthermore, Beclin-1 protein expression did not correlate with overall survival of the patients (Figure 3). In contrast, a high intensity in immunochemical staining of LC3B predicted poor prognosis (Figure 4). Likewise, negative or weak LC3B protein expression displayed a similar survival curve as that of high LC3B levels. The results also showed that low and moderate levels of LC3B expression had a significant increase in survival when compared with those of high LC3B levels (Figure 4).

3.2. Prognostic Values of CD133 and LC3B Expressions in Astrocytoma. A previous study has shown that cancer stem-like cells are resistant to radiation therapy or chemotherapy, and enrichment in these cells is indicative of poor prognosis [30]. Since the expression of LC3B protein was shown to correlate with resistance of radiation- and chemotherapy (Table 1), we assessed the expression of CD133, an astrocytoma cancer stem-like cell marker, together with that of LC3B in overall survival. Figure 5 shows representative immunochemical staining sections for CD133 with weak, low, moderate, and high intensities. The overall survival of patients with high-level expression in both CD133 and LC3B was 38 mon, and those with weak expression in both CD133 and LC3B was 170 mon (Figure 6). These results showed that high-level expression of both CD133 and LC3B was indicative of poor prognosis in astrocytoma ($P < 0.05$).

4. Discussion

Glioblastoma multiforme (GBM), or grade IV astrocytoma, is the most frequently found class of malignant primary brain tumor and one of the most aggressive forms of cancer. As a consequence, median survival after diagnosis is usually just 12 mon [31]. Standard therapy for the management GBM includes surgical resection, focal radiotherapy, and treatment with alkylating agents such as temozolomide [32, 33]. Unfortunately, these therapeutic approaches increase the survival of GBM patients only modestly. Thus, extensive studies have focused on identifying new pathways and/or molecular markers that are predictive of poor prognosis and resistance to radiotherapy/chemotherapy in this class of patients.

There are several key findings presented in this study. Increased expression of LC3B, an autophagy marker, was found to be correlated with radiotherapy/chemotherapy as well as with poor survival in astrocytoma patients. Furthermore, increased expression of LC3B together with enhanced levels of CD133, a cancer stem-like cell marker, also correlated significantly with poor prognosis. Previous studies have shown that chemical induction of autophagy enhances chemosensitivity and radiosensitivity in papillary thyroid cancer [34]. Song et al. reported that autophagy inhibitors may make liver cancer stem cells (LCSCs) more sensitive to the tumor microenvironment and be useful in improving

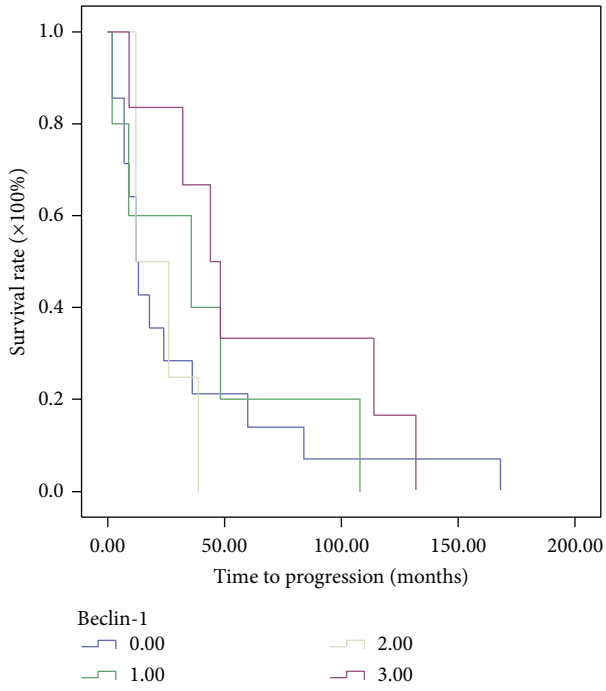

Figure 3: Assessment of the relationship between Beclin-1 protein expression and overall survival of astrocytoma patients using Kaplan-Meier method with log-rank test. No statistically significant correlation was found.

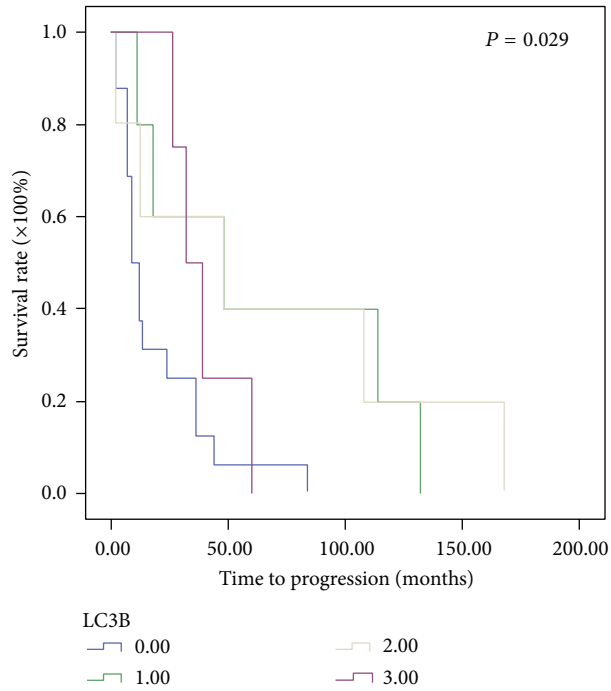

Figure 4: Assessment of the relationship between LC3B protein expression and overall survival of astrocytoma patients using Kaplan-Meier method with log-rank test. High intensity of LC3B immunohistochemical staining was shown to predict poor prognosis. Similar survival curve was also found with negative or weak LC3B protein expression. By contrast, low and moderate levels of LC3B expression had a significant increase in survival when compared with those of weak or high LC3B levels.

anticancer treatments [35]. Likewise, induction of autophagy in glioma-initiating cells by rapamycin also increased their sensitivity to radiation [36].

Autophagy constitutes the basic catabolic mechanism that involves the degradation of unnecessary or dysfunctional cellular components by lysosomes [12]. Autophagy has the ability to protect cells against metabolic stress by removing damaged or aged organelles, toxic metabolites, or intracellular pathogens [37–40]. Previous studies reported that inhibition of autophagy by 3-methyladenine (3-MA) and Atg7 siRNA enhances 5-FU induced cytotoxicity in human colorectal cancer cells [34]. Autophagy suppression also enhances the therapeutic efficacy of cisplatin and 5-FU in esophageal and colon cancer cells, respectively [41, 42].

These results drastically differ from our finding that high levels of autophagy marker LC3B led to a significant correlation with resistance to radiation- and/or chemotherapy. In our study, enhanced autophagy was a result of disease progression rather than due to chemical induction as reported by other investigators [34, 36]. In addition, the cell type used in the present study was clearly different from those utilized in other studies [34, 36] as GBM is highly resistant to chemotherapy and radiotherapy. Further investigation is needed to clarify this matter. Unexpectedly, Beclin-1, another autophagy maker, did not exhibit properties similar to those of LC3B in the current study. The reasons for this difference are not known at the present.

Huang et al. reported that the expressions of LC3B-II and Beclin-1 were reduced in GBM due to a downregulated autophagic capacity [27]. Consistent with these results, we also found that high-level expression of LC3B was associated with poor prognosis. Our results suggest that a reduction in autophagy may lead to more advanced astrocytoma although their correlation did not reach statistical significance. The role autophagy plays in tumor development is complicated [15]. It can suppress tumor development during early stages of tumorigenesis. However, autophagy can also promote further tumor development in established tumors. Our results showed that high levels of LC3B expression, or enhanced autophagy, correlated with poor prognosis are consistent with the role of autophagy as tumor promoter.

Recently, GBM cancer stem-like cells have been shown to participate in the formation of the tumor endothelium, increase in the invasiveness of the tumor, and lead to resistance to radiotherapy [43, 44] through various mechanisms. Our results showed that a high-level expression of CD133, a cancer stem-like cell marker, together with a high-level expression of LC3B, was indicative of poor prognosis in astrocytoma (Figure 6). These results suggest that astrocytoma cancer stem-like cells together with enhanced autophagy may cause resistance to radiation therapy/chemotherapy and that targeting the cancer stem-like cell in astrocytoma may offer a viable therapeutic approach.

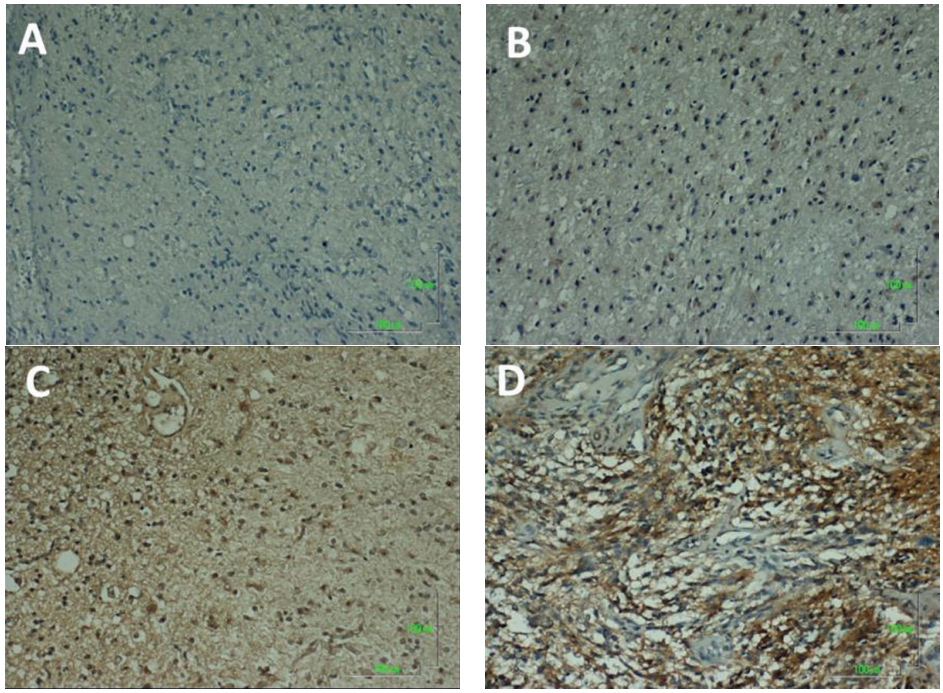

Figure 5: Representative immunohistochemical staining for CD133, a cancer stem-like cell marker, in astrocytoma sections. A: Score 0, negative or weak intensity. B: Score 1, low intensity. C: Score 2, moderate intensity. D: Score 3, high intensity. Magnification, 100x.

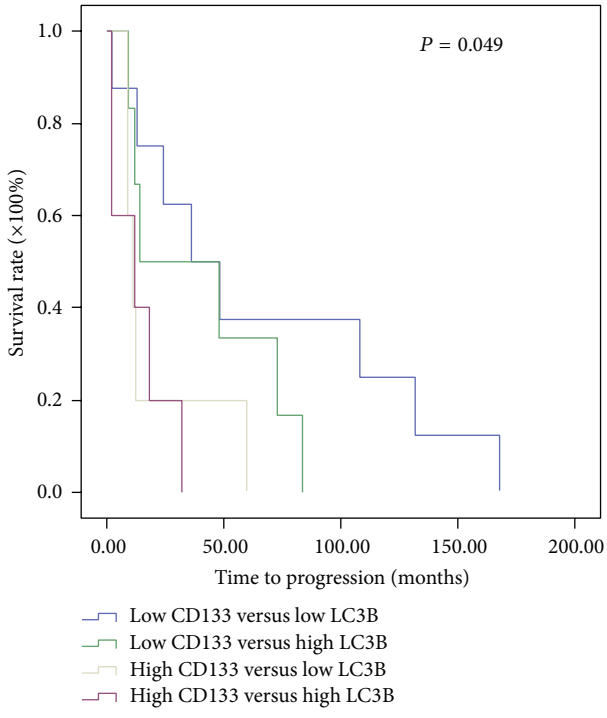

Figure 6: Assessment of the relationship between a combination of CD133 and LC3B protein expressions and overall survival of astrocytoma patients using Kaplan-Meier method with log-rank test. The average overall survival of patients with high-level expression in both CD133 and LC3B was 38 mon, and those with weak expression in both CD133 and LC3B was 170 mon.

Disclosure

This work neither has been published nor is it being considered for publication elsewhere in any form. All authors have read and approved the paper.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 256879, 7 pages
http://dx.doi.org/10.1155/2014/256879

Research Article

Association between Oxidative Stress and Outcome in Different Subtypes of Acute Ischemic Stroke

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Received 27 February 2014; Accepted 7 April 2014; Published 8 May 2014

Academic Editor: Hung-Chen Wang

Objectives. This study investigated serum thiobarbituric acid-reactive substances (TBARS) and free thiol levels in different subtypes of acute ischemic stroke (AIS) and evaluated their association with clinical outcomes. *Methods*. This prospective study evaluated 100 AIS patients, including 75 with small-vessel and 25 with large-vessel diseases. Serum oxidative stress (TBARS) and antioxidant (thiol) were determined within 48 hours and days 7 and 30 after stroke. For comparison, 80 age- and sex-matched participants were evaluated as controls. *Results*. Serum TBARS was significantly higher and free thiol was lower in stroke patients than in the controls on days 1 and 7 after AIS. The level of free thiol was significantly lower in the large-vessel disease than in the small-vessel disease on day 7 after stroke. Using the stepwise logistic regression model for potential variables, only stroke subtype, NIHSS score, and serum TBARS level were independently associated with three-month outcome. Higher TBARS and lower thiol levels in the acute phase of stroke were associated with poor outcome. *Conclusions*. Patients with large-vessel disease have higher oxidative stress but lower antioxidant defense compared to those with small-vessel disease after AIS. Serum TBARS level at the acute phase of stroke is a potential predictor for three-month outcome.

1. Introduction

Stroke is a major cause of morbidity and mortality worldwide [1]. Inflammation and oxidative stress play important roles in acute ischemic stroke (AIS) [2–4] and the close relationship between inflammation and oxidative stress is now well defined [5, 6]. Acute ischemia leads to increased production of free radicals and reactive oxygen species (ROS) in tissue and plasma through several mechanisms [7], including stimulation of N-methyl-D-aspartate (NMDA) receptors [3], mitochondrial dysfunction [8], activation of neuronal nitric oxide synthase (NOS) [9], and migration of neutrophils and leukocytes that can generate superoxide anions [10]. Although its exact mechanism is not clear yet, oxidative stress is a pivotal event in the setting of ischemic stroke and may contribute to stroke outcome [11, 12].

Oxidative stress has been defined as "an imbalance between oxidants and antioxidants in favor of oxidants,

potentially leading to damage" [13]. To assess oxidants, the accumulation of malondialdehyde (MDA), an end-product of peroxidative decomposition of polyenic fatty acids in the lipid peroxidation process, in tissues is indicative of the extent of lipid peroxidation. Measured as thiobarbituric acid-reactive substances (TBARS), MDA is used as an indicator of oxidative damage for several diseases [14].

On the other hand, the antioxidant defense system has been studied in stroke patients as regards enzymes, including superoxide dismutase (SOD) and glutathione peroxidase [15, 16], and nonenzymatic antioxidants like retinol, ascorbic acid, α-tocopherol, carotenoids, and uric acid [3, 17, 18]. Most of these studies are cross-sectional or have short follow-up periods after stroke.

Under the hypothesis that the level of oxidative stress is increased and may be diverse in different subtypes of stroke, this study evaluated longitudinal changes in serum oxidant and antioxidant levels after ischemic stroke to determine their value in predicting short-term outcome. Serial changes of serum TBARS and free thiol were measured in different subtypes during the first month after stroke and the possibility of using these markers for predicting three-month outcome was assessed.

2. Patients and Methods

2.1. Subjects and Design. From August 2010 to July 2012, consecutive patients with AIS who were admitted to the Neurology Department of Chang Gung Memorial Hospital-Kaohsiung were evaluated. Acute ischemic stroke was defined as an acute onset loss of focal cerebral function persisting for at least 24 hours. Diagnosis was based on clinical presentation, neurologic examination, and results of brain magnetic resonance imaging (MRI) with diffusion-weighted imaging (DWI). Patients aged 18–80 years with acute noncardioembolic ischemic stroke were included and divided into two major etiologic subtypes (i.e., large-artery atherosclerosis and small-artery occlusion) according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification [19]. For comparison, 80 age- and sex-matched subjects with no clinical evidence of acute cerebral infarction within one year were enrolled as the control group. The hospital's Institutional Review Committee on Human Research approved the study protocol and all participants provided informed consent.

Patients with cardioembolic stroke, other determined causes and undetermined causes of stroke, and those with underlying neoplasm, end-stage renal disease, liver cirrhosis, and congestive heart failure were excluded. Clinical examination, electrocardiography, and cardiac ultrasound were conducted to exclude cardioembolic stroke. Patients with fever or any infectious disorder within the first week after acute stroke were also excluded.

2.2. Clinical Assessments and Treatment. All of the participants underwent complete neurologic examination. Brain MRI with DWI, extracranial carotid sonography, and transcranial color-coded sonography were performed during the hospitalization. The therapeutic regimens for AIS were based on the American Heart Association (AHA)/American Stroke Association (ASA) guidelines [20]. Neurologic deficits due to stroke were assessed using the National Institutes of Health Stroke Scale (NIHSS). The therapeutic outcomes were evaluated by the modified Rankin Scale (mRS) at three months after stroke. Good outcome was defined as a three-month mRS of 0–2 without any cardiovascular event. Poor outcome was defined as mRS of 3–6 [21].

2.3. Determination of Serum Malondialdehyde Content. Blood samples were collected by venipuncture of forearm veins from patients within 48 hours of the stroke (presented as day 1) and on days 7 and 30 after stroke. Serum MDA was measured using the TBARS assay. The concentration of TBARS was assessed based on the method of Huang et al. [22]. TBARS reagent (1 mL) was added to a 0.5 mL aliquot of serum and heated for 20 minutes at 100°C. The antioxidant, butylated hydroxytoluene, was added before heating the samples. After cooling on ice, the samples were centrifuged at 840 g for 15 min. Absorbance of the supernatant was read at 532 nm. Blanks for each sample were prepared and assessed in the same way to correct for the contribution of A532 to the sample. The TBARS results were expressed as MDA equivalents using 1,1,3,3-tetraethoxypropane.

2.4. Assessment of Serum Free Thiol Content. The ability of antioxidative defense in response to increased oxidative damage was evaluated by measuring the serum level of total reduced thiols because thiols were physiologic free radical scavengers. Serum free thiols were determined by directly reacting thiols with 5,5-dithiobis 2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB). The amount of thiols in the sample was calculated from absorbance, as determined using the extinction coefficient of TNB (A412 = 13,600 M^{-1} cm^{-1}).

2.5. Statistical Analysis. Data were presented as mean ± SEM. Continuous variables, including age, cell count, lipid profile, hemoglobin A1c (HbA1c), blood pressure, and serum free thiol and TBARS, were analyzed by independent t-test among groups. Chi-square test or Fisher's exact test was used to compare proportions among groups. Repeated measures of ANOVA were used to compare serum free thiol and TBARS at different time points (within 48 hours and on days 7 and 30 after stroke).

Scheffe's multiple comparison was used to analyze the intraindividual courses of parameters over time. These were then compared among patients with small-vessel and large-vessel diseases. Multiple logistic regression analyses determined the independent influence of different predictive variables on clinical outcome. Statistical significance was set at $P < 0.05$. All statistical calculations were performed using the SAS software package, version 9.1 (2002, SAS Statistical Institute, Cary, NC, USA).

Table 1: Baseline characteristics and laboratory data between small-vessel and large-vessel disease.

	Small vessel disease ($n = 75$)	Large vessel disease ($n = 25$)	P value
Age (y) (mean ± SD)	61.1 ± 11.5	64.6 ± 8.8	0.17
Sex (male) (n)	60	17	0.27
Hypertension (n)	56	22	0.26
Diabetes mellitus (n)	32	10	0.82
Dyslipidemia (n)	37	12	0.91
Coronary artery disease (n)	5	2	0.82
White blood cells ($\times 10^3$/mL)	7.4 ± 0.3	8.1 ± 0.5	0.22
Red blood cells ($\times 10^6$/mL)	4.8 ± 0.1	4.5 ± 0.1	0.11
Platelet counts ($\times 10^4$/mL)	21.1 ± 0.7	19.8 ± 1.2	0.30
Total cholesterol (mg/dL)	181.2 ± 3.8	194.6 ± 11.6	0.15
LDL-cholesterol (mg/dL)	109.9 ± 3.7	123.4 ± 9.9	0.12
Triglyceride (mg/dL)	134.7 ± 7.7	134.9 ± 13.3	0.99
HbA1c (%)	7.0 ± 0.2	6.4 ± 0.3	0.15
Free thiol (μM/L)	0.90 ± 0.03	0.81 ± 0.06	0.19
TBARS (μM/L)	19.7 ± 1.2	20.7 ± 2.6	0.85

3. Results

3.1. Baseline Characteristics of Patients with Small-Vessel and Large-Vessel Diseases. Of the 120 patients with acute non-cardioembolic ischemic stroke, 20 were excluded for various infections or fever in the first week after acute stroke ($n = 6$), cardioembolic stroke ($n = 5$), end-stage renal disease ($n = 5$), and gastrointestinal bleeding in the acute stage ($n = 4$). The remaining 100 patients included 75 with small-vessel occlusion and 25 with large-vessel atherosclerosis. Based on their baseline characteristics and laboratory data (Table 1), there were no significant differences in vascular risk factors and in white blood cell (WBC), red blood cell (RBC) count, platelet count, and serum levels of total cholesterol, LDL-cholesterol, triglyceride, and HbA1c. Serum concentration of free thiol and TBARS were also not different between the two groups.

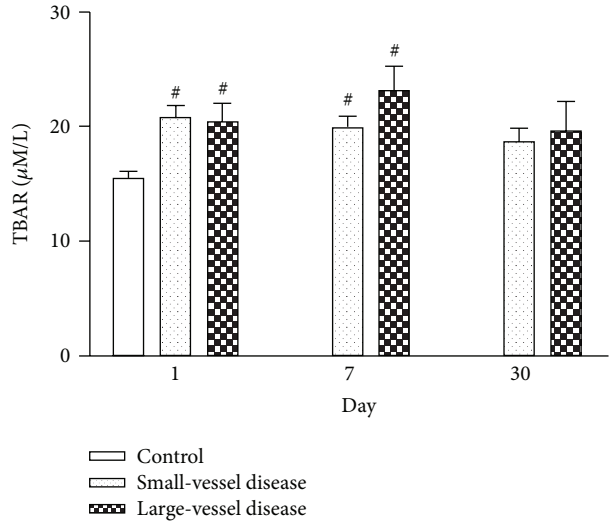

Figure 1: Serial changes in serum TBARS among patients with small-vessel and large-vessel diseases and in the controls at various time points after stroke. $^{\#}P < 0.05$ compared to controls.

3.2. Changes in Serum TBARS and Free Thiol among Patients with Small-Vessel and Large-Vessel Diseases. Serial changes in serum concentration of TBARS among patients groups and in the controls (Figure 1) revealed that the concentration of TBARS was significantly higher in stroke patients than in the controls on days 1 and 7 after AIS ($P < 0.05$). At the three different time points, the levels of TBARS were similar between patients with large-vessel disease and those with small-vessel disease.

Serial changes in serum concentration of free thiol among patients with small-vessel and large-vessel diseases and in the controls (Figure 2) demonstrated that the concentration of free thiol was significantly lower in stroke patients than in the controls on day 1 after AIS ($P < 0.05$). Free thiol concentration was also significantly lower in the large-vessel disease group than in the small-vessel disease group on day 7 after stroke ($P < 0.05$). Thereafter, the level of free thiol gradually increased until it became similar to that of the controls on day 30 after stroke. Repeated ANOVA with Scheffe's multiple comparison showed significantly different free thiol levels between patients with small-vessel disease and those with large-vessel disease at three different time points (within 48 h and on days 7 and 30 after stroke) ($P < 0.05$).

3.3. Factors Predictive of Clinical Outcome. Potential prognostic factors of the 100 stroke patients were listed in Table 2. No one died during the three-month followup, and 80 patients had good outcomes while 20 had poor outcomes. Statistical analysis revealed that stroke subtype, NIHSS score, and serum free thiol and TBARS levels on days 1 and 7 after

Table 2: Prognostic factors in patients with acute ischemic stroke.

	Good outcome ($n = 80$)	Poor outcome ($n = 20$)	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Age (year)	61.2 ± 11.5	65.1 ± 8.0	1.04 (0.99–1.09)	0.16	—	—
Sex (male) (n)	62	15	0.87 (0.28–2.72)	0.81	—	—
Hypertension (n)	61	17	1.77 (0.47–6.68)	0.55	—	—
Diabetes mellitus (n)	32	10	1.50 (0.56–4.01)	0.46	—	—
Hyperlipidemia (n)	38	11	1.35 (0.51–3.61)	0.62	—	—
Cardiac disease (n)	5	2	1.67 (0.30–9.30)	0.80	—	—
NIHSS score on admission			1.35 (1.16–1.58)	<0.001	1.55 (1.11–2.16)	0.01
Stroke subtype (large/small)			21.0 (6.26–70.4)	<0.001	0.01 (0.001–0.33)	0.008
Small vessel disease	70	5			—	—
Large vessel disease	10	15			—	—
With statin therapy	35	11	1.49 (0.56–4.00)	0.46	—	—
Laboratory data on admission					—	—
White blood cells ($\times 10^3$/mL)	7.4 ± 0.2	8.3 ± 0.6	1.17 (0.96–1.43)	0.12	—	—
Hemoglobin (g/dL)	13.8 ± 0.2	13.6 ± 0.3	0.91 (0.69–1.22)	0.53		
Red blood cells ($\times 10^6$/mL)	4.8 ± 0.1	4.5 ± 0.1	0.50 (0.24–1.07)	0.07	—	—
Platelet counts ($\times 10^4$/mL)	21.2 ± 0.6	18.9 ± 1.4	0.99 (0.98–1.00)	0.12	—	—
Total cholesterol (mg/dL)	185.7 ± 4.6	180.0 ± 8.6	0.99 (0.98–1.01)	0.58	—	—
LDL-cholesterol (mg/dL)	114.2 ± 4.2	109.6 ± 7.7	0.99 (0.98–1.01)	0.62	—	—
HDL-cholesterol (mg/dL)	43.8 ± 1.0	45.6 ± 3.0	1.02 (0.97–1.07)	0.48	—	—
Triglyceride (mg/dL)	136.3 ± 7.6	128.3 ± 13.8	0.99 (0.99–1.01)	0.63	—	—
HbA1c (%)	6.8 ± 0.2	7.3 ± 0.6	1.14 (0.90–1.45)	0.28	—	—
Systolic BP (mmHg)	147.1 ± 2.8	140.1 ± 4.4	0.99 (0.97–1.01)	0.28	—	—
Diastolic BP (mmHg)	84.5 ± 1.5	81.1 ± 2.8	0.98 (0.94–1.02)	0.31	—	—
Free thiol on admission (μM/L)	0.92 ± 0.03	0.70 ± 0.07	0.04 (0.01–0.32)	0.002	—	—
TBARS on admission (μM/L)	19.9 ± 0.72	24.1 ± 3.0	1.05 (0.99–1.11)	0.06	—	—
Free thiol on day 7 (μM/L)	0.96 ± 0.04	0.66 ± 0.08	0.12 (0.03–0.52)	0.005	—	—
TBARS on day 7 (μM/L)	18.4 ± 0.77	29.1 ± 2.8	1.14 (1.07–1.22)	<0.001	1.37 (1.14–1.65)	0.001
Free thiol on day 30 (μM/L)	1.00 ± 0.04	0.88 ± 0.11	0.29 (0.05–1.75)	0.78	—	—
TBARS on day 30 (μM/L)	19.7 ± 1.24	20.4 ± 2.4	1.01 (0.95–1.07)	0.82	—	—

Abbreviations: BP: blood pressure; HbA1c: hemoglobin A1c.

stroke were significantly different between the good and poor outcome groups.

Potential variables such as age, sex, stroke subtype, blood pressure, HbA1C, total cholesterol, HDL, LDL, and TBARS and free thiol levels on admission were analyzed using a stepwise logistic regression model. Only the stroke subtype (OR: 0.014, 95% CI: 0.001–0.325; $P = 0.008$), NIHSS score (OR: 1.55, 95% CI: 1.11–2.16; $P = 0.01$), and serum TBARS on day 7 after stroke (OR: 1.37, 95% CI: 1.14–1.65; $P = 0.001$) were independently associated with three-month outcome. Any increase in TBARS concentration by one μM/L worsens the poor outcome rate by 37%.

3.4. Serial Changes in Serum TBARS and Free Thiol between Groups with Good and Poor Outcomes. Changes in serum TBARS between groups with good and poor outcomes (Figure 3) revealed a gross increase in TBARS level in the poor outcome group during the acute stage after stroke. The concentration of TBARS was significantly higher in the poor outcome group than in the good outcome group on day 7 after stroke ($P < 0.05$). These levels gradually decreased thereafter and no significant difference existed between the two groups on day 30 after stroke. Repeated ANOVA with Scheffe's multiple comparison revealed significantly different serum TBARS levels on three different time points between the two groups ($P < 0.05$).

Changes in serum concentration of free thiol among patients with good and poor outcomes, and in the controls (Figure 4) revealed that the concentration of free thiol was significantly lower in the poor outcome group than in the good outcome group on days 1 and 7 after stroke ($P < 0.05$). Repeated ANOVA with Scheffe's multiple comparison showed significantly different free thiol levels between the good and poor outcome groups at three different time points (within 48 h and on days 7 and 30 after stroke) ($P < 0.05$).

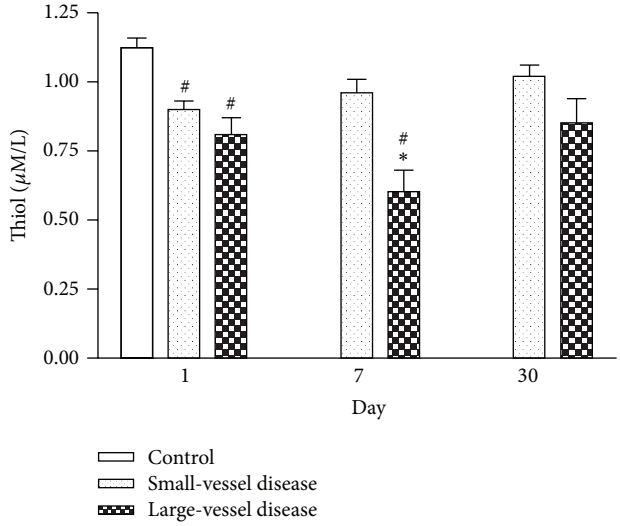

Figure 2: Serial changes in serum free thiol among patients with small-vessel and large-vessel diseases and in the controls at various time points after stroke. $^{*}P < 0.05$ compared to the small vessel group; $^{\#}P < 0.05$ compared to the controls.

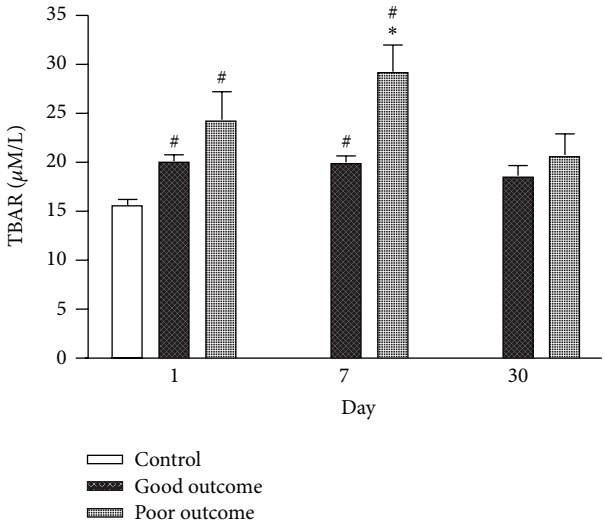

Figure 3: Serial changes in serum TBARS among patients with good and poor outcomes and in the controls at various time points after stroke. $^{*}P < 0.05$ compared to the small vessel group; $^{\#}P < 0.05$ compared to the controls.

4. Discussion

The present study has four major findings. First, patients with AIS in the acute phase had significantly higher TBARS and lower free thiol levels than the controls. Second, the level of free thiol is significantly lower in patients with large-vessel disease than in those with small-vessel disease on day 7 after stroke. Third, the higher TBARS and lower free thiol levels in the acute phase of AIS is associated with poor outcome. Lastly, the most important finding in this study is that TBARS level on day 7 after stroke is an independent predictive factor of three-month outcome.

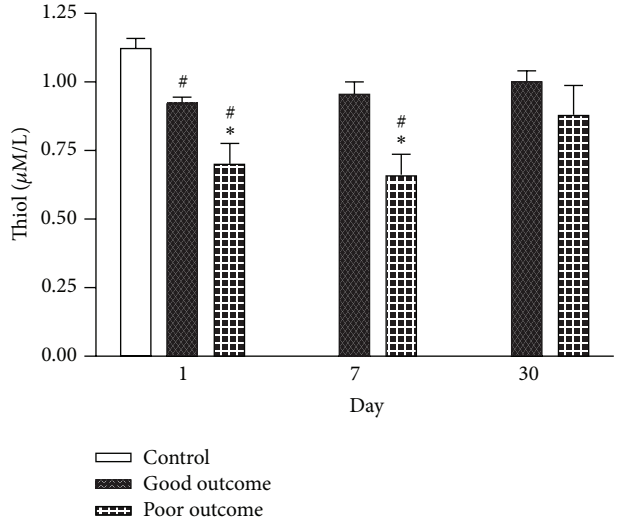

Figure 4: Serial changes in serum free thiol among patients with good and poor outcomes and in the controls at various time points after stroke. $^{*}P < 0.05$ compared to the small vessel group; $^{\#}P < 0.05$ compared to the controls.

The results on the MDA level changes over time in stroke patients are controversial. Some researchers observed higher erythrocyte MDA levels in the very early phase of stroke, with subsequent decline in the levels of the controls [23], while others report an MDA increase only in some days after acute stroke [24]. Gariballa et al. found that TBARs levels were constantly higher in AIS patients compared to those of the controls [15]. Consistent with a previous study [25], the longitudinal observation here shows higher levels of TBARS in stroke patients than in controls upon hospital admission, and these levels persist in the next 7 days. Thereafter, TBARS levels gradually decrease to the level as controls a month after the stroke. The discrepancy in results may be due to methodological factors, including sample handling, storage, and preparation prior to the performance of the biomarker assay. Moreover, stroke is a pathologically heterogeneous disease and baseline risk factors may be etiologically different.

Furthermore, the current study demonstrates that antioxidant levels, measured by free thiol, are much lower in patients with large-vessel cerebral infarction than in those with small-vessel infarction on day 7 after stroke. The level of free thiol gradually increases until the difference is no longer significant one month after the stroke. These suggest that oxidant/antioxidant balance is related to the different pathogenesis in the two major subtypes of noncardioembolic stroke. The pathogenesis of small-vessel infarction is lipohyalinosis [26], while atherothrombosis is the major cause of large-vessel cerebral infarction [27]. Thus, different subtypes of ischemic stroke have different pathogenesis, with consequences on oxidative stress.

Oxidative stress is an important contributor to the pathophysiologic sequelae of stroke. A correlation of MDA level with infarct size, clinical stroke severity, and patient outcome has been observed [15, 25]. Since plasma level of oxidized LDL (Ox-LDL) is thought to reflect the oxidative status of the whole body, a previous study has shown that higher Ox-LDL

level in the acute phase of AIS is an independent predictor of poor outcome three months after stroke [28]. The results here reveal that the level of TBARS on day 7 but not day 1 after stroke is much higher in patients with poor outcome than in those with good outcome (Figure 3). Moreover, TBARS level on day 7 after stroke is an independent predictive factor of three-month outcome. These findings suggest that oxidative stress is progressive after stroke and contributes to further neurologic damage in particular cases.

On the other hand, antioxidant vitamin concentrations are associated with neurologic damage and stroke prognosis [29, 30]. The antioxidant defense system, measured by SOD activity, is inversely correlated with infarct size and the severity of neurologic damage [15, 16]. The data here also confirms that the lower antioxidant (free thiol) level in the acute phase of stroke is associated with poor outcome.

Although other inflammatory biomarkers like high-sensitivity C-reactive protein (hs-CRP) and leukocytes have been reported to be useful in predicting clinical outcome after stroke [31, 32], there has been no head-to-head study to date that compares these markers in terms of predictive value. A previous research demonstrates that statin therapy reduces serum hs-CRP level and oxidized LDL in patients after AIS [33]. A prospective study is warranted to evaluate the predictive value of these biomarkers on stroke outcome.

Some limitations of this study should be acknowledged. First, the measurement of only few biomarkers of oxidative damage cannot be considered a valid tool for exploring a multifaceted, complex oxidant/antioxidant imbalance after acute stroke. Second, the oxidant/antioxidant balance of stroke patients may be influenced by a multitude of parameters, including age, sex, smoking habit, alcohol consumption, physical activity, and vitamin supplementation. Third, oxidative stress may be influenced by other drugs (e.g., antiplatelet, angiotensin II type 1 receptor blockers, and antidiabetics). Since the use of these drugs depends on the preference of the attending physician, this may cause potential bias in statistical analysis and in drawing conclusions. Nonetheless, the sample size is not large and the number of variables considered in the stepwise logistic regression analysis is small. Hence, the maximum likelihood estimates of the coefficients are valid in the analysis.

In conclusion, patients with AIS have significantly higher TBARS and lower free thiol levels than healthy controls. The level of free thiol is significantly lower in patients with large-vessel disease than in those with small-vessel disease in the acute phase of stroke. Serum TBARS on day 7 after stroke is an independent predictive biomarker of three-month stroke outcome.

Conflict of Interests

All of the authors declared that they have no conflict of interests.

Acknowledgments

This study was supported by grants from the National Science Council Research Project (99-2314-B-182-055-MY2). The authors wish to thank Doctor Gene Alzona Nisperos who provided language help and writing assistance for this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 598762, 10 pages
http://dx.doi.org/10.1155/2014/598762

Research Article

Elevated Serum Vascular Cell Adhesion Molecule-1 Is Associated with Septic Encephalopathy in Adult Community-Onset Severe Sepsis Patients

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Received 26 February 2014; Revised 11 April 2014; Accepted 11 April 2014; Published 6 May 2014

Academic Editor: Hung-Chen Wang

Background and Aim. Septic encephalopathy (SE) is a common complication of severe sepsis. Increased concentrations of circulating soluble adhesion molecules are reported in septic patients. This study aimed to determine whether serum adhesion molecules are associated with SE. *Methods*. Seventy nontraumatic, nonsurgical adult patients with severe sepsis admitted through ER were evaluated. Serum adhesion molecules were assessed for their relationship with SE, and compared with other clinical predictors and biomarkers. *Results*. Twenty-three (32.8%) patients had SE. SE group had higher in-hospital mortality (40% versus 11%, $P = 0.009$) and their sVCAM-1, sICAM-1, and lactate levels on admission were also higher than non-SE group. By stepwise logistic regression model, sVCAM-1, age, and maximum 24-hours SOFA score were independently associated with septic encephalopathy. The AUC analysis of ROC curve of different biomarkers showed that sVCAM-1 is better to predict SE. The sVCAM-1 levels in the SE group were significantly higher than those of the non-SE group at three time periods (Days 1, 4, and 7). *Conclusions*. Septic encephalopathy implies higher mortality in nontraumatic, nonsurgical patients with severe sepsis. VCAM-1 level on presentation is a more powerful predictor of SE in these patients than lactate concentration and other adhesion molecules on admission.

1. Introduction

Septic encephalopathy (SE) is a common complication of severe sepsis and septic shock. An estimated 9–71% of patients with sepsis exhibit symptoms of encephalopathy [1–3], including consciousness disturbance, impaired cognitive function, personality changes, and lack of concentration or somnolence [4, 5]. Several mechanisms have been proposed, including oxidative stress [6], increased cytokine and proinflammatory factor [7], mitochondrial dysfunction, apoptosis [8], decreased cerebral blood flow [9], endothelium activation [10], and blood-brain barrier breakdown [11], as well as any combination of the abovementioned etiologies. By itself, SE is pivotal in determining sepsis mortality [12] and makes clinical evaluation more complex since patients cannot express themselves well. Although SE has been described as a reversible syndrome, studies indicate long-lasting cognitive and depressive disturbances in patients

after the sepsis resolves [13]. Early accurate detection of septic encephalopathy not only makes clinical physician more alert about the sepsis status and aggressive treatment but also decreases unnecessary examination and movement of patient.

To date, useful biomarkers in predicting SE are still limited. Several studies used S-100B protein, a marker of astrocytes activation and injury, as a marker for brain injury in SE [14, 15]. However, its results were not promising. Five studies demonstrated that elevated S-100B protein correlated with the development of SE, but two other studies found no correlation and all their sample sizes were small [16, 17]. On the other hand, studies using animal models showed that some proinflammatory cytokines or cell adhesion molecules could be potential biomarkers that induce SE, but their effects on human beings are still not demonstrated [18–20].

In human studies, increased concentrations of circulating soluble adhesion molecules have been reported in patients with systemic inflammatory response syndrome, septic shock, and cardiovascular diseases [21, 22]. Increased concentrations of adhesion molecules have also been associated with multiple organ dysfunction, disease severity, or death [23]. Since sVCAM-1 and sICAM-1 have been involved in leukocyte-endothelium cell crosstalk at the blood-brain barrier [24], we wanted to know their relationship with SE. This prospective study aimed to determine the roles of serum adhesion molecules and conventional biomarkers in predicting SE among adult severe sepsis patients.

2. Patients and Methods

2.1. Study Population. This is a secondary analysis of prospective collected data on the time course of levels of adhesion molecules in severe sepsis and septic shock patients and the association of these biomarkers with SE. Over an 18-month period (January 2011 to June 2012), patients aged ≥20 years who were admitted through emergency room (ER) of Kaohsiung Chang Gung Memorial Hospital (CGMH), a 2482-bed acute-care teaching hospital in southern Taiwan providing both primary and tertiary referral care, were screened every weekday for severe sepsis and septic shock according to specific criteria and were enrolled in the study within 24 hours after identification. The hospital's Institutional Review Committee on Human Research approved the study, and all of the patients provided informed consent.

Severe sepsis on ER admission was defined according to the American College of Chest Physicians/Society of Critical Care Medicine criteria, which included the following: (a) suspicion or confirmed infection; (b) two or more manifestations of systemic inflammatory response syndrome; and (c) at least one sepsis-induced acute organ dysfunction or signs of hypoperfusion. All of the patients who met these three criteria were eligible to enroll in the study. Septic shock was defined as severe sepsis associated with hypotension not controlled by vascular expansion but requiring vasopressive agents to maintain SBP > 90 mmHg [25].

Patients were excluded if they had one of the following: (1) traumatic etiology; (2) previous surgical treatment; (3) underlying hematologic diseases or those under chemotherapy; (4) pregnancy; (5) central nervous systems disorders with various levels of conscious disturbance before arriving at the ER; and (6) history of exposure to drug, toxic substances, alcohol, industrial agents, heavy metals, or any substance known to cause consciousness change. We excluded these patients to eliminate the influence of inflammation reaction other than sepsis on the biomarkers and reduced the potential factors that would affect the diagnosis of SE.

2.2. Clinical Assessment and Treatment. The patient information collected was demographic data, Acute Physiology and Chronic Health Evaluation (APACHE) II score, Sequential Organ Failure Assessment (SOFA) score, and Charlson Comorbidity Index (CCI) score, which were calculated during the first 24 hours of admission to assess the severity of organ dysfunction. Basic laboratory tests, lactate concentration, B-type natriuretic peptide, and inflammatory markers, including plasma C-reactive protein (CRP) and procalcitonin, were taken on ER admission. The course of various organ dysfunctions and supportive treatments like vasoactive drugs, ventilator, and steroid therapies were also recorded.

2.3. Definition. Comorbidity was defined as preexisting disorder before severe sepsis event. Stroke history in this study only referred to the patients who still could maintain clear conscious level after ischemic or hemorrhagic stroke event. Charlson comorbidity scoring system was used to assess the severity of comorbidity [26]. The source of infection was classified as one of the following: lower respiratory tract, urinary tract, skin and musculoskeletal soft tissue, central nervous system, or intra-abdomen infection which included liver abscess, spontaneous bacterial peritonitis, biliary tract, peritonitis, and primary bacteraemia with unknown focus. For the grading of disease severity, we use both 24 h APACHE II and 24 h SOFA score which were calculated according to their laboratory data and clinical parameters [27, 28]. And mortality in this study means in-hospital mortality. Ventilator treatment within 24 hours means that the patient needed ventilator support to help him or her maintain adequate oxygenation and the event happened within 24 hours of ER admission.

2.4. Septic Encephalopathy. The patient's conscious level was recorded by the Glasgow Coma Scale (GCS) and mental status at least twice daily. Symptoms of SE included somnolence, stupor, coma, confusion, disorientation, agitation, irritability, and decreased level of GCS. Encephalopathy was confirmed if the patient had two or more of the aforementioned symptoms for more than 72 hours, regained consciousness after treatment, or deteriorated and died. Continuous sedative medication was never used, even in mechanically ventilated patients, although short-duration sedatives were used if patients did not cooperate for the treatment and examination. Patients with obvious etiologies of consciousness change other than SE during treatment were excluded. These include severe hypoglycemia event, intracranial hemorrhage, status

epilepsy, acute ischemic stroke, hyponatremia, and cardiopulmonary resuscitation related hypoxic encephalopathy.

2.5. Assessment of Infectious Biomarkers. All tests were conducted by the quality-controlled central laboratory of CGMH. Concentrations of CRP were determined by enzyme immunoassay (EMIT; Merck Diagnostica; Zurich, Switzerland), while PCT was measured using enzyme-linked fluorescent assay (VIDAS; BioMerieux; Ponte a Ema, Italy). Serum lactate levels were measured using a serum-based assay catalyzed by lactate oxidase (UniCel Integrated System; Beckman Coulter INS; Boulevard, Brea, CA).

2.6. Blood Sampling and Assessment of Serum Adhesion Molecules. Blood samples of serum adhesion molecules were collected on the first day of enrollment (Day 1). Additional samples were obtained on Days 4 and 7. Blood samples were collected by venipuncture into Vacutainer SST tubes. Blood was allowed to clot in room temperature for a minimum of 30 minutes, and the clot was removed by immediate centrifugation at 3,000 rpm for 10 min at 4°C. All serum samples were collected after centrifugation, isolated, and stored at −80°C in multiple aliquots. Serum sICAM-1, sVCAM-1, sE-selectin, sL-selectin, and sP-selectin levels were determined by commercially available ELISA (R&D Systems, Minneapolis, MN, USA). In the assay, standards, controls, and unknown samples were incubated in microtitration wells that were coated with marked antibodies (i.e., anti-ICAM-1, VCAM-1, P-selectin, E-selectin, and L-selectin). After incubation and washing, the wells were treated with another anti-Ag detection antibody labeled with enzyme horseradish peroxidase (HRP).

After second incubation and washing, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added, and the enzymatic turnover rate of the substrate was determined by dual wavelength absorbance measured at 450 and 620 nm. Absorbance was directly proportional to the concentration of antigens present. A set of antigen standards was used to plot a standard curve of absorbance versus antigen concentration, from which antigen concentrations in the unknowns were calculated.

Samples for serum adhesion molecules were collected and measured immediately. It takes 5 hours to measure serum levels. Other serum samples were collected after centrifugation, isolated, and stored at −80°C in multiple aliquots in accordance with our study design [29].

2.7. Statistical Analysis. Data were presented as mean ± SD or n (%) accordingly. Comparisons between septic encephalopathy and no encephalopathy groups were made by Mann-Whitney U test, while proportions among groups were compared by using χ^2 test or Fisher's exact test. Repeated measures of ANOVA were used to compare serum adhesion molecules at three different time points after severe sepsis. Analysis of covariance (ANCOVA) was used to compare groups after controlling for potential confounding variables. Spearman correlation analysis was used to test the correlation of serum adhesion molecule levels and traditional infection makers with the severity of sepsis, including maximum 24 h SOFA score and maximum 24 h APACHE II score.

Stepwise logistic regression was used to evaluate the relationship between significant variables and septic encephalopathy, with adjustments for other potential confounding factors. Only variables strongly associated with septic encephalopathy ($P < 0.05$) were included in the final model. Receiver operating characteristic (ROC) curves were generated to determine a cut-off level for significant variables for SE. Areas under the ROC curves (AUCs) were calculated for each parameter and compared. All statistical analyses were conducted using the SAS software package, version 9.1 (2002, SAS Statistical Institute, Cary, NC).

3. Results

3.1. Baseline Characteristics of the Study Patients. Seventy-five adult severe sepsis and septic shock patients were enrolled, but five patients were excluded after finding the etiology of consciousness change. Two of them had severe hypoglycemia, and the other 3 patients had severe hyponatremia, basilar artery occlusion as revealed by brain magnetic resonance imaging, and status epilepsy by electroencephalography, respectively. In the 70 patients, there were 22 females and 48 males, with an average age of 64.3 years. During admission, 23 patients experienced SE and 47 patients did not. Among the 23 SE events, 14 (61%) occurred at the time of enrollment, 6 (26%) were within 24 h after enrollment, and 3 (13%) were after 24 h but within 3 days of enrollment. The 14 patients who received brain computed tomography (CT) examination all had nonspecific findings. Four patients died after SE, but another five patients had returned to clear consciousness before occurrence of death.

Baseline characteristics, including comorbidities, clinical presentations, hospital mortality, and disease severity index including shock within 24 h, mechanical ventilation treatment within 24 h, and maximum 24 h APACHE II and 24 h SOFA scores between the SE and non-SE groups, were listed in Table 1. In this study, the patient with a stroke history was more likely to have SE (odds ratio (OR) 5.6, $P = 0.03$). In the disease severity index, the SE group had higher APACHE II and SOFA scores (21.3±5.5 versus17.5±5.7, $P = 0.01$; 8.2±2.4 versus 5.4 ± 3.1, $P < 0.001$, resp.), which meant more organ dysfunction. In-hospital mortality and ventilator treatment within 24 hours were also both higher in SE patients (40% versus 11%, $P = 0.009$; 57% versus 24%, $P = 0.008$, resp.).

The sources of infection and laboratory data of the two groups were listed in Tables 2 and 3, respectively. There was no significant difference between infection source and culture result. Serum lactate (50.5 ± 37.6 versus 32.6 ± 20.3, $P = 0.05$), sICAM-1 (1028.2 ± 525.2 versus 764.8 ± 504.9, $P = 0.03$), and sVCAM-1_{Day1} (3048.1 ± 1261.1 versus 1969.0 ± 1129.5, $P = 0.001$) were the only three markers with significant difference between the SE and non-SE groups.

3.2. Effect of Infection Markers and Serum Adhesion Molecules on Sepsis Severity. Based on the statistical results (Spearman

Table 1: Baseline characteristics of septic encephalopathy (SE) and non-SE groups in severe sepsis patients.

	SE group $n = 23$	Non-SE group $n = 47$	P value
Age (y) (mean ± SD)	68.0 ± 11.2	62.6 ± 13.6	0.15
Male/female	17/6	31/16	0.59
Underlying diseases [n (%)]			
Diabetes mellitus	10 (44)	15 (32)	0.43
Hypertension	8 (35)	22 (47)	0.44
Liver diseases/alcoholism	6 (26)	6 (13)	0.19
Chronic lung disease	3 (13)	11 (23)	0.36
Stroke[a]	7 (30)	4 (9)	0.03*
Coronary artery disease	0 (0)	6 (13)	0.17
Cancer	6 (21)	10 (21)	0.76
Chronic renal disease	2 (9)	1 (2)	0.25
Clinical presentations (mean ± SD)			
Systolic BP	108.0 ± 39.0	108.0 ± 47.0	0.91
Diastolic BP	72.1 ± 24.2	64.2 ± 22.8	0.20
Pulse rate	103 ± 26	112 ± 25	0.26
Respiratory rate	20 ± 3	21 ± 4	0.25
Shock within 24 hours [n (%)]	15 (65)	34 (74)	0.58
Ventilator treatment within 24 hours [n (%)]	13 (57)	11 (24)	0.008*
Disease severity index (mean ± SD)			
Maximum 24 h APACHE II score	21.3 ± 5.5	17.5 ± 5.7	0.01*
CCI score	3.6 ± 2.6	3.2 ± 3.0	0.44
Maximum 24 h SOFA score	8.2 ± 2.4	5.4 ± 3.1	<0.001*
Steroid treatment [n (%)]	11 (48)	21 (45)	1.00
In-hospital mortality [n (%)]	9 (40)	5 (11)	0.009*

SD: standard deviation; BP: blood pressure; APACHE: Acute Physiology and Chronic Health Evaluation; CCI: Charlson Comorbidity Index; SOFA: Sequential Organ Failure Assessment.
[a]Stroke only referred to the patients who still could maintain clear conscious level after ischemic or hemorrhagic stroke event.
*$P < 0.05$.

correlation coefficient, P value), sICAM-1_{Day1} level ($\rho = 0.36$, $P = 0.003$), sVCAM-1_{Day1} level ($\rho = 0.404$, $P = 0.001$), sE-$selectin_{Day1}$ ($\rho = 0.364$, $P = 0.002$), procalcitonin ($\rho = 0.347$, $P = 0.004$), and lactate ($\rho = 0.379$, $P = 0.001$) had correlation with maximum 24 h SOFA score. sE-$selectin_{Day1}$ ($\rho = 0.284$, $P = 0.02$), sVCAM-1_{Day1} ($\rho = 0.287$, $P = 0.018$), and lactate ($\rho = 0.441$, $P < 0.001$) had correlation with maximum 24 h APACHE II score. Traditional infection markers, CRP, were neither related to SOFA nor to APACHE II score.

3.3. Prediction of Septic Encephalopathy. Serum sVCAM-1_{Day1} level, sICAM-1_{Day1} level, serum lactate level, 24 h SOFA score, 24 h APACHE II score, stroke history, and ventilator treatment within 24 hours were significantly higher in SE patients and could be used as clinical predictors. However, after using both forward and backward stepwise logistic regression model with all the predictors plus age and sex, only sVCAM-1_{Day1} level ($P = 0.009$, 0.02), age ($P = 0.002$, 0.011), and SOFA score ($P = 0.007$, 0.002) were independently associated with SE.

The effectiveness of infection markers in predicting SE in the ER setting was evaluated by assessing the area under curve (AUC) of each biomarker's ROC curves. The AUCs for each marker were calculated (Table 4; Figure 1). The AUC for CRP, procalcitonin, lactate, E-$selectin_{Day1}$, sICAM-1_{Day1}, and sVCAM-1_{Day1} levels was 0.561 ($P = 0.423$), 0.616 ($P = 0.130$), 0.647 ($P = 0.052$), 0.593 ($P = 0.219$), 0.664 ($P = 0.031$), and 0.760 ($P = 0.001$), respectively. sVCAM-1_{Day1} level had the highest AUC, reflecting good discrimination. Our suggestion of sVCAM-1_{Day1} cut-off value for predicting SE was 1900 ng/mL for the better sensitivity (sensitivity of 81.8%, specificity of 61.9%, positive predictive value of 51.2%, and negative predictive value of 87.4%).

3.4. Time Course of Serum Vascular Cell Adhesion Molecule Levels. Serum sVCAM-1 levels obtained at different days (Figure 2) revealed that sVCAM-1 level gradually decreased in both groups. In all three different testing points, the SE group had significantly higher sVCAM-1 level than the non-SE group using Mann-Whitney U test (3048.1 ± 1261.1 versus 1969.0 ± 1129.5, $P = 0.001$; 3011.5 ± 1158.1 versus

Table 2: Sources of infection in septic encephalopathy (SE) and non-SE groups.

Sources of infection	SE group $n = 23$	Non-SE group $n = 47$	P value
Respiratory tract infection	8 (36.4)	19 (40.4)	0.955
Urinary tract infection	4 (18.2)	9 (19.1)	
Intra-abdominal infection	4 (18.2)	10 (21.3)	
Soft tissue infection	5 (22.7)	7 (14.9)	
Unknown origin	1 (4.5)	2 (4.3)	
Concurrent bacteremia episode	13 (56.5)	21 (44.7)	0.192
Causative pathogens			0.56
Gram-negative			
Escherichia coli	5 (21.7)	6 (12.8)	
Klebsiellapneumoniae	2 (8.7)	8 (17.0)	
Proteus mirabilis	1 (4.3)	1 (2.1)	
Burkholderiapseudomallei	1 (4.3)	0 (0)	
Salmonella enteritidis	1 (4.3)	0 (0)	
Gram-positive			
Streptococcus pneumoniae	0 (0)	2 (4.3)	
β-Hemolytic Streptococcus group A	1 (4.3)	2 (4.3)	
Staphylococcus aureus	2 (8.7)	2 (4.3)	

Table 3: Laboratory data of the septic encephalopathy (SE) and non-SE groups in severe sepsis patients.

	SE group $n = 23$	Non-SE group $n = 47$	P value
WBC/DC (mean ± SD)			
WBC ($\times 10^9$/L)	15.0 ± 10.0	17.7 ± 12.0	0.13
Segment (%)	76.7 ± 20.3	81.8 ± 17.3	0.38
Band (%)	8.5 ± 12.7	3.2 ± 4.8	0.06
Lymphocyte (%)	7.7 ± 7.9	10.4 ± 17.4	0.96
Biochemistry (mean ± SD)			
Glucose (mg/dL)	210 ± 162	171 ± 94	0.91
Creatinine (mg/dL)	2.9 ± 1.9	2.3 ± 2.5	0.06
Lactate (mg/dL)	50.5 ± 37.6	32.6 ± 20.3	0.05*
Total bilirubin (mg/dL)	3.3 ± 3.0	2.2 ± 2.7	0.09
BNP (pg/mL)	991 ± 1127	742 ± 1024	0.21
Inflammatory markers (mean ± SD)			
CRP (mg/L)	231 ± 112	206 ± 137	0.40
Procalcitonin (ng/mL)	32.5 ± 33.4	35.6 ± 59.4	0.12
sICAM-1_{Day1} (ng/mL)	1028.2 ± 525.2	764.8 ± 504.9	0.03*
sVCAM-1_{Day1} (ng/mL)	3048.1 ± 1261.1	1969.0 ± 1129.5	0.001*
sP-$selectin_{Day1}$ (ng/mL)	98.8 ± 42.5	98.0 ± 23.0	0.77
sL-$selectin_{Day1}$ (ng/mL)	1208.2 ± 777.5	933.9 ± 311.9	0.10
sE-$selectin_{Day1}$ (ng/mL)	214.2 ± 111.9	117.8 ± 127.6	0.22

WBC: white blood cells; DC: differential count; CRP: C-reactive protein; BNP: B-type natriuretic peptide; sE-selectin: soluble E-selectin; sICAM-1: soluble intercellular adhesion molecule-1; sL-selectin: soluble L-selectin; sP-selectin: soluble P-selectin; sVCAM-1: soluble vascular cell adhesion molecule-1.
* $P < 0.05$.

Table 4: AUC for biomarkers in diagnosing septic encephalopathy by ROC curve analysis.

	AUC	95% confidence interval	*P* value
CRP	0.561	0.416–0.705	0.423
Procalcitonin	0.616	0.479–0.752	0.130
Lactate	0.647	0.494–0.800	0.052
sVCAM-1	0.760	0.638–0.881	0.001*
sICAM-1	0.664	0.532–0.795	0.031*
sE-selectin	0.593	0.452–0.734	0.219

AUC: area under the curve; CRP: C-reactive protein; sVCAM-1: soluble vascular cell adhesion molecule-1; sE-selectin: soluble E-selectin; sICAM-1: soluble intercellular adhesion molecule-1; ROC: receiver operating characteristic.
* $P < 0.05$.

1532.6 ± 1005.2, $P < 0.001$; 2376.4 ± 1017.9 versus 1597.6 ± 1048.0, $P = 0.027$, resp.). By repeated measures of ANOVA, serum VCAM-1 levels between the two groups at three different time points, Days 1, 4, and 7, were significantly different ($P = 0.011$).

4. Discussion

The present study examines the association between SE and circulatory cell adhesion molecules and produces the following major findings. First, the level of serum sVCAM-1, sICAM-1, and lactate on presentation, 24 h SOFA score, 24 h APACHE II score, underlying diseases of stroke, and ventilator treatment within 24 hours were significantly higher in the SE group of severe septic patients than in the non-SE group. Second, VCAM-1 level on presentation is a more powerful predictor of SE in severe sepsis patients than lactate concentration and other adhesion molecules on admission by stepwise logistic regression and AUC analysis. Lastly, sVCAM-1 levels in the SE group were significantly higher than those of the non-SE group at three time periods (Days 1, 4, and 7).

In this study, SE is closely related to the severity and mortality of severe sepsis and septic shock. The 24 h APACHE II score, SOFA score, serum lactate level, ventilator treatment within 24 hours, and mortality rate in SE of severe septic patients are significantly higher than those of non-SE patients. The longer duration of SE also implies higher mortality since four patients died within three more days of SE. Our result is compatible with other studies [30]. In another study, they also found that mortality related to SE increased from 16% to 63% when the GCS decreased from 15 to less than 8 [12]. The increased mortality in SE group could partially explained by increased severity of sepsis. But further evaluation of the direct effect of brain injury to the systemic response may need to clarify it.

Patients with SE had also higher incidence of early respiratory failure. In clinical practice, patients with conscious disturbance are at risk of respiratory failure since they have less airway protection and respiratory drives. Certain periods of hypoxemia before respiratory failure may also cause more brain injury. Tissue hypoperfusion can lead to elevated serum lactate level and increased acidosis, which can explain part of the mechanisms of SE by their correlation.

Cell adhesion molecules regulate endothelial function by activating leukocyte recruitment and tissue inflammation. Endothelium dysfunction may be central to the development of sepsis-induced multiple organ failure [31]. Soluble form of VCAM-1, ICAM-1, and E-selectin are present in plasma and reflect cellular inflammatory status and correlate with endothelial dysfunction [32]. In the present study, all three kinds of cell adhesion molecules and serum lactate correlate with SOFA score. They can represent the degree of organ damage in severe sepsis and septic shock patients.

Brain has a unique blood-brain barrier that regulates the brain capillary blood flow and thus precisely maintains the brain internal microenvironment [33]. But endothelial activation may result in the breakdown of the blood-brain barrier [34]. Increased permeability of the blood-brain barrier has been demonstrated in experimental models of sepsis [35]. The brain MRI of SE patients also reveals vasogenic edema of the brain parenchyma [36, 37]. Hofer's study using septic animal models reveals early changes in the integrity of the blood-brain barrier in the central nervous system. Increased cerebral ICAM-1 expression may be an early factor involved in these pathogenic events [20]. In Hamed's study, children with sepsis-induced encephalopathy have elevated serum and cerebrospinal fluid levels of sICAM-1, NO, and S100B compared to those with sepsis only [38]. Both studies used only sICAM-1 as the biomarkers.

In our study, both sICAM-1 and sVCAM-1 had correlated with SE. Serum sVCAM-1 and sICAM-1 are also correlated with each other in this study. Previous study has revealed that many signaling events attributed to ICAM-1 engagement appear to be similar to VCAM-1 [39]. It is difficult to disentangle the two CAMs effects. In cardiovascular disease study, sICAM appears to be a general marker of proinflammation and may be used as a risk factor. But sVCAM-1, which is not expressed in baseline conditions, is rapidly induced by injury and can emerge as a strong risk predictor of existing disease [40]. Compared to sICAM-1 and E-selectin, it is sVCAM-1 that has the strongest correlation with cardiovascular-related future death and cardiovascular events [41]. Just like our study, sVCAM-1 is also a better predictor than other adhesion molecules in SE patients.

4.1. Limitations. Although this study demonstrates that serum VCAM-1 level on presentation is a more powerful

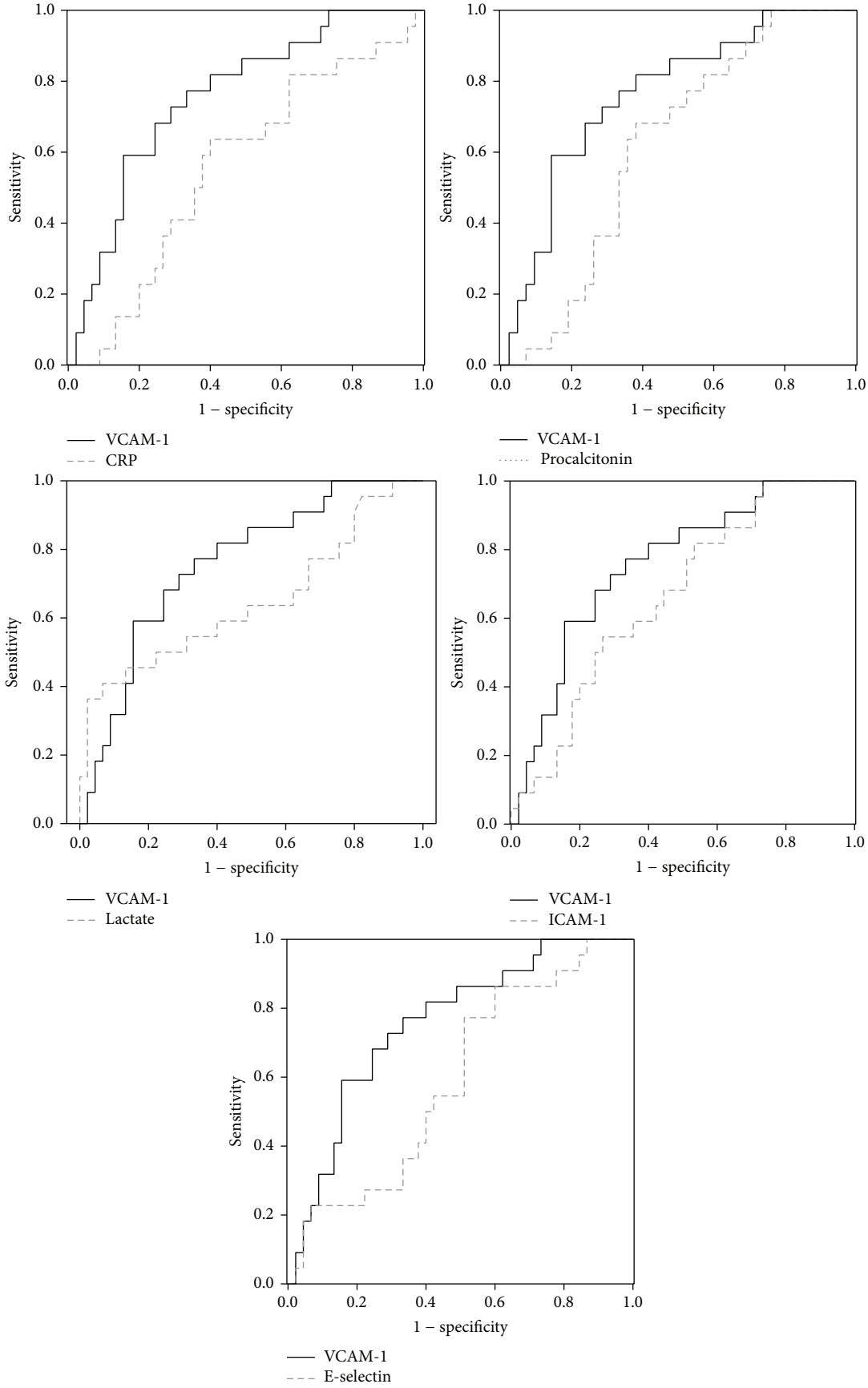

Figure 1: The comparison ROC curve of various biomarkers with sVCAM-1 for predicting septic encephalopathy.

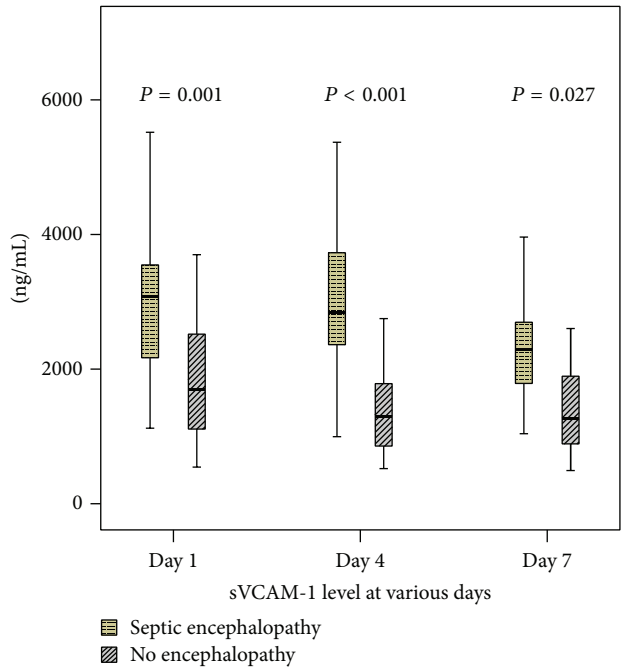

Figure 2: Comparison of serum VCAM-1 level between septic encephalopathy (SE) and non-SE groups in various days.

predictor of SE in severe sepsis patients than lactate concentration and other adhesion molecules on admission, this study has several limitations. First, the occurrence of SE can be at the time of presentation or after the stay at the ER and both the duration and severity of SE were different in each patient. Its relationship with levels of serum cell adhesion molecules cannot be exactly evaluated in every SE patient. Second, although only nontraumatic, nonsurgical septic patients are enrolled and patients with underlying brain pathologies, cognitive decline, and continuous sedative medication use have been excluded, not all of the severe sepsis patients received both neuroimaging studies and electroencephalography studies. The findings may underestimate the "true" frequency of SE in septic patients in this study. Lastly, the choice of therapeutic strategy for sepsis (e.g., use of steroids and choice, dosage, and duration of antibiotics) may be different for each patient based on the preference of the attending physician. This may cause potential bias in patients' outcome and serum biomarkers levels.

5. Conclusion

In conclusion, this study demonstrates that SE implies higher mortality in severe septic patients and VCAM-1 level on presentation is a more powerful predictor of SE than lactate concentration and other adhesion molecules on admission.

Ethical Approval

The Institutional Review Committee on Human Research of Chang Gung Memorial Hospital approved the study.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Chih-Min Su participated in the design of the study and drafting of the paper. Chia-Te Kung, Sheng-Yuan Hsiao, Tsung-Cheng Tsai, Hsien-Hung Cheng, Wen-Neng Chang, Ya-Ting Chang, Nai-Wen Tsai, and Hung-Chen Wang participated in the sequence alignment and clinical evaluation of patients. Wei-Che Lin interpreted the imaging studies. Yi-Fang Chiang, Ben-Chung Cheng, and Yu-Jih Su performed the statistical analysis. Chia-Te Kung and Cheng-Hsien Lu conceived the study, participated in its design and coordination, and helped draft the paper. All authors read and approved the final paper. Drs. Cheng-Hsien Lu and Chia-Te Kung contributed equally to this work.

Acknowledgments

This work was supported by Grants from Chang Gung Memorial Hospital (Chang Gung Medical Research Project CMRPG891341) and NHRI-EX101-10142EI. The authors wish to thank Dr. Gene Alzona Nisperos for editing and reviewing the paper for English language considerations.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 861812, 7 pages
http://dx.doi.org/10.1155/2014/861812

Research Article

Association between Autoimmune Rheumatic Diseases and the Risk of Dementia

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Received 24 February 2014; Revised 21 April 2014; Accepted 22 April 2014; Published 30 April 2014

Academic Editor: Cheng-Hsien Lu

Aim. Autoimmune rheumatic diseases (ARD) are characterized by systemic inflammation and may affect multiple organs and cause vascular events such as ischemic stroke and acute myocardial infarction. However, the association between ARD and increased risk of dementia is uncertain. This is a retrospective cohort study to investigate and compare the risk of dementia between patients clinically diagnosed with ARD and non-ARD patients during a 5-year follow-up period. *Methods*. Data were obtained from the Longitudinal Health Insurance Database 2000 (LHID2000). We included 1221 patients receiving ambulatory or hospitalization care and 6105 non-ARD patients; patients were matched by sex, age, and the year of index use of health care. Each patient was studied for 5 years to identify the subsequent manifestation of dementia. The data obtained were analyzed by Cox proportional hazard regression. *Results*. During the 5-year follow-up period, 30 ARD (2.48%) and 141 non-ARD patients (2.31%) developed dementia. During the 5-year follow-up period, there were no significant differences in the risks of any type of dementia (adjusted hazard ratio (HR), 1.18; 95% CI, 0.79–1.76) in the ARD group after adjusting for demographics and comorbidities. *Conclusions*. Within the 5-year period, patients with and without ARD were found to have similar risks of developing dementia.

1. Introduction

Autoimmune rheumatic diseases (ARD) such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren syndrome, progressive systemic sclerosis (PSS), polymyositis (PM), dermatomyositis (DM), vasculitides (including polyarteritis nodosa, Kawasaki disease, hypersensitivity angiitis, Wegener's granulomatosis, giant cell arteritis, and Takayasu arteritis), and Behçet's disease are chronic diseases that are characterized by progressive and systemic inflammation.

Patients with ARD have a higher mortality rate and a greater risk of developing cancer than the general population [1–5]. ARD may affect multiple organs and cause vascular events such as ischemic stroke, acute myocardial infarction, and peripheral arterial occlusive disease [6–10]. Multiple epidemiological studies have shown that ARD is a risk factor

for dementia and other neurological conditions such as stroke and seizure [6, 9, 11]. However, some studies have reported contrary results that ARD had a lower prevalence of dementia and decreased risk of AD [12–15]. Studies that reported ARD as a significant risk factor for dementia exhibited small case series or lacked a definitive diagnosis for dementia [16, 17]. Therefore, the association between ARD and dementia has not been fully established thus far [12–18].

This study aimed to determine the association between ARD and increased risk and incidence of dementia using the Longitudinal Health Insurance Database (LHID2000) in Taiwan and to determine if such an association could be explained based on medical comorbidities or ARD.

2. Methods

2.1. Database, Standard Protocol Approval, Registration, and Patient Consent. This study used data from the LHID2000, which is a subset of the National Health Insurance Research Database (NHIRD) in Taiwan. Taiwan began its National Health Insurance (NHI) program on March 1, 1995, in order to provide comprehensive health care to all citizens of Taiwan. As of December 2007, 22.6 million individuals, representing 98% of the Taiwanese population, had been enrolled in the program [8, 19]. The data on NHI claims include comprehensive demographic data, dates of clinical visits, and diagnostic codes, as well as details of prescriptions, examinations, and procedures, which are entered into the NHIRD; it is maintained by the Bureau of National Health Insurance, Taiwan. Scientists can access the data for research purposes. The LHID2000 consists of information on the original claims and registration files of 1,000,000 individuals, randomly sampled from the NHIRD in 2000. Taiwan National Health Research Institutes (TNHRI) (http://nhird.nhri.org.tw/) publishes the information on the creation of the database online. There are no significant differences in gender, age, or the amount of insurance payments between the individuals registered in LHID2000 and the individuals in the NHI program. More than 600 studies have been published using the data obtained from the LHID2000. The Institutional Review Board exempted this study from a full review.

This is a retrospective cohort study. Patients with ARD were identified from the catastrophic illness registry in the LHID2000. In Taiwan, ARD patients are eligible for a catastrophic illness certificate after a rheumatology specialist makes the diagnosis based on clinical manifestations, laboratory data, and the criteria set by the American College of Rheumatology, which is reviewed by rheumatologists commissioned by the NHI. Individuals with these certificates are eligible for government subsidies, which include discounted outpatient and inpatient copayments [1–8]. Thus, the catastrophic illness patient data is highly accurate and reliable.

We identified patients with an ARD diagnosis using the International Classification of Diseases, ninth revision, Clinical Modification (ICD-9-CM) code (714.0 for rheumatoid arthritis (RA), 710.0 for systemic lupus erythematosus (SLE), 710.2 for Sjögren syndrome, 710.1 for progressive systemic sclerosis (PSS), 710.3 for polymyositis (PM), 710.4 for dermatomyositis (DM), vasculitides (446.0 for polyarteritis nodosa, 446.1 for Kawasaki disease, 446.2 for hypersensitivity angiitis, 446.4 for Wegener's granulomatosis, 446.5 for giant cell arteritis, and 446.7 for Takayasu disease), and 136.1 for Behçet's diseases) from the registry of catastrophic illnesses from 2000 to 2005. Patients found to have ARD prior to 2000 were excluded from the study. Additionally, patients diagnosed with the following conditions prior to the index use of health care facilities were excluded from the study: 290.0 (senile dementia, uncomplicated); 290.1 (presenile dementia); 290.2 (senile dementia with delusional or depressive features); 290.3 (senile dementia with delirium); 290.4 (arteriosclerotic dementia); 294.1 (dementia in conditions classified elsewhere); 331.0 (Alzheimer's disease, AD); 331.1 (pick disease); and 331.2 (senile degeneration of brain) [20]. A total of 1221 patients with new diagnoses of ARD from 2000 to 2005 were selected for the study.

Subjects for the comparison cohort were selected from the remaining records of patients in the LHID2000 registry. First, we excluded patients with previous NHI ambulatory claim records or inpatient records indicating any ARD diagnosis between 1996 and 1999. Then, we randomly selected 6105 beneficiaries (5 comparison subjects were randomly selected for every patient with ARD) matched in terms of sex, age (<30, 30–49, and ≥50 years), and the year of index use of health care services. We defined the first ambulatory care visit occurring in the year of index health care use as their index health care use. Patients who were diagnosed with incident dementia prior to their clinic visits were excluded from the comparison cohort group. There were a total of 7326 patients enrolled in the study. In order to identify patients who subsequently suffered from dementia (ICD-9-CM codes from 290.0 to 290.4, 294.1, and from 331.0 to 331.2), every patient was followed up until 2010 using data on their index health care visit.

2.2. Statistical Analysis. The primary aim of this study was to identify patients who had received ambulatory care or undergone hospitalization for any type of dementia. We used Pearson's χ^2 tests to compare differences between patients with and without ARD at baseline in terms of demographic characteristics (age, sex, and geographical location of the patient's residence (Northern, Central, Eastern, and Southern Taiwan)) and certain comorbidities (diabetes, hyperlipidemia, hypertension, cerebrovascular disorders, heart failure (HF), and atrial fibrillation (AF)) [21–23]. These comorbidities were considered only if the condition occurred in an inpatient setting or during 2 or more ambulatory care claims within 1 year, prior to the index ambulatory care visit.

Patients without ARD were designated as the reference group. The crude and adjusted hazard ratios (HR) for the association between ARD and dementia during the 5-year follow-up period were estimated using univariate and multivariate Cox proportion hazard regression. The demographic characteristics and selected comorbidities were corrected for determine the adjusted HR. All models were examined for violation of the proportional hazards assumption and the results showed no violations of the proportionality assumption. A stratified Cox proportional hazard regression was

performed to compare the relationship between ARD and dementia in various ARD groups. The associations of ARD with dementia were further analyzed according to dementia subtypes. The dementia subtypes were as follows: AD (ICD-9-CM code 331.0), vascular dementia (ICD-9-CM code 290.4), and unspecified dementia (ICD-9-CM codes 290.0–290.3, 294.1, and 331.1-331.2). The HR values and 95% confidence intervals (CI) were computed with a statistical significance of 0.05. All statistical analyses were conducted using the SAS statistical package (SAS System for Windows, version 9.1, SAS Institute Inc., Cary, NC, USA).

3. Results

We identified 1221 ARD and 6105 non-ARD cases in our analysis. The data description for the sampled subjects is presented in Table 1. There were no significant difference in age or sex between the ARD and non-ARD patients, after matching for gender, age in the 5-year interval, and year of index health care use. However, among subjects with ARD, the female-to-male ratio and age distribution varied according to the ARD subtype. The prevalence of ARD was higher in female subjects (79.4%). In decreasing order, the female-to-male ratios were as follows: SLE (87.3%), Sjögren syndrome (86.7%), RA (75.8%), PSS (74.3%), PM/DM (71.9%), Behçet's disease (60.0%), and Vasculitides (55.6%). Most patients with RA, SS, and PM/DM were older than 50 years. Patients with PSS and Behçet's disease were aged 30–49, and SLE patients were aged less than 30. ARD patients had a higher prevalence of HF (8.93 versus 5.44%, $P < 0.001$) and AF (0.90 versus 0.62%, $P = 0.028$) than patients without ARD, after matching for age and sex.

The crude and adjusted HRs for dementia during the 5-year follow-up period after index health care use are shown in Table 2. Out of 7326 patients, 171 patients experienced dementia during the 5-year follow-up period, including 30 patients (2.48% of the patients with ARD) from the study cohort and 141 patients (2.31% of the patients without ARD) from the comparison cohort. The univariate Cox proportional hazard regressions showed that the HR for dementia in ARD patients within the 5-year period was 1.10 (95% CI, 0.74–1.63; $P = 0.63$) compared to that in non-ARD patients. We further analyzed the HR value after adjusting the data for diabetes, hyperlipidemia, hypertension, cerebrovascular disorders, heart failure, and atrial fibrillation. The HR value of 1.18 (95% CI, 0.79–1.76; $P = 0.41$) was obtained. The HRs for dementia in both cohorts with the ARD subtypes RA, SLE, and SS were 1.42, 0.71, and 1.31, respectively. The adjusted HRs for RA, SLE, and SS were 1.20, 2.86, and 0.81, respectively. Table 2 also presents HR of dementia between cohorts according to different age groups. Stratified Cox proportional hazard regressions yielded the following results for the 5-year period: in the age group of <30 years, the HR of patients with ARD was 1.29 (95% CI, 0.81–1.57; $P = 0.56$), in comparison with the non-ARD patients; in the age group of 30–49 years, the HR of ARD patients was 1.31 (95% CI, 0.67–1.63; $P = 0.34$). The HR for TBI patients older than 50 years was 1.08 (95% CI, 0.76–1.52; $P = 0.59$). Furthermore, we found that the adjusted HR for dementia within the 5-year period in ARD patients younger than 30 years was 1.23 (95% CI, 0.76–1.61; $P = 0.62$); the corresponding value in ARD patients aged 30–49 was 1.29 (95% CI, 0.73–1.59; $P = 0.62$); and in patients older than 50 years, the HR values for TBI patients were 1.21 (95% CI, 0.81–1.77; $P = 0.44$).

Table 1: Comparison of demographic characteristics and comorbidities of ARD and non-ARD patients.

Variable	ARD $N = 1221$		Comparison $N = 6105$		P
	n	%	n	%	
Sex					1
Male	251	20.6	1255	20.6	
Female	970	79.4	4850	79.4	
Age, year					1
<30	228	18.7	1140	18.7	
30–49	433	35.5	2165	35.5	
≥50	560	45.9	2800	45.9	
Geographic region					0.003
Northern	507	41.5	2900	47.5	
Central	299	24.5	1200	19.7	
Southern	336	27.5	1713	28.1	
Eastern	79	6.47	292	4.78	
Comorbidities					
Diabetes	116	9.50	538	8.81	0.294
Hyperlipidemia	187	15.3	896	14.7	0.655
Hypertension	209	17.1	1054	17.3	0.814
Stroke	28	2.29	119	1.95	0.623
Heart failure	109	8.93	332	5.44	<0.001
Atrial fibrillation	11	0.90	38	0.62	0.028

Table 3 shows the HR analysis for dementia subtypes in both cohorts. The HRs for vascular dementia in both cohorts for ARD and RA were 0.86 and 1.66, respectively. The adjusted HRs for ARD and RA were 0.99 and 1.52, respectively. The HRs for unspecific dementia in both cohorts for ARD, RA, SLE, and SS were 1.21, 1.51, 0.84, and 1.54, respectively. The adjusted HRs for ARD, RA, SLE, and SS were 1.28, 1.27, 3.48, and 0.91, respectively.

4. Discussion

There has been interest in the potential association between ARD and neuropsychiatric affections, including cognitive dysfunction, headache, mood disorder, cerebrovascular changes within intracranial vessels, seizures, polyneuropathy, anxiety, and psychosis [24, 25]. The primary findings of our study showed that the ARD and comparison groups had similar risks of developing dementia during the 5-year follow-up. We observed that 2.48% of ARD patients experienced dementia (30 patients), and 2.31% of non-ARD patients (141 patients) developed dementia. However, there were no significant differences in the risks of dementia in the ARD group after adjusting for demographics and comorbidities.

Table 2: Dementia risk among sampled patients of different age groups during the 5-year follow-up period from index health care utilization ($N = 7326$).

Dementia	Comparison $N = 6105$	All $N = 1221$	RA $N = 637$	SLE $N = 300$	SS $N = 173$
Total (%)	141 (2.31)	30 (2.48)	20 (3.14)	5 (1.67)	5 (2.89)
Crude HR (95% CI)	1.00	1.10 (0.74–1.63)	1.42 (0.89–2.27)	0.71 (0.29–1.74)	1.31 (0.54–3.20)
P value		0.63	0.24	0.46	0.46
Adjusted HR (95% CI)	1.00	1.18 (0.79–1.76)	1.20 (0.75–1.92)	2.86 (1.16–7.05)	0.81 (0.33–1.99)
P value		0.41	0.56	0.02	0.64
Age group					
<30	7 (0.11)	2 (0.16)	1 (0.16)	1 (0.33)	0 (0.00)
Crude HR (95% CI)	1.00	1.29 (0.81–1.57)	1.22 (0.77–1.96)	2.73 (0.68–10.99)	—
P value		0.56	0.36	0.67	
Adjusted HR (95% CI)	1.00	1.23 (0.76–1.61)	1.12 (0.82–2.04)	3.83 (0.89–16.55)	—
P value		0.62	0.53	0.45	
30–49	16 (0.26)	4 (0.33)	3 (0.47)	1 (0.33)	0 (0.00)
Crude HR (95% CI)	1.00	1.31 (0.67–1.63)	1.91 (0.86–2.41)	1.62 (0.71–2.32)	—
P value		0.34	0.48	0.67	
Adjusted HR (95% CI)	1.00	1.29 (0.73–1.59)	1.87 (0.81–2.39)	1.48 (0.88–2.45)	—
P value		0.62	0.53	0.45	
≥50	118 (1.82)	24 (1.80)	16 (2.51)	3 (1.00)	5 (2.89)
Crude HR (95% CI)	1.00	1.08 (0.76–1.52)	1.38 (0.91–2.33)	0.79 (0.34–1.87)	1.52 (0.57–3.75)
P value		0.59	0.27	0.61	0.43
Adjusted HR (95% CI)	1.00	1.21 (0.81–1.77)	1.24 (0.82–1.89)	0.800 (0.34–1.90)	1.79 (0.51–2.03)
P value		0.44	0.47	0.53	0.47

Hazard ratio was calculated by using Cox proportional regression method during the 5-year follow-up period. Adjustments were made for demographic characteristics (age, sex, and the geographical region) and selected comorbidities in patients (diabetes, hyperlipidemia, hypertension, stroke, heart failure, and atrial fibrillation).

Table 3: Crude and adjusted hazard ratios by dementia subtype among sampled patients during the 5-year follow-up from index health care utilization.

Dementia	Comparison $N = 6105$	All $N = 1221$	RA $N = 637$	SLE $N = 300$	SS $N = 173$
Alzheimer's disease					
Yes (%)	9 (0.15)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Crude HR (95% CI)	1.00				
Adjusted HR (95% CI)	1.00				
Vascular dementia					
Yes (%)	12 (0.20)	2 (0.16)	2 (0.31)	0 (0.00)	0 (0.00)
Crude HR (95% CI)	1.00	0.86 (0.19–3.82)	1.66 (0.37–7.42)	—	—
P value		0.43	0.51		
Adjusted HR (95% CI)	1.00	0.99 (0.22–4.45)	1.52 (0.34–6.86)	—	—
P value		0.51	0.37		
Unspecific dementia					
Yes (%)	120 (1.97)	28 (2.29)	18 (2.83)	5 (1.67)	5 (2.89)
Crude HR (95% CI)	1.00	1.21 (0.80–1.82)	1.51 (0.92–2.47)	0.84 (0.34–2.05)	1.54 (0.63–3.78)
P value		0.57	0.33	0.68	0.41
Adjusted HR (95% CI)	1.00	1.28 (0.84–1.93)	1.27 (0.77–2.10)	3.48 (1.40–8.63)	0.91 (0.37–2.26)
P value		0.32	0.24	0.03	0.46

Hazard ratio was calculated using Cox proportional regression method during the 5-year follow-up period. Adjustments were made for demographic characteristics (age, sex, and the geographical region) and selected comorbidities in the patients (diabetes, hyperlipidemia, hypertension, stroke, heart failure, and atrial fibrillation).

The mechanism leading to a high incidence of dementia in patients with ARD is not fully understood. There are several reasons why ARD patients may have an increased risk of developing dementia. Patients with ARD have a well-documented risk for subclinical and clinical cardiovascular diseases such as stroke and myocardial infraction [6–9]. Epidemiological studies have shown that the risk of dementia increases with cardiovascular abnormalities such as coronary heart disease, heart failure, and atrial fibrillation. Thus, ARD can increase the incidence of dementia [16, 17]. In addition, ARD is a chronic inflammatory disease, which is characterized by progressive and systemic inflammation. Such inflammation is associated with an increased risk for cognitive decline due to a dysfunction in the hypothalamic-pituitary-adrenal axis (HPA) [26–28].

Our results, however, did not support the theory that ARD is a potential risk factor for dementia. Trysberg et al. showed that the low levels of amyloid β found in SLE patients seemed to be a direct consequence of diminished production of amyloid precursor protein, probably mediated by heavy anti-inflammatory/immunosuppressive therapy [29–31]. In Taiwan, most ARD patients who had a catastrophic illness certificate were treated with anti-inflammatory drugs for musculoskeletal symptoms unless there were specific contraindications. To further investigate the effect of anti-inflammatory drugs, we should have compared the risk of developing dementia in patients using anti-inflammatory/immunosuppressive therapy with that of patients who are not using these drugs. This study was unable to make such a comparison, as it did not have a prospective randomized control design. Additionally, in Taiwan, it is unacceptable to stop the treatment of ARD patients with catastrophic illness certificate. Therefore, the ARD group with anti-inflammatory/immunosuppressive therapy is less likely to develop dementia during the 5-year follow-up when compared to the control group.

After adjusting for sociodemographic characteristics, region of residence, and selected comorbidities, SLE diagnosis was independently associated with an adjusted HR of 2.86 and 3.48, respectively. Most SLE patients were aged less than 30 in our study. Young onset dementia more likely has a genetic or metabolic disease. These findings suggest that patients with SLE seem to have higher cognitive dysfunction after long-term follow-up.

Our study had several limitations. First, the sample size was not large enough. A huge sample size is necessary to detect differences in dementia for patients with or without ARD, considering the overall low incidence of dementia and the lower incidence attributed to ARD. Therefore, the difference in incidence rate between ARD and comparison groups is low; the statistical power is too low to detect the difference. Second, the NHI database included data on patients that had treatment for ARD and dementia, but it did not indicate parameters such as clinical severity and laboratory data (serum cholesterol levels, titers of antiphospholipid antibodies, or rheumatic factor). Marital status, body mass index, smoking habits, intelligence, and education level are also associated with ARD and dementia [10–12]. However, data on these variables were not available and could not be adjusted for in the analysis. Third, dementia is more likely to occur in the elderly; therefore, selecting younger patients may affect the age-related risks. All ARDs were included in the present study. Thus, patients with different forms of ARDs had varying ages. Both RA and SS cohorts consisted of older patients. The future analysis of each type of ARD may yield results that are more concise. However, age is not a risk factor for dementia after performing a stratified subgroup analysis. Forth, patients with ARD have an increased risk for cancers and greater mortality than the general population [1–4]. If there were more numbers of deaths among patients with ARD, it would cause an underestimation of the association and increase the risk of dementia. Fifth, ARD diagnosis was based on ICD-9-CM database. The duration or subtypes of ARD were hard to know. For example, Sjögren's syndrome is classified into primary and secondary diseases. Primary Sjögren's syndrome presents alone in the absence of any other autoimmune disorders and only impacts the salivary and lacrimal glands. Secondary disease refers to Sjögren's syndrome in individuals who have another autoimmune disease, such as RA or SLE. It is difficult to know primary or secondary Sjögren's syndrome within the ICD-9-CM-derived database. Lastly, AD is not the most common dementia subtype in our study. The most common forms of dementia are AD, vascular dementia, frontotemporal dementia, semantic dementia, and dementia with Lewy bodies. However, patients with dementia can apply the catastrophic illness registry only when patients with a dementia diagnosis using the ICD-9-CM code 290 in Taiwan. This situation causes vascular dementia or Alzheimer's disease underestimation. Therefore, it is hard to know what subtype of dementia is related to ARD in our study.

5. Conclusions

Our study suggests that patients with ARD have similar chances of developing dementia during the 5-year follow-up compared to the control group. Although evidence suggests that ARD is not a risk factor for dementia, a lot of limitations preclude formal conclusions. Care must be taken before extrapolating these results further due to some limitation, such as the low difference in incidence rate between ARD and comparison groups. Therefore, further research is needed to address this issue.

Abbreviations

ARD: Autoimmune rheumatic diseases
DM: Dermatomyositis
HR: Hazard ratio
ICD-9-CM: International Classification of Diseases, Ninth Revision Clinical Modification
HPA: Hypothalamic-pituitary-adrenal axis
LHID2000: Longitudinal Health Insurance Database 2000
NHI: National Health Insurance
NHIRD: National Health Insurance Research Database

PM: Polymyositis
PSS: Progressive systemic sclerosis
RA: Rheumatoid arthritis
SLE: Systemic lupus erythematosus
TNHRI: Taiwan National Health Research Institutes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This study is based, in part, on data obtained from the National Health Insurance Research Database provided by the Bureau of National Health Insurance, Department of Health, Taiwan, and managed by the National Health Research Institutes. The interpretations and conclusions contained herein do not represent those of the Bureau of National Health Insurance, Department of Health, or the National Health Research Institutes. This study was supported in part by Department of Health, Executive Yuan (Taiwan Grant nos. DOH101-TD-B-111-004 and DOH101-TD-C-111-005), and the National Cheng Kung University and E-da hospital (Grant no. NCKUEDA10303).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 129796, 7 pages
http://dx.doi.org/10.1155/2014/129796

Research Article

Predictors of Memory and Processing Speed Dysfunctions after Traumatic Brain Injury

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Received 7 February 2014; Accepted 17 March 2014; Published 29 April 2014

Academic Editor: Hung-Chen Wang

Background. The aims of this study were to evaluate the predictive value of admission Glasgow Coma Scale (GCS) scores, duration of unconsciousness, neurosurgical intervention, and countercoup lesion on the impairment of memory and processing speed functions six months after a traumatic brain injury (TBI) based on a structural equation modeling. *Methods*. Thirty TBI patients recruited from Neurosurgical Department at the Kaohsiung Medical University Hospital were administered the Wechsler Memory Scale-III (WMS-III) and the Wechsler Adult Intelligence Scale-III processing speed index to evaluate the memory and processing speed functions. *Results*. The study showed that GCS scores accounted for 40% of the variance in memory/processing speed. No significant predictive effects were found for the other three variables. GCS classification at the time of TBI seems to correspond moderately to the severity of memory/processing speed dysfunctions. *Conclusions*. The present study demonstrated that admission GCS score is a robust predictor of memory/processing speed dysfunctions after TBI. The results should be replicated with a large sample of patients with TBI, or be extended by examining other potential clinical predictors.

1. Introduction

Traumatic brain injury (TBI) which often occurs in adolescents and young adults remains a major issue for public health. The physical and cognitive deficits following TBI often disrupt important developmental processes [1] and psychosocial problems [2, 3]. Therefore, identification of predictors of cognitive recovery from TBI at the acute stage is important in setting realistic expectations of patients' recovery as well as mobilizing appropriate medical and community resources to address patients' needs.

The most commonly reported cognitive dysfunctions in patients with TBI are disturbances in memory and processing speed functions that can persist for years after injury [3–5]. Several premorbid and injury severity factors have been identified to pose a substantial impact on the cognitive sequelae of head injury including age, educational level, presence or absence of neurosurgical intervention, and Glasgow Coma Scale (GCS) scores upon admission [6–9]. Previous studies are toward employing multivariate techniques to predict outcome. The multivariate approaches employed have included variations of multiple regressions, in some cases focusing on a few variables and in others assessing a broader range of predictors. However, conventional multiple regression analysis fails to take measurement errors associated with psychological constructs (i.e., cognition) into account, which

Table 1: Demographic and clinical characteristics of study participants.

	Total sample (n = 30)	Mild TBI (n = 7)	Moderate TBI (n = 9)	Severe TBI (n = 14)
Age (years)	32.8 ± 14.2	42.4 ± 14.3	29.1 ± 13.7	30.4 ± 13.2
Male [n (%)]	24 (80.0)	6 (85.7)	7 (77.8)	11 (78.6)
Education (years)	11.2 ± 2.4	10.7 ± 2.4	11.2 ± 2.7	11.5 ± 2.3
MMSE scores during the study	27.0 ± 3.6	26.3 ± 5.4	27.1 ± 4.1	27.4 ± 2.0
GCS score at admission	9.7 ± 3.4	14.1 ± 0.7	11.0 ± 1.3	6.6 ± 1.5
Time from injury to cognitive testing, mo	17.4 ± 14.2	8.6 ± 2.8	19.5 ± 13.6	20.6 ± 16.6
Duration of unconsciousness				
<8 days	20 (66.7)	7 (100)	7 (77.8)	6 (42.9)
>7 days	13 (33.3)	0 (0)	2 (22.2)	8 (57.1)
Presence of emergent craniotomy [n (%)]	17 (56.7)	0 (0)	5 (55.6)	12 (85.7)
Loss of consciousness [n (%)]	27 (90.0)	5 (71.4)	9 (100)	13 (92.9)
Side of brain damage [n (%)]				
Right brain	15 (50.0)	4 (57.1)	5 (55.6)	6 (42.9)
Left brain	11 (36.7)	1 (14.3)	3 (33.1)	7 (50.0)
Bilateral	4 (13.3)	2 (28.6)	1 (11.1)	1 (7.1)
Types of brain injury [n (%)]				
Closed head injury	21 (70.0)	7 (100)	5 (55.6)	9 (64.3)
Open head injury	9 (30.0)	0 (0)	4 (44.4)	5 (35.7)
Hemorrhagic locations				
Intracerebral	3 (10.0)	1 (14.3)	0 (0)	2 (14.3)
Subarachnoid	7 (23.3)	4 (57.1)	3 (33.3)	0 (0)
Epidural	2 (6.7)	0 (0)	0 (0)	2 (14.3)
Subdural	5 (16.7)	0 (0)	1 (11.1)	4 (28.6)
Multiple sites	13 (43.3)	2 (28.6)	5 (55.6)	6 (42.9)
Presence of countercoup lesion [n (%)]	16 (53.3)	3 (42.9)	6 (66.7)	7 (50.0)

GCS: Glasgow Coma Scale; MMSE: Mini-Mental State Examination; TBI: traumatic brain injury.

can result in estimates of effects that are highly biased due to the influence of error. In addition, multiple regression tests a predictive model with only one dependent variable (i.e., a single test score) which generally does not provide adequate representation of constructs of interest because of imperfect reliability and validity [10].

The structural equation modeling (SEM) is a technique used to specify and estimate models of linear relationships among measured and latent variables [11]. SEM is a superior approach to multiple linear regression analysis as it examines the constructs at the latent level, which provides a more accurate account of the relationships because the relations between theoretically error-free constructs rather than error-prone observed composite variables are estimated [12]. The present study was performed to validate several clinical variables as predictors of memory/processing speed functioning in patients with TBI, using structural equation modeling (SEM).

2. Materials and Methods

2.1. Participants. A total of 30 patients (24 males, 6 females) with mean age of 32.8 years (range: 16–65 years) with TBI were recruited from neurosurgical outpatient clinic at the Kaohsiung Medical University Hospital in this study. Patients were eligible for the study if they were aged between 16 and 65 years to allow applicability of all available norms of the Chinese versions of the Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) [13] and the Wechsler Memory Scale-Third Edition (WMS-III) [14], were 6 months postonset, and had a Mini-Mental State Examination (MMSE) [15] score > 23 and a Glasgow Coma Scale score of 15 at study inclusion. Patients with multitrauma (e.g., extremity fracture, thoracic injury, etc.), evidence of a prior history of focal brain diseases (e.g., stroke, tumor), serious acute medical illness (heart or renal failure), significant motor impairment, or previous history of dementia, psychiatric disease, Parkinson's disease, or drug and alcohol abuse were excluded. Participants' head injury severity was categorized as "mild," "moderate," or "severe" based on the GCS scores at the time of injury. Mild TBI was defined as a loss of consciousness for no greater than 30 minutes and an initial GCS score of 14 to 15, moderate TBI as a GCS score of 9–13, and severe TBI as a GCS score of 3–8 after resuscitation [16]. The demographic and clinical characteristics of the participants are summarized in Table 1.

2.2. Neuropsychological Assessments. The processing speed index of the Chinese version of the Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) [13] and the Chinese version of the Wechsler Memory Scale-Third Edition (WMS-III) [14] were used to assess the cognitive impairments after TBI in the domains of processing speed and memory.

Table 2: Mean WAIS-III and WMS-III index scores, standard deviations, z-tests, and rates of impairment for entire sample.

Indexes	Means	SD	z-test statistic		
			z	P	Power
WAIS-III					
Processing speed	82.0	17.4	−6.57	0.00	1.00
WMS-III					
Auditory immediate	86.5	14.0	−4.93	0.00	1.00
Visual immediate	81.9	19.3	−6.61	0.00	1.00
Immediate memory	82.2	17.1	−6.49	0.00	1.00
Auditory delayed	90.4	18.1	−3.51	0.00	0.88
Visual delayed	84.3	20.4	−5.72	0.00	1.00
Auditory recognition delayed	91.8	14.8	−2.98	0.00	0.74
General memory	85.9	17.3	−5.14	0.00	1.00
Working memory	89.9	22.0	−3.68	0.00	0.91

WAIS-III: Wechsler Adult Intelligence Scale-Third Edition; WMS-III: Wechsler Memory Scale-Third Edition; SD: standard deviation.

The MMSE was used to evaluate general cognitive function in five domains, including orientation to time and place, attention and calculation, registration, short-term recall, and language. The total score ranges from 0 to 30 with a score below 24 indicating cognitive impairment.

2.3. Procedure. Demographic, past history, and injury related data were collected via patient interview and examination of the hospital record. All imaging studies were interpreted by a neurosurgeon (ALK) blinded to the findings of the cognitive examination.

A well-trained research assistant administered and scored the WAIS-III, WMS-III, and MMSE in accordance with the standardized procedures as outlined in the manuals. This study was approved by the Kaohsiung Medical University institutional review board. Written informed consent was obtained from all participants.

2.4. Statistical Analysis. One-sample z test was conducted to assess the differences in Wechsler scales among the TBI patients (mild to moderate group and severe group) and standardization samples of the Wechsler scales. Independent variables included in this analysis were the WAIS-III processing speed index and WMS-III visual immediate and delayed, auditory immediate and delayed, auditory recognition delayed, and working memory indices.

Analysis of Moment Structures (AMOS) software, version 5.0 [17], was used to determine the independent clinical factors associated with memory/processing speed functions. Because the sample size of our study was relatively small, we employed several alternative measures of global fit—the comparative fit index (CFI), nonnormed fit index (NNFI), and root mean square error of approximation (RMSEA). The cut-off values used to assess the adequacy of model fit were determined according to the criteria of MacCallum and Austin [10]. Nonsignificant paths were trimmed from a model described with a series of multiple regression analyses. The fit of the respecified model was tested before being provisionally accepted.

3. Results

3.1. Patient's Performances on the WAIS-III and WMS-III. Four of the nine WAIS-III and WMS-III index scores fell below the normative mean. Of these, visual immediate index had the lowest mean scores. The indexes below the mean were auditory immediate and delayed, auditory recognition delayed, general memory, and working memory. Overall, our result revealed significant impairment in all of the indices, with the processing speed, visual immediate and immediate memory indexes being the most impaired (Table 2).

Multivariate analysis of variance (MANOVA) was performed to test the severity of TBI injury associated with the WAIS-III and WMS-III performances, and the severity of the injury was significantly associated with WAIS-III and WMS-III performances ($P < 0.01$). To clarify the influence on WAIS-III and WMS-III performances, ANOVAs analyses were performed for seven significant factors.

After the ANOVA analysis, it was determined that patients with mild to moderate TBI group scored significantly higher than severe TBI group on auditory immediate ($P = 0.001$) and auditory delayed ($P < 0.001$) indices. Figure 1 illustrates the distribution of index scores for the two TBI groups. The discriminant analysis yielded one canonical discriminant function (Wilks' lambda = 0.44; $\chi^2 = 20.07$; df = 7; $P < 0.01$), accounting for 100% of the discriminating variance. Four variables contributed to the classification of a patient as having mild to moderate TBI, with a standardized discriminant coefficient >0.5, visual immediate (−1.68), visual delayed (1.27), auditory immediate (0.67), and auditory delayed (0.59). The classification results showed that 80% of the original group cases were correctly classified. Group membership was correctly predicted for 78.6% of the patients with severe TBI and 81.3% of the patients with mild to moderate TBI.

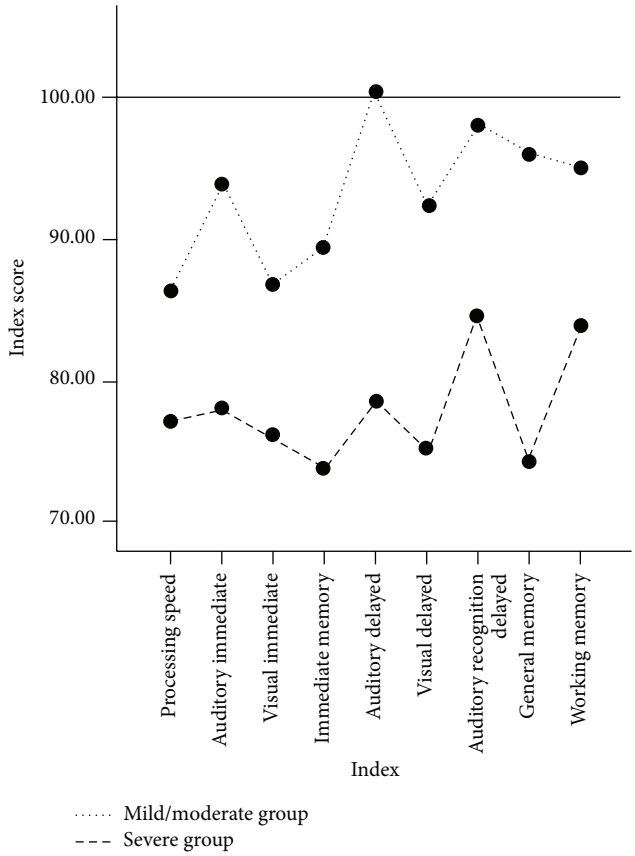

Figure 1: Distribution of index scores for groups with mild to moderate and severe traumatic brain injury.

3.2. Influence of Brain Injury Severity on Memory and Processing Speed. The measurement component of our model was first created, in which the latent variable of memory/processing speed functions was specified by the manifest variables processing speed/working memory, auditory memory, and visual memory. The processing speed/working memory variable was made up of the sum of age-corrected scaled scores for four subtests (letter-number sequencing, spatial span, digit symbol-coding, and symbol search). Working memory is one of the skill components demanded in processing speed. Auditory memory variable was composed of the sum of scaled scores for the immediate and delayed trials of 2 auditory subtests (logical memory and verbal paired associates) as well as the scaled score of the auditory recognition delayed total score. Visual memory variable was composed of the sum of scaled scores for the immediate and delayed trials of 2 visual subtests (faces and family pictures).

Standard goodness-of-fit statistical criteria indicated an excellent fit of the measures to their intended construct (χ^2 = 1.81, P = 0.40, CFI = 1.00, NNFI = 1.01, RMSEA = 0.00). The standardized regression coefficients, which are used to compare the relative importance of the independent variables, for the auditory memory, visual memory, and processing speed/working memory were 0.93, 0.72 and 0.60, respectively. Collectively, these results suggested that our memory/processing speed model was adequately operationalized by successfully identifying variables and latent factor that were clearly related.

Table 3: Intercorrelations among injury severity variables.

	GCS group	Length of coma group	Neurosurgical intervention
GCS group	—		
Length of coma group	−0.50[a]	—	
Neurosurgical intervention	0.67[a]	−0.62[a]	—
Countercoup lesion	0.02	−0.24	−0.01

GCS: Glasgow Coma Scale.
[a]$P < 0.01$.

SEM regression analysis was performed to test the independent clinical factors (countercoup lesion, three GCS groups, length of coma >7 days versus ≤7 days, and neurosurgical intervention) associated with memory/processing speed latent construct. Significant association was demonstrated for the four independent clinical factors and memory/processing speed latent construct (Table 3). However, using the regression component of our model (Model 1), poor association was demonstrated for the four independent clinical factors and the variance in memory/processing speed (χ^2 = 16.97, P = 0.20). These four predictors together accounted for 44% of the variance in memory/processing speed (R^2 = 0.44).

To clarify the influence of four independent clinical factors on memory/processing speed and eliminate the confounding effect from other clinical variables, individual regression coefficients analyses were performed for the four significant factors. After the analyses, only GCS group was significantly associated with memory/processing speed (Table 4). A graphic display of this regression model is depicted in Figure 2. As shown in the figure, correlations among GCS group, length of coma group, and neurosurgical intervention were moderate, ranging from −0.50 to 0.67. After removing three nonsignificant paths, global fit of the respective model (Model 2) (CFI = 1.00, NNFI = 1.01, RMSEA = 0.00) markedly improved over Model 1. The squared multiple correlation of memory/processing speed was 0.40, suggesting that GCS grouping explained 40% of memory/processing speed's variance.

4. Discussion

To the best of our knowledge, predicting memory outcome using a comprehensive measure of various aspects of memory has never been published. Moreover, our study is not only examining predictive model for different memory functions one at a time but also is enabling data analysis and interpretation in a holistic fashion. GCS reflects the integrity of neuronal function of the brain stem and both cerebral cortices and is widely used as a classification measure of the severity of brain injury [18]. Previous studies [7, 19] showed that the GCS score was significant and independent factors for predicting memory/processing speed dysfunction; our multivariate analysis of the patient data also determined that

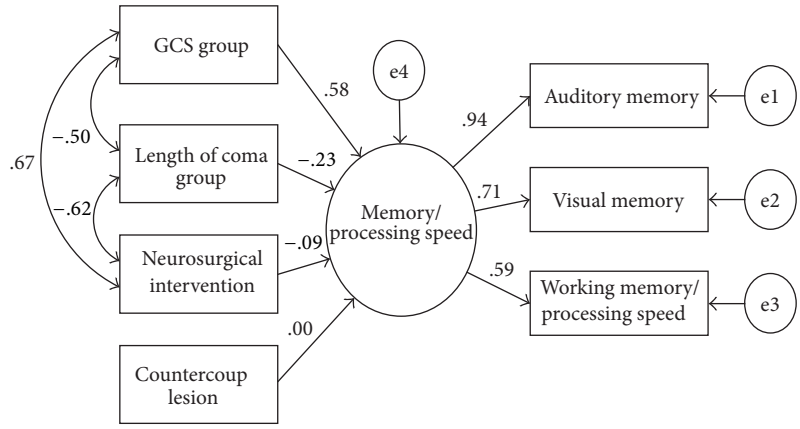

Figure 2: The main structural equation model tested, along with standardized parameter estimates. The rectangles denote observed, endogenous variables or indicators (severity variables and WAIS-III and WMS-III indices), while ellipses to the right of indicators represent measurement errors. The ellipse to the left of the observed variable designates exogenous, latent factor. The values next to the longer single-headed arrows are standardized factor loadings, and the values next to the curved double-headed arrows are correlation coefficients.

Table 4: Parameter estimates for regression models describing effects of injury severity variables on memory/processing speed.

Regression models	Parameter estimates				
	Unstandardized	Standardized	Standard error	Critical ratio	*P* value
Model 1					
Memory/processing speed ← GCS group	93.09	0.50	38.60	2.41	0.02
Memory/processing speed ← length of coma group	−111.15	−0.35	65.89	−1.69	0.09
Memory/processing speed ← surgery	−53.34	−0.18	70.05	−0.76	0.45
Memory/processing speed ← countercoup lesion	35.99	0.12	48.53	0.74	0.46
Model 2					
Memory/processing speed ← GCS group	13.36	0.63	3.23	4.13	0.00

GCS: Glasgow Coma Scale.

only GCS grading was a significant factor. Although the impact of GCS was implied in this study, 21% of patients with severe TBI were mistakenly classified into mild to moderate TBI category, whereas 13% of mild to moderate TBI patients were incorrectly classified into severe TBI category. In other words, only 44% of the variance in memory/processing speed construct was explained by 4 TBI severity variables. Thus, our results indicate that factors other than the severity of coma scale may contribute to the low correspondence to the severity of memory/processing speed impairments. Difficult distinguishing of posttraumatic amnesia and coma induced by sedation, small sample size, and suboptimal pooling of the patients with mild and moderate TBI may contribute to this phenomenon, but further study is necessary to determine this.

Traumatic brain injury patients frequently report neurological and psychological symptoms following acute traumatic brain injury. The etiology of these symptoms remains unknown, partly because the symptoms are not specific to TBI, being found in the other clinical conditions and normal individuals. Even though good screening programs are not available of such disease, adequate widespread information could lower the GCS at diagnosis. Length of coma is a commonly reported risk factor for neuropsychological outcome [10, 15, 20]. However, in the present study it was not found to be a significant risk factor. A reason for these discrepancies may be the narrow range of length of coma in our TBI sample, ranging from 0 to 15 days with the exception of one case whose length of coma was 42 days. Another possible reason is that the trauma patterns of these patients are less infiltrative and initially involve the superficial brain tissues, causing conscious disturbance; thus, no neuropsychological dysfunction would be evident on presentation.

Surgical intervention is considered by many investigators to be the best treatment of choice for TBI. Recent retrospective results show that modern anti-increase intracranial pressure agents, combined with good drainage, appear to be associated with a better outcome, including neuropsychological dysfunctions. However, our result demonstrated that surgical intervention had no positive impact on memory/processing speed function in patients with brain injury. Short follow-up time of these patients, associated with the limited number of patients, could explain this observation.

To date, few studies have been published concerning the relationship between presence of a countercoup lesion and memory/processing speed impairments. Ommaya [21] demonstrated that countercoup was associated with the memory/processing speed impairments and served as an

unfavorable prognostic factor. However, it remains controversial whether countercoup brain injury could be a significant risk factor for memory/processing speed impairments. In this study, the proportion of patients with countercoup was comparatively low; therefore, it would be absolutely necessary to include a larger number of patients who were accurately diagnosed by CT scan and then followed for a longer period.

To our knowledge, few investigators have analyzed the issues about the differences in visual versus verbal memory impairments as a result of head trauma, and their conclusions remain controversial; some studies reported that visual memory (immediate and delayed) indices were the most impaired in TBI patients [22, 23], whereas others that cited auditory memory indices were the most impaired in TBI sample. For example, Axelrod et al. [24] concluded that WMS-III visual indexes display greater sensitivity to brain dysfunction than the auditory indexes. Because of the small sample size of this study, it is not clear whether the hemispheric laterality has a more detrimental effect on modality-specific (visual versus verbal) memory dysfunctions than GCS group, or vice versa.

In an earlier work by Hoskison et al. [25] prefrontal injury was associated with memory and processing speed dysfunction. In this important study, the authors utilized a preclinical model involving cortical impact injury of rats. Unfortunately, in the clinical setting, traumatic brain injury is seldom as uniform or controlled. In the current study, we did include patients with mild, moderate, and severe TBI. Overall, 53.3% of patients in this study exhibited a countercoup injury. As such, the majority of patients had injury involving two different lobes. In addition, intervening diffuse axonal injury would likely have been observed on MRI sequences designed to evaluate such injury. We believe the current patient population represents a clinically more realistic albeit heterogeneous TBI population. The current study's relationship between GCS and memory/processing speed outcome validates the findings of prior preclinical studies but does so in a clinically representative TBI patient population. Future studies could focus on a more homogenous TBI patient population for evaluation. Also, further studies with larger numbers of TBI patients who with either left, right, or bilateral hemispheric lesions are needed to discern the relative influences of these two variables. Another interesting theme to emerge from our study is the discrepancy between immediate and delayed memory scores. Our study demonstrates a more severe impairment of delayed than immediate memory. This result conforms to those of other studies [23, 26] and may imply that TBI patients generally have retrieval deficit.

The countercoup lesion describes the damage that occurs away from the impact area, suggesting that a shock wave traverses the skull [26]. This secondary reaction can often cause more damage than the initial impact, as shearing of internal tissues and blood vessels leads to further bruising, bleeding, and swelling to the brain. Nevertheless, the effect of this severity variable has not been fully explored in relation to cognitive or functional outcome in patients with TBI.

The present study had several limitations. First, a selection bias could have been present because the study population was small. Second, the present study was retrospective study, and the follow-up time for patients still was short. Third, we could not completely differentiate between duration of unconsciousness due to injury compared to sedation induced coma. Taken together, a multi-institutional prospective study with a large number of patients would be required to confirm the present finding. Furthermore, basic biologic research would be needed to explain the contradictory effects of severity of brain injury on the risk and prognosis of neuropsychological dysfunctions.

In conclusion, our study demonstrated that GCS grading correlates significantly to memory/processing speed outcome but moderately corresponds to severity of memory/processing speed impairments. That is, 21% of severe TBI patients scored in the same range as patients with mild to moderate TBI on memory and processing speed tasks. Our predictive model offers a good vantage point from which similar models can be constructed to cater for the specific nature of outcome measures of interest in future studies.

Conflict of Interests

The authors have no conflict of interests or financial disclosures.

Acknowledgment

No part of this paper has been published/presented elsewhere.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 506458, 6 pages
http://dx.doi.org/10.1155/2014/506458

Research Article

Differential Hypermethylation of Death-Associated Protein Kinase Promoter in Central Neurocytoma and Oligodendroglioma

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Received 10 February 2014; Accepted 16 March 2014; Published 27 April 2014

Academic Editor: Hung-Chen Wang

Background. Central neurocytoma and oligodendroglioma are rare tumors of the central nervous system. However, diagnosis between these two types of tumors is challenging due to their many cytological and histological similarities. Death-associated protein kinase (DAPK) is a calcium/calmodulin-regulated serine/threonine protein kinase involved in many apoptosis pathways, and repressed expression of DAPK by promoter hypermethylation has been found in a variety of human cancers. The purpose of this study was to assess DAPK protein expression and promoter hypermethylation in central neurocytoma and oligodendroglioma. *Method*. Central neurocytoma and oligodendroglioma samples were obtained from age- and sex-matched patients. DAPK protein expression was performed using immunohistochemical assays in formalin-fixed, paraffin-embedded sections. DAPK promoter hypermethylation was carried out using bisulfite-modified genomic DNA in methylation-specific PCR followed by separation in agarose gels. *Findings*. A statistically significant difference ($P = 0.021$) in DAPK promoter hypermethylation between central neurocytoma (76.9%) and oligodendroglioma (20%) was observed. High levels of DAPK protein expression were generally found in oligodendroglioma (90%), compared with 38.5% in central neurocytoma ($P = 0.054$; not statistically significant). There was an inverse correlation between DAPK protein expression and DAPK promoter hypermethylation in the cohort of 23 patients ($P = 0.002$). *Conclusions*. The results show that DAPK promoter hypermethylation and repressed expression of DAPK protein were more common in central neurocytoma than in oligodendroglioma. Thus, DAPK promoter hypermethylation could be useful for differential diagnosis between these two types of tumors, whereas DAPK protein expression might be less predictive. The role of DAPK promoter hypermethylation in the pathogenesis of central neurocytoma warrants further study.

1. Introduction

Central neurocytomas are rare tumors of the central nervous system, comprising only 0.1–0.5% of all brain neoplasms [1, 2]. Generally, central neurocytoma affects young adults with the tumors most frequently localizing in the supratentorial ventricular system and demonstrating calcification on computed tomography (CT) images [3, 4] although various cases of extraventricular neurocytoma have also been reported [5–9]. Despite a substantial advancement in the diagnosis and management [10–12] since its initial description reported in 1982 [13], central neurocytoma is still often confused with other tumors of the central nervous system, especially oligodendrogliomas.

Oligodendrogliomas occur primarily in the cortex and white mater of the cerebral hemispheres of adults in their

fourth and fifth decades of life, while projection into the ventricles has also been found [14]. Histologically, central neurocytomas and oligodendrogliomas are characterized by sheets of monotonous cells with round nuclei surrounded by clear cytoplasm [15]. Thus, differentiation between oligodendroglioma and neurocytoma is challenging [16], and a definite diagnosis for these two types of tumors requires other complementary evaluations. Genetically, loss of heterozygosity on chromosomes 1p and 19q has been unequivocally found in the majority of oligodendroglioma patients [2, 17], whereas such codeletion in subjects with neurocytomas is still a matter of debate. Fujisawa et al. found no allelic loss on chromosomes 1p and 19q in central neurocytomas [17], while Rodriguez et al. and Tong et al. reported that 1p19q loss was seen in the majority of patients with extraventricular and central neurocytomas, respectively, although common regions of deletion could not be identified [18, 19]. A more convincing differentiation between central neurocytomas and oligodendrogliomas has been provided by immunohistochemical studies. Expression of Olig2 is seen in all oligodendrogliomas, whereas none or little expression of this transcription factor in central neurocytoma has been found [20, 21]. On the other hand, expression of the neuronal marker synaptophysin is observed in nearly all patients with central neurocytomas but rarely found in oligodendrogliomas [3, 11, 22]. Nevertheless, differentiation between central neurocytomas and oligodendrogliomas based on biochemical studies has not received much attention.

Oligodendroglioma cells can actively induce neuronal damage by releasing molecules able to inhibit neurite sprouting and to eventually cause apoptotic neuronal death [23, 24]. As for central neurocytoma, there is still no such published report aiming for the involving cell death pathway. The important roles of protein kinases in various cancers have long been recognized [25]. Death-associated protein kinase (DAPK) is a calcium/calmodulin-regulated serine/threonine protein kinase involved in many apoptotic pathways [26, 27]. Repressed expression of DAPK by promoter hypermethylation has been found in a variety of human cancers, such as colorectal carcinoma [28], soft tissue leiomyosarcoma [29], bladder cancer [30], and ulcerative colitis-associated carcinoma [31], to name a few. However, there has been no publication concerning the role of DAPK in central neurocytoma or oligodendroglioma. The purpose of this study was to assess DAPK protein expression and promoter hypermethylation in central neurocytoma and oligodendroglioma.

2. Materials and Methods

2.1. Patients. This study was approved by the Kaohsiung Medical University Hospital Review Board. Central neurocytomas and oligodendrogliomas were obtained from age- and sex-matched patients (ranged from 15 to 47 yr; 8 males and 5 females in each group) treated at the Kaohsiung Medical University Hospital. The specimens were diagnosed by H&E stain under light microscopy and immunostaining of synaptophysin and glial fibrillary acidic protein. All the 13 central neurocytomas are located intraventrically. All the 10 oligodendrogliomas are low grade tumors. The diagnosis was confirmed by physicians. Consents were received from all patients. Each tissue was divided into two equal parts, one for DNA extraction and the other for immunohistochemical staining.

2.2. DNA Extraction and Bisulfite Modification. Tissue samples from central neurocytoma and oligodendroglioma patients were digested with proteinase K at 56°C overnight, and genomic DNA was isolated by phenol-chloroform extraction using a commercially available kit according to the manufacturer's procedures. Approximately 2 μg of tumor DNA was further modified by sodium bisulfite to convert unmethylated cytosines to uracils, and the modified DNA was eluted into buffer EB (Qiagen, Hilden, Germany). This bisulfite conversion and clean-up of genomic DNA were performed using the EpiTect Bisulfite kit (Qiagen). Purified DNA was used immediately as a template for methylation-specific polymerase chain reaction (PCR) described below or stored at −70°C until use.

2.3. Methylation-Specific PCR. Approximately 0.2 μg of modified DNA was added to a PCR solution containing 1x PCR buffer, 1.25 mM $MgCl_2$, 0.25 mM dNTP, 0.5 μM PCR primers, and 1.25 U of GoTaq DNA polymerase (Invitrogen) in a total volume of 25 μL. The forward and reverse primer sequences used for methylated DNA were 5′-GGATAGTCGGATCGAGTTAACGTC-3′ and 5′-CCCTCCCAAACGCCGA-3′, respectively, whereas the forward and reverse primer sequences used for unmethylated DNA were 5′-GGAGGATAGTTGGATTGAGTTAATGTT-3′ and 5′-CAAATCCCTCCCAAACACCAA-3′, respectively [32]. The CpGenome Universal Methylated DNA (Chemicon Int.) was used as positive control, and water was utilized as negative control. Amplification was carried out in a 2720 Thermal Cycler (ABI) at 95°C for 10 min followed by 35 cycles at 95°C for 4 s, 60°C for 60 s, and 72°C for 60 s. Afterwards, a 10 min extension was allowed at 72°C. The PCR products were then separated on 2% agarose gels and visualized after staining with ethidium bromide. Hypermethylation of DAPK genes was defined when DNA bands were detected in the agarose gel using PCR products generated from methylated primers or from both unmethylated and methylated primers. On the other hand, nonmethylation of DAPK genes was defined only when DNA bands were visible using PCR products obtained from unmethylated primers.

2.4. Immunohistochemical Staining. For immunohistochemical staining, tissues were fixed in formalin, embedded in paraffin, and cut into 5 μm sections. They were then stained with hematoxylin and eosin and were evaluated to determine the extent of tumor cells presented in the sections using a light microscope. Subsequently, the samples were washed with PBS and incubated with anti-DAPK antibody (Santa Cruz) at a 1 : 100 dilution for 1 hr at room temperature. Afterwards, slides were washed for 30 min in PBS and incubated with secondary antibody (Dako Code K5007). Specimens were again washed with PBS, incubated with peroxidase-labeled streptavidin (DAB; Dako Code K3468) for measurement of the intensity of immunoreactivity.

Each section was given two independent scores, namely, the extent of tumor cells in the sample and the intensity of immunoreactivity, by an investigator blinded to the experiment. A score of 0 (zero) was assigned to a section if the extent of tumor cell was <1%, whereas scores of 1, 2, and 3 were given to sections with 1%–10%, 11%–50%, and >50% tumor cells, respectively. Likewise, a section received a score of 0 (zero) when the intensity of the slide was similar to the background level. Intensity scores of 1, 2, and 3 were assigned to sections with weak, moderate, and strong intensity of immunoreactivity, respectively. The values of these two independent parameters were multiplied to generate the final score for each section (ranging from 0 to 9) according to a published procedure [29]. A final score of <4 in a sample was considered as low DAPK expression, while a score of ≥4 was regarded as high DAPK expression.

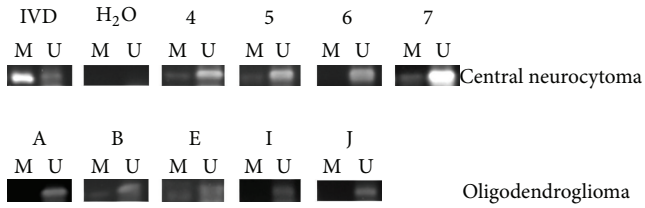
2.5. Statistical Analysis. All results were expressed as mean ± SEM. An analysis of variance (ANOVA) followed by Fisher's exact test was performed to determine statistical significance between two groups. A P value of <0.05 was considered statistically significant.

3. Results

3.1. DAPK Promoter Hypermethylation. DAPK promoter hypermethylation was observed in both central neurocytoma and oligodendroglioma (Figure 1(a)). Interestingly, 76.9% of central neurocytoma samples displayed DAPK promoter hypermethylation, while only 20% of oligodendrogliomas showed such an effect (Figure 1(b)). The difference was found to be statistically significant ($P = 0.023$).

3.2. DAPK Protein Expression. As mentioned in the methods, DAPK protein expression was assessed by both the extent of tumor cells and the intensity of immunoreactivity [29]. These two parameters were graded numerically (0–3), and their product was used to determine the level of protein expression. Representative slides from negative, low, and high DAPK protein expression are shown in Figure 2. Table 1 summarizes the results of DAPK protein expression in patients with central neurocytoma and oligodendroglioma. A high level of DAPK protein expression was common in oligodendroglioma and was seen in 90% of patients. In contrast, only 38.5% of samples from central neurocytoma displayed high levels of DAPK protein expression. However, this difference observed between central neurocytoma and oligodendroglioma was not statistically significant ($P = 0.054$).

3.3. Correlation between DAPK Promoter Hypermethylation and Protein Expression. An effort was made to correlate DAPK promoter hypermethylation and DAPK protein expression in all of the central neurocytoma and oligodendroglioma samples evaluated. It was found that samples with low levels of DAPK protein expression always exhibited high levels of DAPK promoter hypermethylation. This was seen in 9 out of 9 cases (Table 2) from 1 oligodendroglioma and 8 central neurocytoma samples. In contrast, in the majority of samples with high levels of DAPK protein expression, unmethylated DAPK promoter was detected (11 out of 14 cases or 78.6% from 9 oligodendrogliomas and 5 central neurocytomas) (Table 2). These results showed that there was an inverse correlation between DAPK protein expression and DAPK promoter hypermethylation in the cohort of 23 patients ($P = 0.002$).



(a)

Type of tumor	Methylation status		P^a
	–	+	
Central neurocytoma ($n = 13$)	3	10	0.021
Oligodendroglioma ($n = 10$)	8	2	

[a] P was determined by Fisher's exact test

(b)

Figure 1: DAPK promoter hypermethylation in central neurocytoma and oligodendroglioma. Genomic DNA from patients with central neurocytoma or oligodendroglioma were extracted and analyzed for DAPK promoter hypermethylation as described in the methods. The upper panel shows representative results of the methylation-specific PCR products analyzed in agarose gels. IVD, *in vitro* methylated DNA used as positive control; H_2O, negative control; M, methylated DAPK promoter gene; U, unmethylated DAPK promoter; and underlined numbers and letters, codes for patients. The methylation status of DAPK promoter in all patients is summarized in the lower panel. Eighty percent of central neurocytoma samples showed DAPK promoter hypermethylation. The result is statistically different from that in oligodendrogliomas, where only 20% of samples had methylated DAPK promoter.

Table 1: Summary of the results of DAPK protein expression in central neurocytoma and oligodendroglioma.

Type of tumor	DAPK protein expression		P
	Low	High	
Central neurocytoma ($n = 13$)	8	5	0.054
Oligodendroglioma ($n = 10$)	1	9	

DAPK protein expression in the formalin-fixed, paraffin-embedded sections from central neurocytoma and oligodendroglioma was determined by two parameters, that is, the extent of tumor cells and the intensity of immunoreactivity. A numerical grade (0–3) from each parameter was assigned to every section, and the product of the two numbers was used to assess the level of DAPK protein expression as described in Section 2. A final score of <4 in a sample was considered as low DAPK expression, while a score of ≥4 was regarded as high DAPK expression. No statistically significant difference was found in the distribution of DAPK protein expression between central neurocytoma and oligodendroglioma.

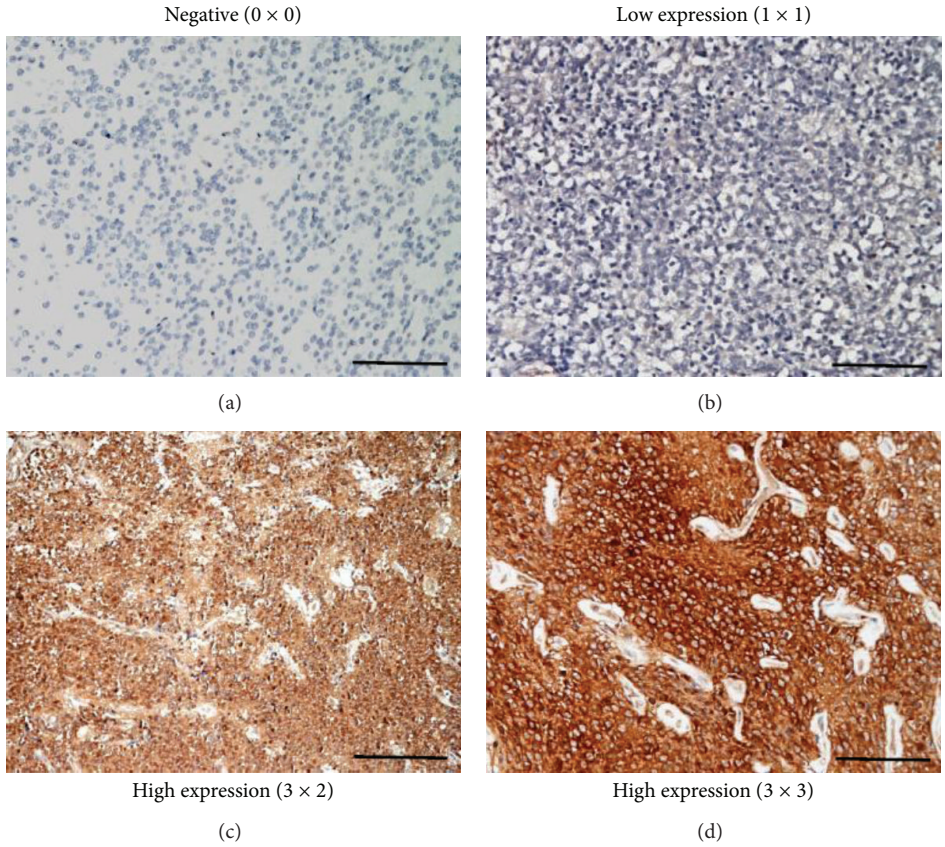


Figure 2: DAPK protein expression. The expression of DAPK protein in central neurocytoma and oligodendroglioma was assessed by both the extent of tumor cells and the intensity of immunoreactivity. These two parameters were graded numerically (0–3) as described in the methods. Representative slides from negative, low, and high DAPK protein expression are shown. Numbers (3 × 2, e.g.) in each parenthesis indicate assigned grade for extent of tumor cells × assigned grade for intensity of immunoreactivity. Scale bar, 100 μm.

Table 2: Correlation between DAPK protein expression and DAPK promoter hypermethylation in combined central neurocytoma and oligodendroglioma samples.

DAPK protein expression	Methylation status		P
	−	+	
Low (n = 9)	0	9	0.002
High (n = 14)	11	3	

DAPK protein expression and DAPK promotor hypermethylation were determined as described in Section 2. Results obtained from both the central neurocytoma and oligodendroglioma samples were combined in this analysis. In 9 out of 9 cases (100%), low levels of DAPK protein expression exhibited high levels of DAPK promoter hypermethylation. In contrast, in samples with high levels of DAPK protein expression, only 21.4% (3 out of 14 cases) showed high levels of DAPK promoter hypermethylation. These results showed the DAPK protein expression and DAPK promoter hypermethylation correlated inversely in the cohort of 23 patients (P = 0.002).

4. Discussion

Central neurocytoma and oligodendroglioma are rare tumors of the central nervous system. However, diagnosis between these two types of tumors is challenging due to their many cytological and histological similarities [15, 16, 33]. Subsequent genetic discoveries on the loss of heterozygosity in chromosomes 1p and 19q from the majority of oligodendroglioma patients [2, 17] as well as immunohistochemical studies showing differential expression of Olig2 [20, 21] and synaptophysin [3, 11, 22] in oligodendroglioma and central neurocytoma, respectively, have greatly helped the diagnosis. The most significant finding reported herein is the differentiation between central neurocytomas and oligodendrogliomas using biochemical methods. DAPK promoter hypermethylation was found in 80% of central neurocytomas but in only 20% of oligodendrogliomas (Figure 1). Although a high level of DAPK protein expression was common in oligodendroglioma (90%), this value is not significantly different from the 40% found in central neurocytoma. Thus, DAPK promoter hypermethylation could be useful for differential diagnosis between these two types of tumors, whereas DAPK protein expression might be less predictive.

Upon analysis of data obtained from both central neurocytoma and oligodendroglioma samples, there was an inverse correlation between DAPK protein expression and DAPK promoter hypermethylation (Table 2). These results are consistent with the findings from other studies showing DAPK promoter hypermethylation leads to a concomitant

loss of DAPK protein expression in various cancers [26, 34]. Further analysis reveals that the repressed expression of DAPK protein derived mainly from central neurocytomas (8 out of 9 cases) (Table 1). The results suggest that DAPK promoter hypermethylation and repressed expression of DAPK protein are more common in central neurocytoma than in oligodendroglioma. It implies that DAPK promoter hypermethylation may play a role in the pathogenesis of central neurocytoma. Therefore, it is envisaged that agents capable of reversing this hypermethylation process may be novel drugs for the treatment of central neurocytoma.

Besides being localized centrally, neurocytomas have also been found extraventricularly [5–9]. It would be of interest to investigate whether or not DAPK promoter hypermethylation and reduced DAPK protein expression also apply to neurocytomas of these origins. A positive outcome would allow a more general statement on the differentiation between neurocytomas and oligodendrogliomas biochemically. Further studies are needed to clarify this matter.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 275178, 9 pages
http://dx.doi.org/10.1155/2014/275178

Review Article

Immune Responses in Parkinson's Disease: Interplay between Central and Peripheral Immune Systems

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Received 13 February 2014; Accepted 16 March 2014; Published 13 April 2014

Academic Editor: Cheng-Hsien Lu

The etiology of Parkinson's disease (PD) is complex and most likely involves numerous environmental and heritable risk factors. Recent studies establish that central and peripheral inflammation occurs in the prodromal stage of the disease and sustains disease progression. Aging, heritable risk factors, or environmental exposures may contribute to the initiation of central or peripheral inflammation. One emerging hypothesis is that inflammation plays a critical role in PD neuropathology. Increasing evidence suggest that activation of the peripheral immune system exacerbates the discordant central inflammatory response and synergistically drives neurodegeneration. We provide an overview of current knowledge on the temporal profile of central and peripheral immune responses in PD and discuss the potential synergistic effects of the central and peripheral inflammation in disease development. The understanding of the nature of the chronic inflammation in disease progression and the possible risk factors that contribute to altered central and peripheral immune responses will offer mechanistic insights into PD etiology and pathology and benefit the development of effective tailored therapeutics for human PD.

1. Introduction

Parkinson's disease (PD) is a progressive, age-related, neurodegenerative disorder. Clinically, it is characterized by motor symptoms, such as rigidity, bradykinesia, postural instability, gait disorder, and tremor [1]. However, nonmotor symptoms, such as hyposmia, gastrointestinal abnormalities, and autonomic dysfunction are increasingly accepted as integral parts of PD clinical manifestations and often precede the classical motor symptoms [2]. Pathologically, selective neurodegeneration in the nigrostriatal circuit, presence of dysregulated immune activation, and the occurrence of Lewy bodies (LB) in central and peripheral nervous systems are observed in PD [3]. The cause of nigral neurodegeneration in PD and its underlying mechanisms remain elusive; however, involvement of inflammatory events has been postulated to contribute to neuronal loss. Indeed, inflammation has been linked to many other age-related chronic neurodegenerative diseases, including Alzheimer's disease [4], amyotrophic lateral sclerosis [5, 6] and Huntington's disease [7, 8]. Triggering factors of inflammation may be dysregulation of inflammatory pathways (e.g., immune alteration associated with aging or genetic vulnerability), pathogens (e.g., bacterial or viral infection), environmental toxins (e.g., pesticides), and protein aggregates (e.g., α-synuclein (α-Syn)).

There has been considerable debate in the field as to whether inflammation is a driving force in neurodegeneration or simply represents a response to neuronal death. Here, we describe the temporal profile of altered immune responses, including central and peripheral inflammation in the disease progression, present evidence indicating the interaction between the central, and peripheral inflammation in both sporadic and familial PD, and discuss recent data supporting the key role of inflammatory responses in the initiation and progression of disease pathology.

2. Central Inflammation in PD

Microglia-associated central inflammation is a pathological hallmark of PD. Initial evidence of the involvement of

inflammation in the progression of PD stems from a postmortem study over twenty years ago, which demonstrated the presence of activated microglia in the substantia nigra pars compacta (SNpc) of a PD patient [9]. Since then, an abundance of clinical and animal studies supports the role of activated microglia and increased levels of inflammatory mediators such as cytokines, chemokines, and ROS in the pathology of PD [10–14].

The central inflammatory process (activation/proliferation of microglia/ astrocyte and secretion of proinflammatory cytokines and free radicals) could be an event secondary to the neurodegenerative process and, in turn, exacerbates the progression of cell death. However, under certain circumstances, inflammation could be a primary event that leads to the neurodegenerative process. A clinical study that uses positron emission tomography to study activated microglia in the brain of idiopathic PD patients shows the absence of significant longitudinal changes in microglia activation over time suggesting that brain inflammation occurs early in PD process [15]. We observe early inflammation in transgenic mice with overexpression or mutation (A53T and A30P) of α-Syn, characterized by microglial activation and increased expression in proinflammatory cytokines, prior to dopaminergic neuronal cell death and motor disorder [16, 17]. Another study using intranigral injection of lipopolysaccharide (LPS) on the dopaminergic system of the rat shows that LPS initially induces microglial activation in a short time (within 2 days) and subsequently causes a progressive degeneration of the dopaminergic system in the long term (up to one year after LPS injection) [18], suggesting that microglia-mediated inflammation underlies the neuronal cell death in the SN.

Activated microglia exert their neurotoxic effects by releasing proinflammatory cytokines such as TNFα, IL-1β, IL-6, and IFNγ and free radicals including ROS and NO, as well as inflammatory mediators such as prostaglandins E2 (PGE2), leading to nigral cell damage and death. Enhanced expression of TNFα, IL-1β, IL-6, and INFγ has been shown in basal ganglia as well as cerebrospinal fluid of PD patients [19–21]. TNFα and IL-1β are robust activators of NF-κB and contribute to neuronal cell death by triggering apoptotic transduction pathway [22–24]. Additionally, TNFα, IL-1β, and INFγ induce potent activation of iNOS [25], presumably mediated by a low-affinity IgE receptor CD23, which is expressed exclusively on glial cells in the SN of PD patients [26]. iNOS is responsible for NO production, contributing to neuronal toxicity [27]. Collectively, cytokine/CD23-dependent activation of iNOS in microglia may be involved in the cascade of events leading to dopaminergic neuronal degeneration [28]. Moreover, TNFα and IL-1β can upregulate COX2, resulting in the production of PGE2 and induction of an intraneuronal toxic effect directly on dopaminergic neurons [29–31]. Given that dopaminergic neurons in the SN are relatively sensitive to "stress" [32] and that there is a large population of microglia in the SN in comparison to other CNS regions [33], inflammation is a crucial step in the pathogenic cascade leading to neurodegeneration.

Central inflammation pertains to all forms of PD, both genetic and idiopathic. Aging is a major factor for both genetic and idiopathic PD. Recent evidence suggests that aged brain resides in a chronic state of neuroinflammation, characterized by increased reactivity upon immune stimulation and low-grade production and central cytokines [18, 34–36]. One working hypothesis suggests that this hyperreactivity is due to priming of brain microglial cells. Microglia in aged brains become "primed," exhibiting increased expression of MHC class II, scavenger receptor CD68 [37, 38], CD11b, and CD11c integrins [34, 39], Toll-like receptor TLR4 [40], and costimulatory molecule CD86 [41] and being capable of adopting a potent neurotoxic and proinflammatory phenotype [42]. Subsequent to peripheral innate immune stimulation, microglia in aged brains respond with an exaggerated inflammatory response compared to younger cohort [37, 43]. The precise priming stimulus of aging has yet to be identified. Some studies have suggested that IFN-γ concentrations are increased in the aged brain [44], which may implicate IFN-γ as a candidate molecule for microglial priming. To this end, the treatment of human microglial cell cultures with IFN-γ results in microglial activation, as evidenced by increased production of reactive oxygen species [45]. This scheme resembles the activation process of peripheral macrophages, wherein the classical macrophage activating factor, produced by stimulated Th1 lymphocytes and NK cells, is IFN-γ [46]. Other studies indicate that aging is associated with a reduction in anti-inflammatory cytokines including IL-10 and IL-4 [47, 48].

Astrocytes play equally important immunomodulatory role in maintaining CNS homeostasis. Altered astrocytic function is now recognized as a primary contributing factor to an increasing number of neurological diseases. With age, astrocytes have a more inflammatory profile. For instance, there is increased expression of astrocytic glial fibrillary acidic protein (GFAP) in the brain of aged rodents and humans [49, 50]. In addition, vimentin, an intermediate filament protein, also increases with aging in humans [51]. The age-related increases in GFAP and vimentin are similar to the activated astrocytic profile associated with inflammation and traumatic CNS injury [52]. There are many potential consequences of a more inflammatory astrocyte in the aged brain. First, astrocytes communicate directly with microglia, so an inflammatory astrocyte phenotype may elevate the amplitude and duration of microglia-mediated neuroinflammation [53]. Second, astrocytes play the most substantial role in maintaining an intact blood-brain barrier (BBB) [54]. Age-related changes in astrocytes can affect BBB permeability, especially under inflammatory conditions and neurodegenerative diseases [5, 55]. Third, astrocytes secrete chemokine monocyte chemoattractant protein-1 (MCP-1) which is a key chemokine involved in the recruitment of peripheral monocytes [56]. In summary, recent studies have shed some light on astrocyte-mediated neuroinflammation in neurodegeneration, and future research on astrocyte pathophysiology is expected to provide new perspectives on neurodegeneration and potential therapeutic strategies.

Several PD-linked genetic mutations are associated with increased glial activation in mediating chronic PD progression. A genetic dysfunction of α-Syn coupled with increased neuroinflammation can potentiate each other, driving chronic progression of neurodegeneration. Increasing

experimental findings point to clear roles for α-Syn in the central inflammation in PD: (I) overexpression or mutation of α-Syn in the dopaminergic neurons leads to neuroinflammatory responses in α-Syn transgenic animals [16, 17]; (II) direct intranigral injection of α-Syn results in the production of proinflammatory cytokines and microglial activation in mouse brain [57]; (III) in the MPTP mouse model, nitrated form of α-Syn (N-α-Syn) is shown to drain into cervical lymph nodes and to elicit an antigen-specific T cell response. N-α-Syn specific effector T cells exacerbate microglial activation and DA neurodegeneration [58, 59]. Collectively α-Syn in SN DA neurons may elicit a self-propelling cycle of microglial activation and overproduction of inflammatory mediators in SN, leading to PD-associated dysfunction and spreading to neighboring neurons [60]. The mechanisms by which α-Syn initiates central inflammation remain to be determined. Recent data support the hypothesis that α-Syn is released from neurons into the interstitial space where the protein would be available to directly stimulate microglial activation via the scavenger receptor CD36 and the prostaglandin E2 receptor subtype 2 (EP2) [16, 17, 61, 62].

Mutations in Leucine-rich repeat kinase 2 (LRRK2) contribute to both idiopathic and familial forms of PD. LRRK2 expression is readily detected in multiple immune cells including B-lymphocytes, monocytes, dendritic cells, and microglial [63, 64], which suggests a potential role for LRRK2 in the immune system. PD-linked LRRK2 mutation (R1441G) increases proinflammatory cytokine release from activated primary microglial cells [65]. Furthermore, LRRK2 R1441G stabilizes cyclooxygenase 2 RNA and increases inflammatory response in idiopathic and genetic PD fibroblast [66]. In addition, LRRK2 in microglia plays a key role in the phagocytosis of neuronal elements [67]. In contrast, LRRK2 deficiency attenuates LPS-induced mRNA and/or protein expression of inducible nitric oxide synthase, TNF-α, IL-1β, and IL-6 [68]. Taken together, these results support LRRK2 as a positive regulator of inflammation in microglia, and disease-related LRRK2 mutations may shift the microenvironment of the brain to favor neuroinflammation.

Loss-of-function mutations in the E3 ligase Parkin give rise to a rare form or autosomal recessive parkinsonism [69]. Although mice deficient in Parkin do not display nigral degeneration, chronic administration of low-dose LPS trigger very similar neuroinflammatory and oxidative stress responses in the SNpc of both WT and Parkin-deficient mice; only the latter develops delayed and selective degeneration of DA neurons in SNpc but not in VTA [70]. These findings suggest that Parkin loss changes sensitivity to specific inflammatory mediators and increases vulnerability to inflammation-induced degeneration. Additional studies will need to establish whether and how Parkin-deficient glia is "primed" and respond aberrantly to exacerbate neurodegeneration.

The altered level or activity of certain gene products in CNS cells may contribute to central inflammatory response as well, although the mutation of these genes has not been identified in familial PD. For instance, the GPEX consortium reports a PD meta-analysis of gene expression data indicating that the mitochondrial master regulator, peroxisome proliferator-activated receptor gamma coactivator-1 alpha, PGC-1α, and related bioenergetic genes, including those encoding NADH ubiquinone oxidase (Complex I), succinate dehydrogenase (Complex II), cytochrome C oxidase (Complex IV), and ATP synthase (Complex V), are down-regulated in affected brain tissue from patients with both symptomatic and subclinical PD [71]. This observation suggests that PGC-1α is emerging as a molecular link between mitochondrial dysfunction and transcriptional dysregulation in PD. *In vivo* studies have shown that PGC-1α knockouts are much more sensitive to the neurodegenerative effects of MPTP, and increased PGC-1α levels protect neurons from oxidative stress *in vitro*, α-Syn-mediated cell death *in vitro*, and MPTP-mediated neuronal degeneration *in vivo* [72, 73]. Interestingly, a long-term study using muscle-specific PGC-1α knockout mice demonstrates that loss of muscle PGC-1α causes age-dependent low-grade, chronic inflammation in white adipose and liver tissue [74]. Whether PGC-1α deficiency in CNS cells will equally cause age-dependent low-grade inflammation in the brain remains unknown but warrant further investigation. Understanding the role of PGC-1α in the central as well as peripheral immune responses will provide new perspective for PD treatment.

3. Peripheral Inflammation in PD

The link between peripheral inflammation and neurodegeneration in PD patients has been revealed in several clinical reports. The clinical evidence for systemic inflammation in PD includes the presence of elevated serum levels of TNFα and TNFα receptor 1 in PD patients compared to control subjects [14, 75, 76]. Also, elevated plasma concentrations of IL-6 correlate with the increased risk of PD [77]. In addition, gut inflammation occurs in PD patients [78–80]. PD patients often suffer infectious disease, and the main causes of death are pneumonia and respiratory infections [81–84]. Furthermore, cytotoxic T lymphocyte (CD4+ and CD8+) has been described to infiltrate the SN of PD patients [85, 86]. The influx of these peripheral cells into the brain parenchyma could indicate a BBB dysfunction in PD patients [87, 88].

The association of peripheral inflammation and PD pathogenesis is further demonstrated in PD animal models. Pregnant rats exposed to intraperitoneal (i.p.) injection of LPS resulted in a decreased number of dopaminergic neurons in the pups when compared to nonexposed controls [89]. Similarly, rat fetuses exposed to LPS are more susceptible to 6-OHDA in adulthood [90, 91]. In adult animals, there is also data that strongly suggests the role of peripheral inflammation in the ongoing PD model. Animals with an increased peripheral inflammatory response after bacterial LPS injection are associated with central dopaminergic hypoactivity [92]. Peripheral inflammation induced by ulcerative colitis worsens the effects induced by intranigral LPS, including dopaminergic neuronal cell loss, microglial inflammation, and alteration in BBB permeability [93]. All previous data indicate a close relationship between the peripheral immune system and the central dopaminergic system.

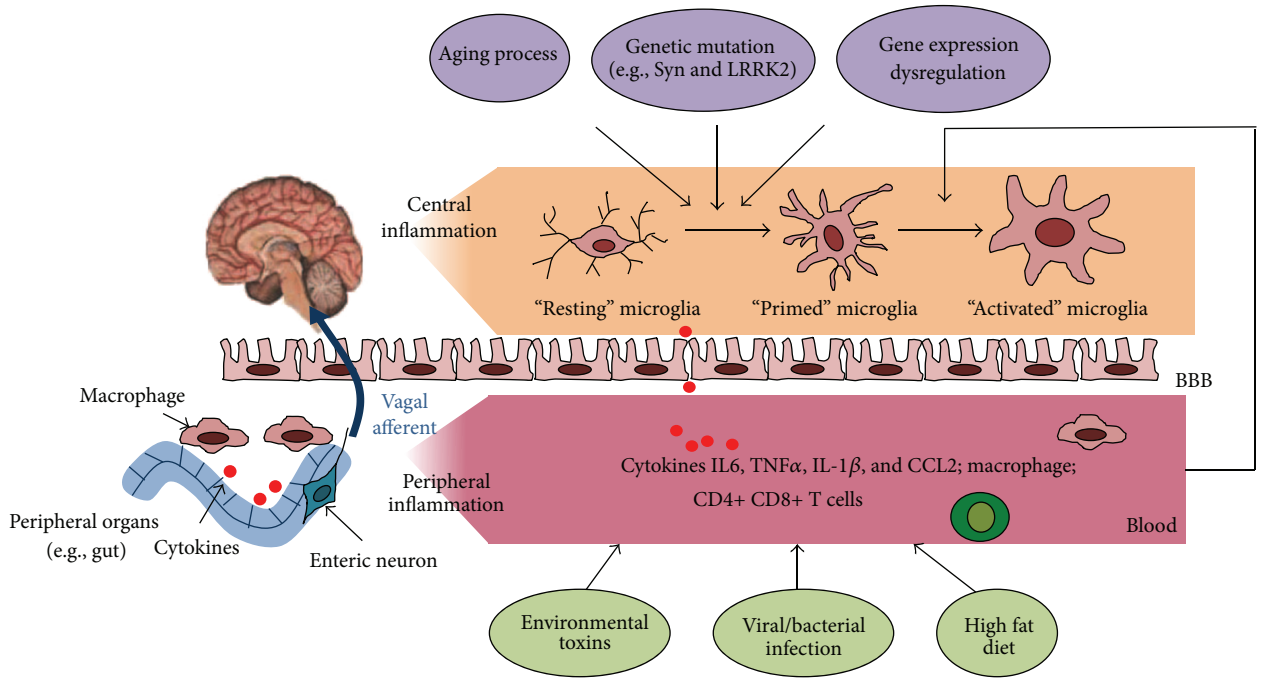

Figure 1: Interplay between central and peripheral immune systems in Parkinson's disease. The aging process, genetic mutation, and/or dysregulation of certain gene expressions serve as a "priming" stimulus for microglia. Upon secondary stimulation (e.g., environmental toxin, viral infection, high fat diet), peripheral inflammation is induced and communicates with brain through neural (vagal afferent) or humoral routs (e.g., cytokines circulation). The primed microglia are further activated and release excessive quantities of proinflammatory cytokines driving neurodegeneration.

It becomes more clear that peripheral inflammation plays a role in early stages of disease initiation and progression, including the development of preclinical nonmotor symptoms (hyposmia, constipation, bladder disorder, sleep disorder, obesity, and depression; Braak stages I and II [94]). Peripheral inflammation appears to accompany the nonmotor symptom of PD. One clinical study observes a significant correlation between serum TNFα levels and the severity of nonmotor symptoms including depression, sleep disturbances, and cognitive dysfunction in PD patients [95]. Gut infection and inflammation, mediated by a gram-negative bacterium, *Helicobacter pylori* (HP), are associated with PD [96, 97]. Moreover, successful treatment of HP has been shown to increase stride length in PD patients, whereas failure to eradicate HP results in significantly worse symptoms [98]. Chronic constipation, which occurs as early as 20 or more years before the onset of motor symptoms of PD, is casually linked to peripheral inflammation. Stomach infections may have early consequences on the enteric nervous system that manifest as gastrointestinal (GI) dysfunction including constipation [99]. Obesity, which is associated with increased risk of developing PD [100], displays increased levels of proinflammatory cytokines including TNFα, IL-1β, IL-6, and CCL2, in adipose tissue [101–103], liver [104], pancreas [105], brain [106], and possibly muscle [107]. Other studies have demonstrated that anti-inflammatory therapy may ameliorate MNS in non-PD related condition [108]. Collectively, strong clinical data support that peripheral inflammation appears to be an early event in the development of PD.

4. Interplay of Central and Peripheral Systems Drives Neurodegeneration

The CNS has been considered as immunologically privileged and protected by the BBB which prevents entry of pathogens and immune cells into the parenchyma. However, recent evidence suggests that the communication between central CNS and periphery is very fluid. BBB breakdown and systemic inflammation appear to play an important role in the pathology of numerous neurodegenerative diseases compromising the vascular unit and inducing leukocyte migration within the brain parenchyma [109]. A systemic infection or injury gives rise to an inflammatory response that communicates with brain. Both neural and humoral routes mediate communication from the site of peripheral inflammation to the brain. The neural route is through the dorsal motor nucleus of the vagus nerve [110]. The humoral route involves circulating cytokines that communicate with the brain via several routes: (i) by saturable transport across the BBB [111]; (ii) by activating endothelial cells and perivascular macrophages [112]; and (iii) through circumventricular organs which lack a functional BBB [113].

Recent studies suggest that activation of the peripheral immune systems exacerbates the discordant central inflammatory response in aged or genetic predisposed brains. For instance, LPS challenge promotes microglial hyperactivity in aged mice, that is, associated with exaggerated induction of both proinflammatory IL1beta and anti-inflammatory IL-10 cytokines [114]. In addition, Gao et al. have established

a two-hit animal model involving α-Syn mutation (A53T) and an environmental trigger (LPS) [115], which reproduces key features of PD and demonstrates synergistic effects of genetic predisposition and environmental exposures in the development of PD. Collectively, the aging process, genetic mutation, and/or dysregulation of certain gene expression serve as a "priming" stimulus for microglia, and upon secondary stimulation (e.g., environmental toxin or viral infection), the primed microglia release excessive quantities of proinflammatory cytokines driving neurodegeneration (Figure 1).

5. Therapeutic Implication

We are at the beginning of understanding the impact of chronic inflammation inside and outside of the brain towards neurodegenerative disorders. The more we are certain about these interactions, the better we would be able to diagnose, manage, and treat Parkinson's disease in a systemic but targeted manner.

Chronic inflammation in both sporadic and familial PD may represent therapeutic opportunities for immunomodulatory interventions in combination with other neuroprotective agents. However, the negative results of nonsteroidal anti-inflammatory drugs in late PD [116] strongly suggest that early immunomodulation is the key in preventing PD onset and progression. Minocycline, a broad-spectrum tetracycline antibiotic, has been tested in experimental models and PD patients. Minocycline effectively crosses the BBB and shows potent anti-inflammatory and neuroprotective effects in neurotoxin models of PD (e.g., MPTP and rotenone) [117]. A randomized, double-blind, Phase II futility clinical trial shows that minocycline offers clinical benefit to early PD patients, which warrants further consideration of minocycline for Phase III clinical trials [118]. Pioglitazone, a synthetic peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, is currently under investigation in a Phase II placebo-controlled clinical trial for the treatment of early PD.

LRRK2 is highly expressed in peripheral macrophage and monocytic cells as well as in central microglia, suggesting a functional role for LRRK2 in the innate immune system [119, 120]. Recent studies show that LRRK2 kinase inhibitors attenuate inflammatory signaling in HIV or LPS-treated microglia [67, 120]. Furthermore, small hairpin RNA targeting LRRK2 can equally inhibit LPS-induced microglial activation [120]. These data strongly suggest that inhibition or attenuation of LRRK2 is a promising therapeutic strategy for anti-inflammatory treatment for PD. However, it should be noted that LRRK2 knockout mice display alterations in exploratory and motor coordination behaviors and cause degeneration in the kidney [121], suggesting the wild-type LRRK2 may be involved in certain important normal physiological functions. Thus, a safe and effective LRRK2 therapeutic strategy using small molecule inhibitors or RNA interference should be specific and target disease-linked mutations.

PGC-1α is a potential new target for anti-inflammatory therapy for PD. Several pharmacological activators have been reported to enhance PGC-1a activity and stimulate mitochondrial biogenesis. PGC-1a activity is mainly controlled by the PPARs, AMPK, and Sirt1 [122]. Hence, pharmacological activators for these proteins have the potential to exert anti-inflammation as well as induce mitochondrial biogenesis through PGC-1α activation. Such activators include fibrates and rosiglitazone (PPAR) [123, 124], metformin [125], pyrroloquinoline quinone (PQQ) [126], and AICAR [127] (AMPK) as well as resveratrol (Sirt1) [128]. As PPAR agonist (fibrates, rosiglitazone) and AMPK activator (metformin, AICAR) are already routinely used in clinical practice for treatment of metabolic syndrome and Type 2 Diabetes, these drugs could be readily translated from animal models to PD patients. Preclinical CNS distribution and efficacy studies using inflammatory animal models of PD (i.e., the two-hit model) will be sufficient to warrant clinical trials on these drugs.

6. Conclusions

Although PD is complex multifactorial disorder with unknown etiology, increasing evidence supports an important role of central and peripheral inflammation in driving PD initiation and progression. Therefore, the current critical need is to identify promising targets for anti-inflammatory therapies, as well as fully understand the potential effects, both positive and negative, of blocking the inflammatory state in the early stage of the disease. Understanding the link between PD genetic variants or altered transcription and specific immune responses is crucial to identify novel therapeutic targets and to devise tailored neuroprotective interventions. As the research on preclinical/subclinical biomarkers for PD advances, anti-inflammation therapies clinical trials will become feasible for those at highest risk for PD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 635923, 7 pages
http://dx.doi.org/10.1155/2014/635923

Research Article

Peripheral Leukocyte Apoptosis in Patients with Parkinsonism: Correlation with Clinical Characteristics and Neuroimaging Findings

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Received 17 January 2014; Accepted 26 February 2014; Published 26 March 2014

Academic Editor: Hung-Chen Wang

Apoptosis of both brain neurons and peripheral blood leukocyte is believed to be an important biomarker for evaluating the functional status of Parkinson's disease (PD). However, their correlation remains unknown. A better understanding of the pathophysiology of neurodegeneration is essential for the treatment and prevention of PD. The present study demonstrated that leukocyte apoptosis is significantly higher in PD patients and is associated with central dopamine neuron loss by using ^{99m}Tc-TRODAT-1 SPECT. The leukocyte apoptosis and striatal dopamine transporter uptake ratios were further associated with increased severity and longer duration of disease. The interaction between brain and systemic inflammation may be responsible for the neurodegenerative disease progression.

1. Introduction

Parkinson's disease (PD) is a movement disorder caused by dopamine (DA) deficiency in the striatum due to DA neuron degeneration in the substantia nigra (SN). The etiopathogeny involves the interaction of environmental and genetic factors [1]. Recently, neuroinflammation has been considered fundamental to the progression of PD [2]. In postmortem analysis of PD patients, activated microglia is found in the SN pars compacta (SNpc) [3]. Elevated proinflammatory substances such as cyclooxygenase 2 (COX2) and cytokines including interleukin-1 beta (IL-1β), interferon-gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) are also found in postmortem PD brains [4–7], suggesting the presence of inflammatory processes [8].

Altered neurovascular functions in PD can lead to increased blood-brain barrier permeability and increased peripheral neutrophil and monocyte infiltration into the SN region, where they play an important role in neuroinflammation [9] and DA neuronal death. Recently, peripheral

inflammation has been considered to have consequences on the degenerative process of DA neurons. In PD, some biochemical alterations affecting neuronal cells have been detected in circulating lymphocytes. Increased oxidative stress is associated with an imbalance between reactive oxygen species (ROS) formation and antioxidant defenses [10] and the presence of DNA damage [11]. Moreover, previous studies show a decreased number of circulating lymphocytes in PD patients [12]. Peripheral blood CD4+ T cells have increased susceptibility to apoptosis with Fas involvement in patients with PD [13]. Interestingly, some of these alterations may be associated with disease severity [14].

^{99m}Tc-[2-[[2-[[[3-(4-chlorophenyl)-8-methyl-8-azabicyclo [3,2,1] oct-2-yl] methyl] (2-mercaptoethyl) amino]ethyl] amino]-ethanethiolato(3-)-N2,N2,S2, S2]oxo-[1R-(exo-exo)] (^{99m}Tc-TRODAT-1) is a specific tracer developed to bind selectively to dopamine transporters in the brain. Studies with TRODAT-1 single photon emission computed tomography (SPECT) allow for an *in vivo* assessment of presynaptic dopaminergic neuron activity of the brain. ^{99m}Tc-TRODAT-1 SPECT is a useful tool for differentiating Parkinsonian disorders [15]. Decreased striatal tracer uptake, indicating loss of DA neurons, can be used to evaluate worsening disease and confirm symptomatic lesions in the early stage of PD [16].

Apoptosis of both brain neurons and peripheral blood leukocyte is believed to be an important biomarker for evaluating the functional status of PD. However, their correlation remains unknown. Better understanding of the pathophysiology of neurodegeneration is essential for the treatment and prevention of PD. The present study hypothesized that leukocyte apoptosis plays an important role in the prognosis of PD. We analyzed the correlations among the peripheral leukocytes apoptosis, striatal neuronal loss on ^{99m}Tc-TRODAT-1 SPECT/CT studies, and clinical presentations.

2. Materials and Methods

2.1. Subjects. Fifty-five PD patients (22 males, mean age 59.9 ± 10.9 years), without a history of other neurologic or psychiatric illness and psychotropic medications, contraindications to Madopar (L-dopa), at the Neurology Department of Chang Gung Memorial Hospital were prospectively enrolled. Patients were included when they had idiopathic PD, diagnosed according to the United Kingdom Brain Bank criteria [17] by an experienced neurology specialist. The time point of the diagnosis of PD was collected from each case, as well as the duration of disease. Disease onset was defined as the time of first recalled motor symptoms, such as tremor, bradykinesia, and rigidity in the pretreatment phase of the disease. Twelve patients never used any anti-Parkinson's medication, while the rest used dopaminergic medication (levodopa and dopamine agonists).

The studies were performed at least 12 h after the last dose of dopaminergic medication (off state). Each patient's disease severity and functional status were evaluated using the Unified Parkinson's Disease Rating Scale (UPDRS), the modified Hoehn and Yahr Staging Scale, and the Schwab and England Activities of Daily Living Scale. The Unified Parkinson's Disease Rating Scale (UPDRS) is the most commonly used scale to follow the longitudinal course of PD [18]. The UPDRS scores are evaluated by interview and clinical observation. The modified Hoehn and Yahr Scale provides a global assessment of severity in Parkinson's disease based on clinical findings and functional disability [19]. It is a commonly used system for describing how the symptoms of Parkinson's disease progress. It is a rating scale measured in an ordinal level and included stages 1 through 5. The higher rates describe an increased severity of the disease. The Schwab and England Activities of Daily Living Scale estimates the abilities of PD patients relative to a completely independent situation. One hundred percent indicates a completely independent patient and 0% indicates an individual in whom vegetative functions are no longer functioning [20].

For comparison, 37 sex- and age-matched healthy subjects (18 males; 62.9 ± 6.3 years) without a medical history of neurologic disease or psychiatric illness, alcohol or substance abuse, or head injury and with similar levels of education were recruited from the hospital. The hospital's Institutional Review Committee on Human Research approved the study and all of the participants or their guardians provided written informed consent.

2.2. Blood Sampling and Assessment of Leukocyte Apoptosis. Blood samples were collected from patients by venipuncture of forearm veins and from the control group upon enrollment. Blood sample analysis was done according to a previous work [21]. Whole blood (100 μL) was stained with 10 μL CD45-phycoerythrin- (PE-) Cy5 (clone J33) for 15 min at room temperature protected from light. The CD45-PE-Cy5 antibody reacts with the CD45 family of transmembrane glycoproteins, expressed on the surface of all human leukocytes, and is a pan-leukocyte marker. Cells were fixed with 5.5% formaldehyde. After washing, permeability was induced with permeability agent (Beckman Coulter) and the remaining erythrocytes were lysed. In this stage, the cells were brought into contact with APO 2.7-PE (clone 2.7A6A3; Immunotech, Marseille, France) for intracellular antigenic determinants. The APO 2.7-PE antibody reacts with the 38-kDa mitochondrial membrane protein (7A6 antigen), which is detectable on nonpermeabilized cells in the late apoptotic state [11]. Mouse immunoglobulin G-PE was used as a control for nonspecific staining. The leukocytes were then analyzed by flow cytometry.

Flow cytometry analysis was performed immediately after staining with an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA) using the EXPO32 ADC software. Five thousand CD45-PE-Cy5+ cells per sample were acquired in combined forward and side scatters and deep-red FL4 fluorescence (CD45-PE-Cy5) leukocyte gate. Leukocyte subtypes were identified according to their CD45 expression intensity. The results were expressed as the percentage of specific fluorescence-positive cells. Apoptotic cells were defined by APO 2.7 positivity. A database coordinator was responsible for monitoring all data collection and entry. All data were checked for any inconsistencies. Intra-assay variability based on repeated measurements of the same blood sample was low.

2.3. ^{99m}Tc-TRODAT-1 SPECT/CT and Region of Interest (ROI) Analysis. Each patient with PD was intravenously injected with a single bolus of 925 MBq (25 mCi) of ^{99m}Tc-TRODAT-1. The image acquisitions were performed after 4 h using a dual-head SPECT/CT equipped with low-energy high-resolution collimators (Symbia T, Siemens Medical Solutions, Erlangen, Germany). Emission data were acquired in a 128 × 128 matrix with 1.45 zoom through 360° rotation (180° for each head) at 3° intervals for 30 s per angle step. Transmission data acquired by low-dose CT without contrast medium were used for attenuation correction and functional-anatomic image fusion.

Low-dose CT images were acquired using the following parameters: 130 kV, 45 mAs (maximum), and 5 mm thick sections. Reconstruction and display of functional-anatomic fusion images were performed on the Syngo MI workplace (Siemens Healthcare, Forchheim, Germany). After FLASH 3D (ordered-subset expectation maximization iterative reconstruction method with 3D collimator beam modeling) reconstruction of the emission data, three-dimensional images of transaxial, coronal, and sagittal slices were obtained. The transaxial images of ^{99m}Tc-TRODAT-1 SPECT/CT were analyzed both visually and semiquantitatively. With the help of anatomical coregistration CT images, ROIs of bilateral striata (including their subregions of caudate and putamen) were defined on composite images of the six highest striatum activity slices. The occipital cortex was drawn in the same way and served as background area (Figure 1). The ROIs' radioactivities were counted and striatal dopamine transporter uptake ratios were calculated as the quotient of the mean counts per pixel in each striatum divided by the mean counts per pixel in the occipital cortex. All images were reviewed by an experienced nuclear physician who was blinded to the patient's information.

2.4. Statistical Analysis. Data were expressed as mean ± SD or median (interquartile range). Univariate analyses used the Student's t-test or the Mann-Whitney test. For categorical variables, the χ^2 test or Fisher's exact test was used as appropriate. Partial Pearson's correlation analysis was used to explore the relationship between leukocyte apoptosis, clinical variables, and dopamine transporter uptake ratios in the PD group, after controlling for age and sex. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using the SPSS software, version 10.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Baseline Characteristics of the Study Patients. The baseline characteristics and laboratory data of both groups (Table 1) showed no significant differences in age, sex, white blood cells, red blood cells, and platelet counts.

3.2. Leukocyte Apoptosis in the PD and Control Groups. The laboratory data, presented as medial value (interquartile range), and the percentage of leukocyte apoptosis of both groups (Figure 2) showed that the percentage of leukocyte apoptosis was significantly higher in PD patients than in controls (1.53 [1.03, 2.17] versus 0.81 [0.57, 1.17], $P < 0.001$). The percentages of apoptosis in the subsets of leukocytes, including neutrophils (0.89 [0.52, 1.37] versus 0.39 [0.24, 0.51], $P < 0.001$), monocytes (4.67 [3.22, 7.45] versus 2.74 [1.73, 4.27], $P < 0.001$), and lymphocytes (0.60 [0.47, 1.00] versus 0.36 [0.21, 0.68], $P < 0.001$), were significantly higher in the PD group ($P < 0.01$) than in controls.

3.3. Correlations of Percentage of Leukocyte Apoptosis, Striatal Dopamine Transporter Uptake Ratios, and Disease Severity of PD. The total leukocyte apoptosis negatively correlated with right striatal dopamine transporter uptake ratio ($\gamma = -0.384$, $P = 0.012$) and duration of disease ($\gamma = -0.293$, $P = 0.039$). The total leukocyte apoptosis and subset of neutrophil apoptosis positively correlated with disease severity in the UPDRS score and the modified H & Y score and negatively correlated with the S & E score. By further analysis, bilateral striatal dopamine transporter uptake ratios were negatively associated with UPDRS II, UPDR III, UPDR total scores, and the modified H & Y score and positively correlated with S & E score (Table 2).

4. Discussions

In the current study, changes in the number and composition of leukocyte subsets in PD suggested enhanced activation of apoptosis, which is consistent with previous findings [13]. The similar results have been extended by showing that the increase in apoptotic leukocytes is associated with striatal dopamine transporter uptake, suggesting a loss of DA neurons compared to healthy individuals. The relationship between leukocyte apoptosis and disease severity explains the increase in the amount of leukocyte subset apoptosis observed in Parkinson's disease and can be associated with the neurodegenerative process.

Apoptotic cell death occurs primarily through three different pathways: the extrinsic death receptor pathway (type I cells), the intrinsic (mitochondrial) pathway (type II cells), and the endoplasmic reticulum (ER) or stress-induced pathway [22]. All of these processes are accomplished or composed by different inflammatory processes. In the present study, higher leukocyte apoptosis level indicates higher peripheral inflammation in PD.

It is important to know whether peripheral inflammation, a very common health problem, can affect the degeneration of nigrostriatal dopaminergic neurons. Studies show that peripheral inflammation can enhance the degeneration of the nigrostriatal dopaminergic system. Systemic IL-1 expression may exacerbate neurodegeneration by an increase in inflammation in SN, as evidenced by the great reactivation of microglia. Studies also suggest that increases in other inflammatory cytokines (i.e., IL-6, IL-2, TNF-α, and IFN-γ) are responsible for this effect [23]. Interestingly, such proinflammatory, immunomodulatory, and anti-inflammatory cytokine alterations involved in apoptosis and enhanced oxidative stress [11] may be related to disease severity [14]. The result here on the high apoptosis of total leukocytes and subset of neutrophils associated with UPDRS

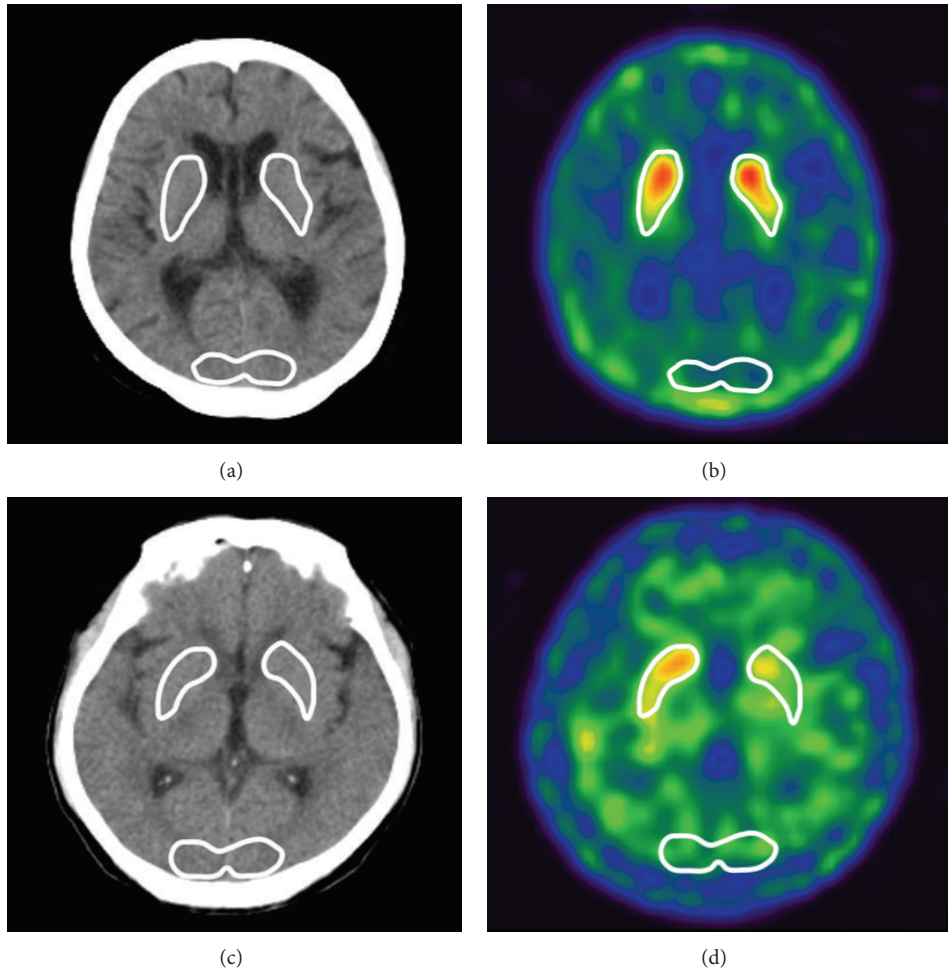

Figure 1: The ROIs of the striatal and occipital cortices (background) on ^{99m}Tc-TRODAT-1 SPECT/CT imaging in patients with (a-b) normal and (c-d) abnormal striatal dopamine transporter uptake. The ROIs of the bilateral striatal and occipital cortices were drawn on the anatomic coregistration CT images (a and c) and transferred them to the transaxial composite slices of ^{99m}Tc-TRODAT-1 SPECT images (b and d). The ROIs' radioactivities were counted for striatal dopamine transporter uptake analysis.

Table 1: Demographic data of patients with PD and controls.

Clinical demographics	PD (n = 55)	Control (n = 37)	P
Age (year) (mean ± SD)	59.9 ± 10.9	62.9 ± 6.3	0.136
Sex (M, F)	22, 33	18, 19	0.089
White blood cells ($\times 10^3$/mL)[#]	5.60 (5.00, 6.80)	6.05 (4.88, 8.43)	0.250
Red blood cells ($\times 10^4$/mL)[#]	4.78 (4.42, 5.08)	4.41 (4.09, 5.00)	0.410
Platelet counts ($\times 10^4$/mL)[#]	232 (200, 306)	223 (173, 276)	0.156
Duration of disease (years)[#]	2.5 (1.0, 5.5)		
UPDRS I[#]	3.0 (1.0, 6.0)		
UPDRS II[#]	10.0 (4.0, 16.0)		
UPDRS III[#]	22.0 (14.0, 34.0)		
UPDRS total[#]	33.0 (20.0, 54.0)		
Modified H & Y[#] (maximum stage is 5)	1.75 (1.0, 3.0)		
S & E[#] (minimum point is 0 suggesting vegetative functions)	90.0 (77.5, 100.0)		
TRODATE R[#]	1.45 (1.24, 1.62)		
TRODATE L[#]	1.37 (1.23, 1.53)		

UPDRS: Unified Parkinson's Disease Rating Scale; modified H & Y: modified Hoehn and Yahr Staging Scale; S & E: Schwab and England Activities of Daily Living Scale.
[#]Median (IQR): IQR: interquartile range.

Table 2: Correlation analysis between leukocyte apoptosis, ^{99m}Tc-TRODAT-1 striatal uptake binding ratio, and clinical variables in the PD group after controlling for age and sex.

	Variables	r	P value
Total leukocyte apoptosis (%)	Striatal dopamine transporter uptake ratios mean	−0.349	**0.020**
	Striatal dopamine transporter uptake ratios R	−0.384	**0.012**
	Striatal dopamine transporter uptake ratios L	−0.252	0.108
	UPDRS I	0.293	**0.041**
	UPDRS II	0.480	**0.000**
	UPDRS III	0.555	**0.000**
	UPDRS total	0.537	**0.000**
	Modified Hoehn-Yahr Staging Scale	0.461	**0.001**
	Schwab and England Activities of Daily Living Scale	−0.463	**0.001**
	Duration of disease	0.293	**0.039**
Neutrophil apoptosis (%)	Striatal dopamine transporter uptake ratios mean	−0.253	0.098
	Striatal dopamine transporter uptake ratios R	−0.272	0.082
	Striatal dopamine transporter uptake ratios L	−0.244	0.120
	UPDRS I	0.120	0.413
	UPDRS II	0.298	**0.037**
	UPDRS III	0.356	**0.012**
	UPDRS total	0.335	**0.019**
	Modified Hoehn-Yahr Staging Scale	0.378	**0.007**
	Schwab and England Activities of Daily Living Scale	−0.318	**0.026**
	Duration of disease	0.145	0.315
Striatal dopamine transporter uptake ratios R	UPDRS III	−0.349	0.019
	UPDRS total	−0.330	0.027
	Modified Hoehn-Yahr Staging Scale	−0.373	0.012
Striatal dopamine transporter uptake ratios L	UPDRS II	−0.295	0.049
	UPDRS III	−0.328	0.028
	UPDRS total	−0.307	0.040
	Modified Hoehn-Yahr Staging Scale	−0.390	0.008
	Schwab and England Activities of Daily Living Scale	0.332	0.026

UPDRS: Unified Parkinson's Disease Rating Scale.

score and disease duration further confirms previous studies. All of these data suggest that the increase in inflammatory parameters in the periphery (blood) due to peripheral inflammation induces an increase in inflammation in SN and, consequently, a synergistic effect on the nigrostriatal dopaminergic system.

^{99m}Tc-TRODAT-1 shows promise as a tracer for the imaging of dopamine transporter [24], which is heavily expressed in the terminals of dopamine neurons that are lost in PD. The specific striatal binding of ^{99m}Tc-TRODAT-1 shows a moderately negative correlation with disease severity and duration. This finding is similar to that of a previous study [16] and suggests that ^{99m}Tc-TRODAT-1 is a useful marker of disease severity in PD, with potential utility for serial monitoring of disease progression. The decrease in striatal dopamine transporter uptake ratio suggests a loss of DA neurons in the substantia nigra.

Further exploring the relationship with the decrease in striatal dopamine transporter uptake ratio reveals a correlation in the increase in peripheral blood total leukocyte apoptosis and a weak correlation in the neutrophil subset. This may be due to the increase of oxidative stress in circulating leukocyte, especially in neutrophils [13], indicating that peripheral inflammation is correlated with dopamine dysfunction/cell loss. The level of neutrophils apoptosis further reflects the disease severity of PD. These findings suggest that circulation neutrophils apoptosis is a useful biomarker to assess disease status, and leukocytes apoptosis may play an important role in the pathogenesis of central neurodegeneration in PD.

Alteration of peripheral T-lymphocyte populations that increase susceptibility to apoptosis in PD has also been demonstrated [25]. In addition, disruption of the BBB with active lymphocyte infiltration to the brain induces inflammation in SN, such as microglial activation and increased proinflammatory cytokines [26]. However, the present results demonstrate no significant correlation between elevated monocyte and lymphocyte apoptosis and between disease severity and duration and striatal dopamine transporter uptake ratio. Immune reactions associated with cell death in SN are hypothesized to occur several years before the onset of symptomatic PD [27] and a series of peripheral immune

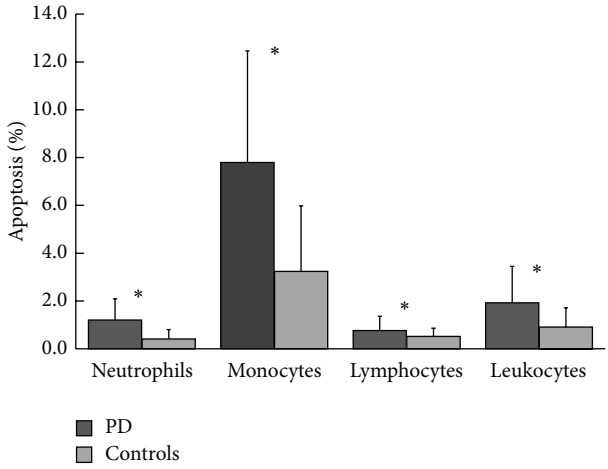

Figure 2: Apoptosis of total leukocytes and their subsets in PD patients and controls. $^{*}P < 0.001$, PD patients versus controls.

alterations may precede the occurrence of immune reaction-related inflammation in the brain. Moreover, lymphocyte proliferation and cytokine production have been proposed to be affected by peripheral dopamine [28] such that levodopa therapy may also have a role in the alteration of lymphocytes populations in PD [29]. The pathophysiology of leukocyte profile alteration in PD is still unclear and further study of the peripheral leukocyte status in preclinical PD and its longitudinal evolution is warranted.

In conclusion, leukocyte apoptosis is significantly high in patients with PD and is associated with decreased striatal dopamine transporter uptake ratio implying central dopamine neuron loss. The interaction between brain and systemic inflammation may be responsible for the progression of this neurodegenerative disease. Investigating the relationship between the central and peripheral nervous system may help find targets for therapeutic interventions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper. The authors declare no competing financial interests.

Acknowledgments

This work was supported by funds from Chang Gang Memorial Hospital (Chang Gang Medical Research Project CMRPG891511 and CMRPG8B0831 to W-C Lin) and from the National Science Council (NSC101-2314-B-182A-078 to W-C Lin).

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