Japanese encephalitis virus infection: effect on brain development and repair

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Japanese encephalitis (JE) is the most prevalent form of viral encephalitis endemic to many parts of Asia where periodic outbreaks take hundreds of lives. It is caused by the JE virus (JEV), belonging to the family Flaviviridae. Recent studies have shown that there is a potential risk of an introduction of JEV into the United States, Italy and other European countries where the threat of this disease was previously unknown, leading to a growing concern that JEV will soon become a global pathogen and cause of worldwide pandemics. JEV infects mostly children, who are in a dynamic state of brain development and the JE survivors are often left with severe neurological sequelae. This may be attributed to the depletion of neural progenitor cells by the virus which in the process suppresses the cycling ability of these cells, preventing their proliferation. This review focuses on this fatal viral infection which poses a major threat in the developing countries, especially among the children, by killing neurons and also depleting the neural progenitor pool with progressive infection. The therapeutic use of the drug Minocycline, a tetracycline derivative in imparting protection against this disease, has also been highlighted which can be a major breakthrough in the treatment of JE.

Keywords: Japanese encephalitis, neural progenitor cells, neuroinflammation, neurogenesis.

Introduction

JAPANESE encephalitis (JE) is a vector-borne viral disease caused by JE virus (JEV) which is a single stranded positive sense RNA virus belonging to the family flaviviridae. This virus is transmitted through a zoonotic cycle between mosquitoes, pigs and water birds. Humans are accidentally infected and are a dead end host because of low level and transient viremia[1]. Countries with proven occurrence of JE are India, Pakistan, Nepal, Sri Lanka, Burma, Laos, Vietnam, Malaysia, Singapore, Philippines, Indonesia, China, maritime Siberia, Korea and Japan[2]. The first clinical case of JE in India was observed in 1955 at Vellore[3]. Since then, many major outbreaks have been reported from different parts of India including states of Bihar, Uttar Pradesh, Assam, Manipur, Andhra Pradesh, Karnataka, Madhya Pradesh, Maharashtra, Tamil Nadu, Haryana, Kerala, West Bengal, Orissa, Goa and union territory of Puducherry, predominantly in the rural areas[4,5]. Although it was once believed to be confined to South Asia, Southeast Asia, East Asia and Pacific, recent reports suggest that its geographical area is spreading, making it one of the most important endemic encephalitis in the world[6]. It is naturally transmitted by the mosquitoes of the genus *Culex* which feed on water birds and larger mammals including pigs, thereby transmitting JEV. The invasion of JEV in new areas in Southeast Asia during the last decades has been mainly associated with the increase of human populations which has led to increasing areas of rice paddies and pig farming[7].

JEV has also shown a clear tendency of expansion with the air transport of infected mosquitoes in higher altitudes of Nepal and into New Guinea, Torres Straight and Northern Australia[8,9]. A study has shown that there is a potential risk of an introduction of JEV into the west coast of the United States where competent vectors and pigs as amplifying vertebrate hosts are available in moderate numbers[10] and there has been evidence of JEV circulation in Italy and other European countries[11]. Birds may also play a critical role of transporting JEV over long distances causing expansion of this virus into virgin areas[12]. An estimated 3 billion people live in countries where JEV is endemic[13] and it affects over 50,000 patients and results in 15,000 deaths annually. These reports indicate that JE has gradually evolved into a disease of global concern.

The high-risk group for JE is 1 to 15-year-old children. Mostly children within the age group of 3–6 years are susceptible to JEV infection in endemic areas; however, it has been found to affect both children and adults in newly affected areas. In humans, the age-specific susceptibility to the virus usually decreases after 14 years of age, probably because of the presence of high levels of neutralizing antibodies resulting from natural exposure and subclinical infection. Thus, JE is a major public health concern in endemic areas and vaccination is the only approach for the control of JE. In spite of having an ad-hoc vaccination programme in the last six decades, 10.5 million children were estimated to have been infected, resulting in 3 million deaths and 4 million long-term disabilities, making JE the most important childhood viral encephalitis[14]. JE has been mainly regarded as a

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disease of children in the endemic areas but in the newly invaded areas, it has been found to affect both the adults and children because of the absence of protective antibodies.

JEV targets the central nervous system (CNS), clinically manifesting with fever, headache, signs of meningeal irritation and altered consciousness and infection can result in high mortality. JEV infects mostly children, who are in a dynamic state of brain development and therefore any insult may have consequences later in life. Survivors of JEV infection are often left with severe neurological sequelae, including motor deficits, cognitive and language impairments and learning difficulties. Neuronal death in JE results from both direct neuronal killing by the virus as well as by a bystander method mediated by microglial activation and robust inflammatory attack[15,16]. Hence, effective CNS repair processes which restore the neuronal loss are crucial for complete recovery from JE. CNS responds to any neuronal loss by differentiating new neurons and astrocytes from resident populations of multipotential neural progenitors cells (NPCs)[17]. These NPCs reside in neurogenic areas of the brain such as the subventricular zone (SVZ) and the dentate gyrus of the hippocampus and have the potential to self-renew over a lifetime[18]. The significance of NPCs in brain repair and regeneration has been supported by accumulating evidence indicating that a large number of newborn neurons can be generated from adult NPCs and integrate into pre-existing neural circuits. As functional roles of adult neurogenesis become more defined, it is evident that adult NPCs may well contribute to the maintenance of physiological tissue homeostasis in the brain[17,19]. JEV leads to massive loss of actively proliferating NPC population from the SVZ by suppressing the cycling ability of these cells. Thus, the ability of JEV-infected SVZ cells to form neurospheres is severely compromised. It primarily targets the critical postnatal age and severely diminishes the NPC pool in SVZ, thereby impairing the process of recovery after the insult. This arrested growth and proliferation of NPCs might have an effect on the neurological consequences in JE survivors.

Neuroinflammation and the regenerative capacity of brain stem cells

In the adult brain, neurogenesis under physiological conditions occurs in SVZ and in the dentate gyrus. These new neurons originate from the existing pool of proliferating neural stem or progenitor cells (NSPCs) which can continuously self-renew and have the potential to generate cells of both glial and neuronal lineages[18]. Although the exact molecular mechanisms that regulate neural stem cell proliferation and differentiation are largely unknown, several factors have been shown to affect neurogenesis.

Several clinical and animal studies have suggested that the principal target cells for JEV in CNS are neurons[15,20]. As a result of infection, there is massive destruction of neurons. During such a pathological condition of CNS involving neuroinflammation, inflammatory mediators such as cytokines and chemokines are known to affect the regenerative capacity of brain stem cells and alter neurogenesis[21]. The link between brain inflammation and neurogenesis and the role of microglia in the modulation of neurogenesis under pathological conditions have been under intense investigation since long. A number of studies have recently revealed that activated microglia have a complex, all-round impact on injury-induced neurogenesis, with both beneficial and deleterious actions[22,23].

It was a common belief that the adult mammalian brain and spinal cord do not regenerate after injury, but recent discoveries have forced a reconsideration of this accepted principle. Recent studies have shown that cell genesis and the synaptic plasticity of the neural stem cells can be influenced by stress, an enriched environment and physical exercise[24,25]. With respect to injury, studies have shown that progenitor cells are capable of proliferation and differentiation into mature myelinating oligodendrocytes in models of acute demyelination[26]. In a model of selective pyramidal cell apoptosis, cortical neurons have been shown to be replaced by dividing progenitor cells[27]. Strikingly, these newborn neurons extend new axons several millimetres through the intact CNS. These findings show that under the correct lesion conditions, CNS stem cells are capable of participating in cell replacement[28].

Neural stem/progenitor cells are susceptible to viral infection

Being one of mitotically active populations in the postnatal/adult brain, the NSPCs have emerged as the potential targets of neurotropic viruses. Brain abnormalities and long-term cognitive deficits in a number of neurodevelopmental disorders have been attributed to virus infection of NSPCs. The regulation of NPC proliferation in response to various insults like infection and inflammation is a topic of extensive research in recent times. A number of studies have shown that Coxsackievirus, a common cause of enteroviral encephalitis in neonates, targets proliferating NPC pool in the neonatal CNS[29,30]. Cytomegalovirus, another leading cause of developmental disorder of CNS, preferentially infects the ventricular and SVZ and impairs the growth and proliferation of NPCs[31,32]. Adult neurogenesis has been reported to be hindered by viral infection, like HIV, which inhibits proliferation of adult NPCs in hippocampus, thereby inducing dementia in HIV patients[33,34]. These reports indicate that the effect of viruses on the susceptible NSC population plays an important role in causing serious neurologic sequelae in various diseases.

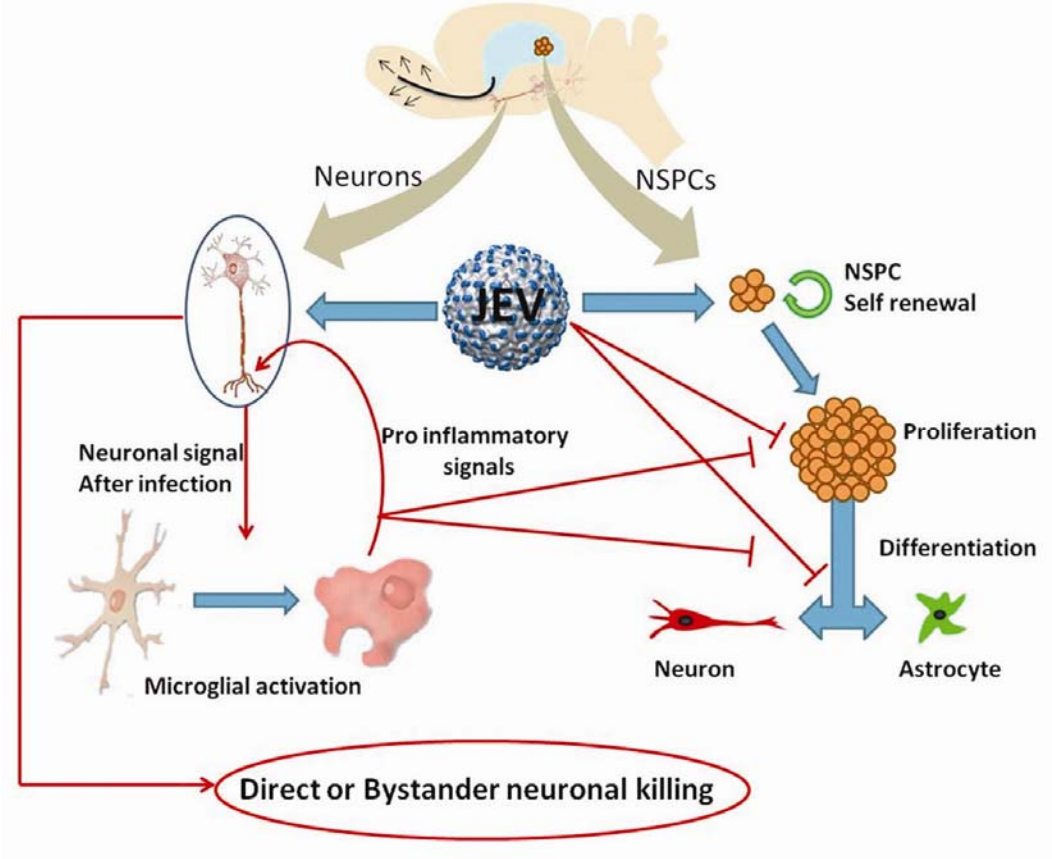

Figure 1. The direct and indirect effect of JEV infection on the fate of NSPCs and the overall pattern of neurogenesis in the subventricular zone. Apart from direct neuronal killing, JEV infection impairs the proliferation and differention of NSPCs, thereby depleting their pool in the SVZ and hampering neurogenesis. JEV-activated microglia produce inflammatory molecules which are also anti-proliferative and anti-neurogenic for NSPCs growth and development.

JEV infects neural progenitor cells and retards their proliferation

Many viruses causing neurodevelopmental damage exhibit an affinity towards NSPCs and alter their developmental fate, thereby affecting the overall neurogenesis. Das and Basu[35] have reported that JEV can infect NPCs and harbour in them. Interestingly, the virus does not induce robust NPC death, but with progressive infection arrests their proliferative ability. This eventually culminates in depletion of NPC pool, which could ultimately lead to long-term neurological sequel in JE survivors. Studies in the murine model of JEV infection have shown that JEV causes global infection of CNS, with high viral load in SVZ and the striatum of mouse pups. It has been found that there is a decrease in the actively proliferating NSPCs in SVZ of infected mouse pups, thereby indicating a diminished NSPC pool upon infection. Moreover, NSPCs cultured from SVZ, the primary neurogenic region of the brain demonstrate that JEV infection decreases the number of colony-forming neurospheres. BrdU (5-bromo-2′-deoxyuridine) incorporation and cell cycle studies of the infected NSPCs have revealed that upon progressive infection they undergo cell cycle arrest at G1 $\rightarrow$ S phase, with increase in the level of checkpoint proteins p21 and p27 (cyclin-dependent kinase inhibitors) and a simultaneous decrease in cyclin D levels which reinforce the fact that JEV infection impairs the self-renewal capacity of NSPCs and thereby depletes their pool in SVZ. This observation has also been supported by clonal neurosphere assays and single cell assays where a remarkable decline in the sphere-generating ability of the JEV infected NPCs has been found.

Besides self-renewal, the other important property of NSPCs which aids in CNS repair/regeneration is their ability to differentiate into any of the three cell types-neurons, astrocytes and oligodendrocytes. *In vitro* studies have also indicated that the ability of JEV-infected NSPCs to form neurons upon differentiation is also severely compromised as compared to control NSPCs. The expression of proneural genes and the terminal neuronal differentiation genes have also been found to be negatively regulated upon JEV infection of NSPCs. These findings provide evidence that JEV infection alters both

the proliferative and differentiation ability of NSPCs and thereby abrogating the replacement of damaged cells and CNS repair.

Effect of JEV-activated microglia on NSPCs

Flaviviruses induce neuroinflammation with typical features of viral encephalitis, including inflammatory cell infiltration, activation of microglia and neuronal degeneration. Microglial activation and the release of pro-inflammatory mediators is the hallmark of JE. Recent studies exploring the effects of inflammatory processes on neuronal survival and neurogenesis have reported bystander death of neurons due to inflammatory molecules secreted by JEV-infected microglia[36].

Typically, infected microglia secrete high levels of pro-inflammatory cytokines TNF-α, IL-6 and MCP-1 and ROS/NO which in high concentrations are neurotoxic and anti-neurogenic[37,38]. The detrimental effects of inflammation on neurogenesis have also been reported in various models of acute and chronic inflammation. Whereas IL-1β and IL-6 have been strongly implicated in decreased NSPC proliferation and neurogenesis[39,40], others like TNF-α exert dual effects depending on the receptor types involved. Action via TNFR1 suppresses NSPC proliferation in adult hippocampus both in normal and diseased brain, whereas TNFR2 improves the proliferation and survival of newly formed hippocampal neurons[41].

Minocycline treatment to JEV-infected animals restores proliferating cells in SVZ

New therapeutic agents are desperately needed for the therapy of acute viral encephalitis in humans. Minocyline, a tetracycline derivative, is one such agent with anti-inflammatory, anti-apoptotic and anti-oxidant properties. Therapy has proved useful in some experimental models of both noninfectious and infectious neurological diseases and also in clinical trials in humans, including acute traumatic cervical spinal cord injury [42].

Many studies have reported the efficacy of this drug against flaviviral infections where it has been shown to inhibit viral replication in cultured human neuronal cells and subsequently prevent virus-induced apoptosis[43]. As tetracycline treatment is clinically well tolerated, this semi-synthetic tetracycline derivative has been frequently studied for its neuroprotective ability against experimental model of JE.

Recent studies have shown that Minocycline confers complete protection in mice following JEV infection. Neuronal apoptosis, microglial activation, active caspase activity, proinflammatory mediators and viral titer have reportedly shown a marked decrease in Minocycline-treated JEV-infected mice on ninth day post-infection. The increased expression of proinflammatory mediators like TNF-α, MCP-1, IL-6, IFN-γ and IL-12 observed following JEV infection has been reported to be remarkably reduced after Minocycline treatment. Minocycline has also been shown to be effective *in vitro*, when JEV-infected neuroblastoma cells were protected from virus-induced death[44].

Minocycline's antioxidative property has also been shown to significantly ameliorate the oxidative stress generated as a result of JEV infection[45] and also imparts protection to the blood brain barrier by decreasing the expression of various adhesion molecules in the brain and downplaying the activity of matrix metalloproteinase 9 (MMP-9) (ref. 46).

Das and Basu[36] have reported that the impairment in neurogenesis caused due to the acute inflammatory milieu created in the SVZ neurogenic niche following JE can be reversed with Minocycline treatment. The efficacy of Minocycline as an anti-inflammatory compound was tested *in vitro* and it was found that the induction of stress signalling and production of cyto/chemokines decrease in JEV-activated microglial cells. These cyto/chemokines secreted by the activated microglia have been shown to cause an arrest in both proliferation and differentiation of the neurospheres. These effects were completely reversed when the JEV-activated microglia were treated with Minocycline.

These findings highlight the role of Minocycline in reducing the inflammatory milieu in the SVZ germinal niche and therefore rescue NSPCs from the toxic effects of the inflammatory mediators. The restoration of SVZ neurogenesis by Minocycline administration in JEV-infected animals may have important implications in promoting brain's regenerative capacity following neuronal loss in JE. Besides exerting neuroprotective effects, Minocycline by virtue of its anti-inflammatory properties stimulates neurogenesis in JEV infection, thereby reinstating its immense therapeutic potential in treatment of JE. This observed protective role of Minocycline in JE has led to the initiation of a randomized phase III clinical trial which is being conducted in Chhatrapati Shahuji Maharaj Medical University[47] (formerly King George's Medical College), Lucknow from August 2012 after approval from the Drug Controller General of India.

Neural stem cell transplantation and brain repair

Stem cell research could lead to the development of radical new therapies for several neurodegenerative diseases that currently lack effective treatments. Owing to their proliferative and differentiation properties, NSCs have recently risen to be a promising resource for novel approaches aiming to repair damaged or lost brain cells in a range of pathologies involving exogenous NSCs transplantation and endogenous NSC activation[48,49]. Over the past few years, there has been continuous progress in

developing strategies to generate various human-derived neurons and glial cells that are needed for cell replacement therapy based on pathology in the respective diseases. Although some scientifically founded clinical trials using stem cells to treat neurodegenerative disorders have already been performed or initiated (e.g. for the rare, fatal, autosomal recessive neurodegenerative disorder Batten disease [http://www.clinicaltrials.gov/ct2/show/NCT00337636?term-batten&rank=4]), no stem cell-based therapy has been proven beneficial for any such condition[50]. Therapeutic approaches using stem cells mainly for neuroprotection by supplying neurotrophic molecules or modulating inflammation are under process in the quest for a cure to these disorders. However, the clinical studies on stem cells to treat neurodegenerative diseases require more basic research for a better understanding of the mechanisms regulating the proliferation, migration, differentiation, survival and function of stem cells and their derivatives.

Conclusion

Japanese encephalitis was until recently considered to be a disease restricted only to the Asian and the developing countries but recent reports show it to be an emerging global threat. This review has aimed at how the JEV infection and the ensuing inflammation modulate the fate of NSPCs and the resultant impairment in neurogenesis possibly results in long-term neurological sequelae in JE survivors. It has tried to highlight the effect of JEV infection on the proliferation and differentiation of NSPCs and overall neurogenesis both directly and indirectly. The discovery of the neuroprotective and anti-inflammatory effects of Minocycline and its potential role as a therapeutic agent has also been a major breakthrough in the treatment of JE. With the recent developments in stem cell research there is a huge potential for new therapies, which can be a huge leap in the treatment of neurodegenerative disorders including Japanese encephalitis.

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