行政院國家科學委員會專題研究計畫　成果報告

B 型流行性感冒病毒感染對胚胎神經細胞增殖、移動、凋亡、和分化之效應分析

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摘要

雖然有大量的流行病學調查顯示，流行性感冒和精神分裂症的發生有相關性，不過其中的致病機轉並不清楚。我們先前以 B 型流感病毒 (B/Taiwan/25/99)感染雞胚胎及懷孕母鼠為研究模式，發現流感病毒可直接攻擊胚胎，並可透過胎盤感染鼠胚，造成中樞神經發育異常和其它畸形發育現象。進一步分析，以感染懷孕母鼠、組織培養、和細胞培養為研究模式，發現流感病毒會透過胎盤直接攻擊胚胎導致眼睛及腦部畸形。在本計畫中，我們再進一步分析 B 型流感病毒感染後對神經嵴細胞增殖、移動、凋亡、和分化之效應。我們發現神經嵴細胞增殖並不會受到 B 型流感病毒影響，以 Hnk-1 及 Sox-10 作為標誌分子定量控制組和感染組雞胚胎於 S9 到 S12 的神經嵴細胞數目，兩組並無顯著差異，且感染組雞胚胎也沒有神經嵴細胞發生顯著細胞凋亡之現象。進一步分析神經嵴細胞之移動和分化效應，可以見到其移動受到顯著影響，受感染雞胚胎之神經嵴細胞移動受限而遲緩，並有廣泛性異位及形成團塊狀，和雞胚胎頭部受 B 型流感病毒感染後的實質細胞團塊類似。神經嵴細胞移動受限之結果導致兩側的背部神經結形成不對稱，其影響周邊神經之效應有待進一步探討。本計畫最有意義結果在證明 B 型流感病毒感染胚胎後，不只是中樞神經發育受損，且也會波及周邊神經，值得以此觀點對層出不窮的新型流感病毒感染進行觀察。

關鍵詞: 流感病毒、胚胎畸形、神經病變、神經嵴細胞、細胞增殖、細胞移動、細胞凋亡、細胞分化

Abstract

Despite epidemiological studies implicated an association of prenatal influenza viral infection with adult schizophrenia, little etiopathogenetic evidence was available to support such hypothesis. We performed experimental influenza B viral infection (B/Taiwan/25/99) using chick and mouse embryos to address the question. Influenza B viral infection infections lead to brain, head and eye teratogenesis, either through direct attacks or indirect maternal immune response that affect through the placenta barrier. In this study, we extended to examine whether the peripheral central nervous system can also be affected, particularly in view of neural crest proliferation, migration, apoptosis, and differentiation. By using Hnk-1 and Sox-10 as markers, we quantified to show that the neural crest cell proliferation and apoptosis appeared not affected by influenza B viral infection. In contrast, the migration and differentiation of neural crest cells were significantly affected by the infections. The migration was generally sluggish as compared to their normal controls. Furthermore, cell mass of displaced neural crest cells were seen, comparable to the observation previously reported in the brain. This affected migration caused asymmetrical formation of dorsal root ganglia, which clearly indicated that influenza viral infection may lead to peripheral nervous system anomalies as well, although the functional detriments remained to be characterized. Our data significantly indicated that the peripheral nervous system, apart from the central nervous system, has to be evaluated in case of novel influenza prevalence.

Key words: influenza virus, teratogenesis, neuronal anomalies, neural crest cell, cellular proliferation, apoptosis, migration, differentiation

Introduction

Although a large amount of epidemiologic surveys pointed to potential effects of influenza viral infection in the etiopathogenesis of schizophrenia, the underlying mechanisms have been largely unknown, and whether influenza infections lead to teratogenesis during early pregnancy has not been documented in detail. We performed experimental influenza B viral infection (B/Taiwan/25/99) using chick and mouse embryos to address the question. Influenza B viral infection infections lead to brain, head and eye teratogenesis, either through direct attacks or indirect maternal immune response that affect through the placenta barrier. In this study, we extended to examine whether the peripheral central nervous system can also be affected, particularly in view of neural crest proliferation, migration, apoptosis, and differentiation.

Results and discussions

In the present study, we further characterize the teratogenic effects of influenza B viral infection using both chick and mouse embryo models. An aliquot of 20 μl of influenza B virus (B/Taiwan/25/99, identity of virus confirmed by nucleic acid sequencing) at 5 x 10^8 p.f.u./ml was used to infect chick embryos in ovo by injection into the sub-blastodermal space before neural tube closure at Hamburger-Hamilton stage 9 [4]. The injections were performed essentially without direct injury to the embryos, as revealed by controls following the same procedures without virus [3]. The infected chick embryos and sham-infected controls were incubated separately, and were allowed to develop until analyses.

We have formerly shown that in the CNS eye and brain malformations were found after the infection, with unilateral distribution of viral RNA in the brain neuroepithelium and in the head mesenchyme, as detected by in situ hybridization with a DIG-labeled RNA probe (Roche, Indianapolis, IN, USA) specific for the HA segment of influenza B genome. The unilateral distribution of viral RNA appeared to be due to single-sided exposure of the embryos to the virus, as chick embryos normally turn during development until a single side of embryo facing the sub-blastodermal space [4]. This unilateral effect allowed for a comparison where the non-infected side was regarded as a *quasi* control within the same embryo.

With this regard, we tried to find unilateral teratogenic effects in the peripheral nervous system in this study. We found that HNK-1 (Lab Vision, Fremont, CA, USA), an early marker for the chick neural crest cells, was also asymmetrically distributed in the neural crest cell areas of the early S9, S10, S11, S12 chick embryos following experimental infections. We further try to detect distributions of apoptotic cells using an in situ apoptosis detection kit (Roche, Indianapolis, IN, USA). Unlike the extensive signals of apoptosis found in the brain region, we observed on no significant increase of apoptotic activities in the neural crest cells, as compared to the sham-infected controls. We then tried to determine whether influenza B virus infection might affect neural crest migration and differentiation. We co-localized, by immunocytochemistry, the viral RNA with signals of HB9 (motor neuron marker) (Developmental Studies Hybridoma Bank, Iowa, IA, USA), GFAP (astrocyte marker), and S-100 (astrocyte marker) (Lab Vision, Fremont,

CA, USA) in the spinal cord of chick embryos after infection. The results showed that both motor neuron and astrocyte lineages could be targeted by the influenza B virus (figure 1; S to V). By using Hnk-1 and Sox-10 as markers, we quantified to show that the neural crest cell proliferation and apoptosis appeared not affected by influenza B viral infection. In contrast, the migration and differentiation of neural crest cells were significantly affected by the infections. The migration was generally sluggish as compared to their normal controls. Furthermore, cell mass of displaced neural crest cells were seen, comparable to the observation previously reported in the brain. This affected migration caused asymmetrical formation of dorsal root ganglia, which clearly indicated that influenza viral infection may lead to peripheral nervous system anomalies as well, although the functional detriments remained to be characterized.

Despite increasingly accumulated epidemiological surveys in recently years pointing to prenatal influenza viral infection as a causal factor of schizophrenia, little evidence regarding the underlying etiopathogenesis was provided to support such hypothesis. Fatemi et al. reported serial observations following maternal exposure of mouse embryos to human influenza virus [6-10]. They found altered expression of synaptosome-associated protein 25 kDa (SNAP-25), nNOS, and Reelin protein in different regions of the developing brain, as well as altered GFAP immunoreactivity in the developing brains of neonatal mice following prenatal viral infection in utero during the secondary trimester. Aronsson et al. [11] reported persistence of viral RNA in the brain of offspring to mice infected with influenza A/WSN/33 during the second trimester of pregnancy, giving evidence of direct access. Whereas Shi et al. [12] found that maternal influenza infection caused marked behavioral and pharmacological changes in the offspring, and at least some of the behavioral changes were probably via an effect of the maternal immune response on the fetus.

We had shown in a previous study that, given direct access by injecting the virus into sub-blastodermal space of chick embryo, influenza B virus (B/Taiwan/25/99) might cause teratogenesis in the eye and brain [3]. The HA segment of viral RNA was directly localized in the neural retina, brain, head surface ectoderm, spinal cord, and lung bud [3]. In the present study, we show altered neural crest migration and extensive apoptosis in the head neuroepithelium and mesenchyme. In addition, viral RNA was detected in the mouse embryos shortly after implantation. The present data demonstrate that transplacental infection may occur, as reported by Aronsson et al. [11] in a mouse model during the second trimester of pregnancy. Furthermore, the present data extend the potential influenza viral access period to the first trimester of pregnancy, shortly after implantation. The transplacental effects of direct viral attack was investigated and the extensive apoptotic activities was shown. Similarly, we isolated maternal serum after viral infection, and found that maternal immune response against the viral particles had exerted similar apoptotic effects on embryonic cells as well as on O2A cells under in vitro conditions. Our result indicated that both effects may be responsible for the teratogenesis of embryos and implied that clinical should deal with both effects simultaneously to prevent any potential aftermath, if any. The teratogenic effects of influenza viral infection are not only supported by studies from animal

models, but also are supported by evidence from postmortem analyses of human fetuses. Nakai et al. [13] investigated glial reaction and apoptosis in postmortem brains of 2 cases of acute necrotizing encephalopathy, 6 cases of influenza encephalopathy, and 5 controls. They found increased apoptosis in neurons and glial cells in four brains with influenza encephalopathy. In addition, they found that the increase in microglia was greater in TUNEL-positive brains than in TUNEL-negative brains [13]. Levine et al. [14] conducted in vitro studies showing that human Schwann cells can be infected with human influenza A virus. Another study by Brask et al. [15] demonstrated the changes in calcium currents and GABAergic spontaneous activity in cultured rat hippocampal neurons after a neurotropic influenza A virus infection. Taken together, these evidences from animal embryo models, human fetuses, and in vitro studies raise concerns of influenza viruse as a teratogenic agent during pregnancy.

Obviously, the underlying mechanisms for the etiopathogenesis of schizophrenia after influenza-viral infections have to be further elucidated. We don't know exactly how apoptosis of head mesenchyme or altered expression of SNAP-25, nNOS, and Reelin proteins in different areas of the developing brain may cause neuronal dysfunctions leading to schizophrenia, nor do we understand the significance of altered GFAP immunoreactivity. Moises et al. [16] hypothesized glial cells as the locus of the genes-environment interactions in schizophrenia, with glial asthenia as an important factor for the genetic liability to the disorder. Their hypothesis may be supported by the previously reported alteration of GFAP (an astrocyte marker) immunoreactivity [10] and increased apoptosis in the glial cells [13]. Tkachev et al. [17] reported that brains with schizophrenia and bipolar disorders exhibited downregulation of key oligodendrocyte and myelination genes, including transcription factors that regulate these genes. Such downregulation may be resulted from the extensive apoptosis after influenza viral infection, as demonstrated in the present study and in the postmortem analysis by Nakai et al. [13]. The effect of direct viral access is to be distinguished from indirect effects from maternal immune responses. Alternatively, both direct and indirect effects, if it is the case, are to be confirmed. More importantly, whether influenza viral infections cause neuropsychiatric disorders and/or neurodegenerative diseases other than schizophrenia remains to be characterized. Another concern will be the effects of influenza viral infection on the peripheral nervous system development, which is also of equal significance and has to be evidenced with animal models to warrant clinical evaluations. In this regard, we have clearly demonstrated that, at least in the animal models used in this present study, the peripheral nervous system can potentially be also affected by influenza viral infections.

To conclude, our data significantly indicated that the peripheral nervous system, apart from the central nervous system, has to be evaluated in case of novel influenza prevalence.

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