行政院國家科學委員會專題研究計畫　成果報告

B 型流行性感冒病毒導致中樞神經發育異常之探討(1/2)

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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爲研究模式，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。 雖然我們直接觀察到病毒 RNA 直接存在於胚胎中，但是我們無法排除上述的異常和畸形現象，可能部份原因是病毒感染母鼠，導致母鼠免疫反應透過胎盤傷害到胚胎，或是傷害到胚胎賴以維生的子宮或胎盤所導致。 另外，目前文獻皆傾向假設神經膠原細胞缺陷導致精神分裂症的發生，但是我們先前發現至少在神經管中，神經膠原細胞和神經細胞兩者皆有病毒 RNA 存在其中，是否兩者受到影響導致精神分裂症的發生有待釐清。 再者，流感病毒或流感病毒所引起的母體免疫反應如何導致神經膠原細胞缺陷，進而引起日後精神分裂症的發生，有待進一步分析。 本計畫以感染懷孕母鼠、組織培養、和細胞培養爲研究模式，發現流感病毒會透過胎盤直接攻擊胚胎導致畸形，也會經由母鼠免疫反應，影響胚胎的中樞神經發育，而二者皆以廣泛的細胞凋亡呈現，不過細胞凋亡並未無針對特定族群。另外，以病毒直接攻擊或是母鼠免疫反應，對 O-2A 細胞導致大量細胞凋亡，但是無直接對分化的影響。

關鍵字: 流感病毒、胚胎畸形原、精神分裂症

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 using chick and mouse embryos to address the question. Both direct viral attacks and maternal humoral immune responses can lead to teratogenesis through placenta barrier. Extensive apoptosis was found in either cases of intervention. Cultured O2A cells subject to direct viral attack and humoral immune response showed similar apoptotic activities, without evident effect on cellular differentiation. We conclude that both transplacental viral attacks and maternal immune responses can lead to influenza B virus-infected teratogenesis. Thus, clinicians may control the teratogenic effects by prevention in both ways.

Keywords: influenza virus, influenza, teratogen, schizophrenia

Although a large amount of epidemiologic surveys pointed to potential effects of influenza viral infection in the etiopathogenesis of schizophrenia [1], the underlying mechanisms have been largely unknown, and whether influenza infections lead to teratogenesis during early pregnancy has not been documented in detail [2].

If influenza viruses are to interfere with embryogenesis, potential pathways are likely to include either directly access to the embryos or indirectly act through detrimental effects against the embryos from maternal immune responses, or both. We had shown in a previous study that, given direct access, influenza B virus (B/Taiwan/25/99) could cause teratogenesis in the early chick embryos [3]. Following experimental infection, gross malformations in the eye and brain were significantly increased at 48 h after infection, in contrast to the sham-infected controls. Direct localization of viral RNA was found in the neural retina, brain, head surface ectoderm, spinal cord, and lung bud, using the RNA probe specifically for the hemagglutinin A segment [3]. Despite that the chick model offers evidence of influenza B viral-induced teratogenesis, it remains largely unknown whether a comparable event occurs in the eutherians, where a placental barrier protects against direct access of viruses to the embryo. Particularly, the placental barrier is not fully formed shortly after implantation, which may pose a potential risk of viral targeting through direct access.

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. The infected embryos showed eye and brain malformations (21/25), 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 (figure 1; A to H). 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 viral-infected embryos. We

found that HNK-1 (Lab Vision, Fremont, CA, USA), an early marker for the chick neural crest cells, was asymmetrically distributed in 16 out of 20 viral-infected chick embryo heads (figure 1; I to P). We further detected distributions of apoptotic cells using an in situ apoptosis detection kit (Roche, Indianapolis, IN, USA). Extensive but unilateral signals of apoptosis were observed on transverse tissue sections of the heads from viral-infected embryos, in contrast to the sham-infected controls (figure 1; Q, R). We then tried to determine whether influenza B virus targeted specific cell lineages of the neuroepithelium. 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).

Another aim of the present study was to know whether transplacental infection could occur shortly after implantation in the mouse and the aftermath of this early targeting. We injected 200 μl of the same virus used for the chick embryo model into tails of pregnant ICR mice at E5.0 (noon of the day a vaginal plug was found was designated as E0.5). The injection resulted in reduction of fetal size (145/172) in viral-infected fetuses at E9.0 (figure 2, A and B). We also found twisted neuroepithelium in the

brain (81/172) and the spinal cord (77/172) of the fetuses after the injection (figure 2, C, indicated by arrows). Between E9.0 to E9.5, viral RNA could be localized in the fetus, notably in the head and the heart, but in some cases viral RNA extended to the other regions (figure 2, D-F). The viral RNA could be detected as early as E6.5 in the extra-embryonic ectoderm (figure 2, G-J). Evident distribution of viral RNA was also detected in the placenta, surface ectoderm and presumptive migratory neural crest cells of embryos, as well as in the amniotic and chorionic membranes (figure 2, J to L).

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.

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Self-assessment of project outcome

We aimed at answering three questions in this project--(I) to distinguish viral-induced maternal effects from direct targeting of virus, or to confirm that effects from both sides are responsible for the viral-induced malformations observed in our previous studies, particularly in the developing CNS; (II) to determine whether differentiation of neural precursor cells is altered with influenza B viral infection and, if this is the case, to understand whether the altered differentiation is through effects directly from viral targeting or indirectly from maternal immune responses against the viral infection; and (III) to understand specifically how the differentiation of the glial lineage is affected by influenza B viral infection. Our results in the present study have answered the first two questions and have thus provided some clinical significance, if these data are applicable to clinical situations. However, the relationships between influenza viral infect and schizophrenia is still not known, and has to be further explored. Despite that, we have submitted current results in two manuscripts to two peer-reviewed international journals.

Figure 1. Teratogenic effects of influenza B viral infection in the chick embryo model. Chick embryos at Hamburger-Hamilton stage 9 were infected with influenza B virus by in ovo injection of 20 μl of influenza B virus (B/Taiwan/25/99) at 5 x 10^8 p.f.u./ml into the sub-blastodermal space. The injection was performed essentially without direct injury to the embryos, as controlled by sham-infections without the virus. The viral-infected and sham-infected embryos were incubated separately before analyses. A to H are from an infected embryo. I to P are from another infected embryo. Q and R are from different embryos. S and U are from the same embryo. T and V are from the same embryo. A to P are at Hamburger-Hamilton stage 15; S to V at stage 25. A, B: an infected embryo exhibited unilateral abnormality in the eye, as indicated by an arrow. The eye primordium in A appeared to be abnormal, without evident lens placode formation comparable to that of B. C, D: unilateral distribution (blue color) of viral RNA in the head region, as shown by the photographs taken on the same focus. The abnormal eye was on the side where viral RNA was located. E to H: tissue sections of the same embryo showing distribution of viral RNA in the head neuroepithelium and mesenchyme (indicated by arrow in H). Viral RNA was not detected in the otic vesicle (indicated by arrow in C). I to L: dorsal (I), ventral (J) and lateral views (K, L) of asymmetrical distribution of HNK-1 (early marker for migrating neural crest cells) positive cells (blue color) in a virus-infected embryo, as

detected by immunohistochemistry. M to P: sections at different levels of the same embryo as shown in I to L, showing asymmetrical distribution of HNK-1 positive cells. Q: unilateral detection of apoptotic cells (green color, indicated by arrow heads) in a viral-infected embryo, in contrast to the even distribution in a sham-infected control in R. S to V: co-localization of viral RNA (blue color) with immunohistochemically positive (brown color) cells of HB-9 (motor neuron marker, in S), S-100 (astrocyte marker, in T), and GFAP (astrocyte marker, in U and V). Abbreviations: ba, branchial arch; fb, forebrain; hb, hindbrain; ht, heart; mb, midbrain; ov, otic vesicle. Scale bars: D and L, 200μm; H and P, 100μm; R, 200μm; T, 100μm.

Figure 2. Teratogenic effects of influenza B viral infection in the mouse embryo model. A preparation of 200 μl of the same virus used for the chick embryo model was injected into tails of pregnant ICR mice at E5.0 (noon of the day a vaginal plug was found was designated as E0.5), followed by analyses at E 6.0, E6.5, E9.0, and E9.5. Sham-infections were performed as controls without virus. A, B: reduction of embryo size in the viral-infected embryos in B as compared to the sham-infected control in A. C: twisted neuroepithelium (arrows) in the head and trunk region in a viral-infected embryo. D, E, F: distribution of viral RNA, notably in the head region and heart of the infected embryos. G, H: viral RNA distribution in wholemount preparations of sham-infected (G) and viral-infected (H) embryos. I, section of the embryo in H, showing viral RNA distribution in the extra-embryonic ectoderm (arrows). J to L: viral RNA distribution (arrows and arrowheads) on a tissue section of viral-infected embryo at E9.5. In K, viral RNA is detected in the placenta, amniotic and chorionic membranes. J is a magnification of the framed area in K, showing localization of viral RNA in the surface ectoderm and presumptive migratory neural crest cells. L is a magnification of an area of the placenta in K. Gestation stages of embryos: A to E, E9.0; F and J to L, E9.5; G and I, E6.0; H, E6.5. Abbreviations: ba, branchial arch; ec, egg cylinder; ee, extra-embryonic ectoderm; etc, ectoplacental cone; ht, heart; pl, placenta. Scale bars: A to F, 500μm; G to I, 50μm; K to L, 100μm.

Figure 1

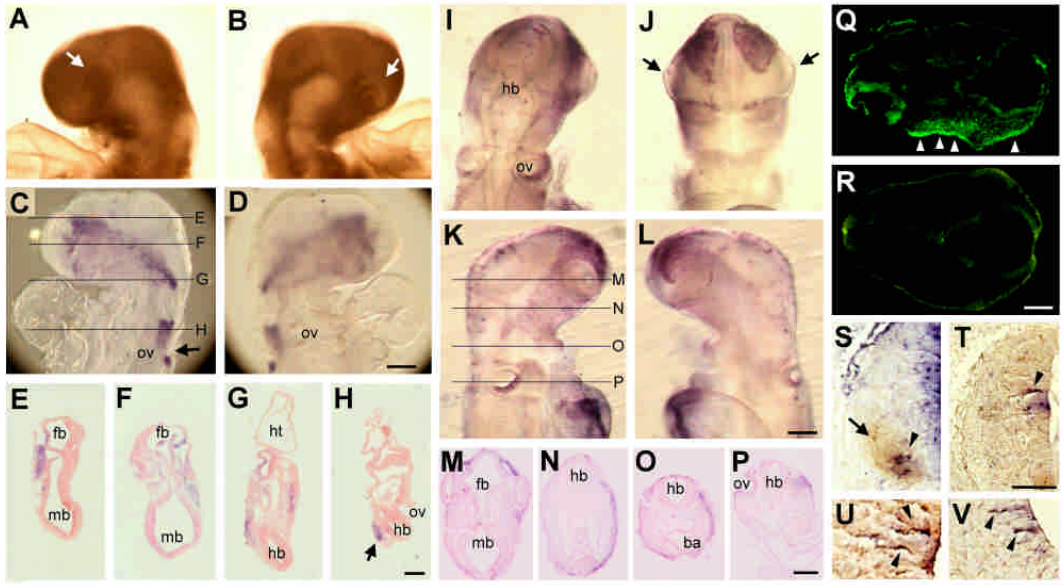

Figure 2