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Intracisternal Administration of Anti-c-Met Antibody Decreases Reelin and Disabled 1 Expression in the Cerebral Cortex; an *in vivo* Study

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Abstract: Hepatocyte growth factor (HGF) and its receptor, c-Met, are widely expressed in the developing brain. HGF enhances cell proliferation and cell growth, stimulates cell migration and motility. Expression of mRNA for HGF has been seen in the rat brain. Neurons and glia produced in the neuroepithelium migrate along radial glial fibers into the cortical plate. Reelin, a glycoprotein which is produced by Cajal-Retzius (CR) cells in the marginal zone directs neuronal migration indirectly via the radial glial cells. It has been demonstrated that Disabled 1 (Dab1) functions downstream of reelin in a tyrosin kinase signal transduction pathway that controls appropriate cell positioning in the developing brain. In this study, intracisternal administration of anti-c-Met antibody on reelin and Dab1 expression in the cerebral cortex has been studied. Using Western blotting, it was shown that the expression of reelin and Dab1 is decreased in response to infusion of antibody when compared to control group. It is concluded that c-Met is essential for reelin and Dab1 expression in the cerebral cortex of the newborn mouse. Moreover, this method may be applied to the other factors and different central nervous system (CNS) regions, allowing identification of molecules involved in neural cell migration.

Key words: c-Met · Hepatocyte growth factor · Reelin · Dab1 · Cerebral cortex · Neural cell migration

INTRODUCTION

There has been considerable recent progress in understanding the processes involved in cerebral cortical development. Attention has been focused on two main area of research. The first of these is brain stem cells, found in the neural tube and ventricular zone of the cerebral cortex, and the mechanisms involved in their proliferation and differentiation into glia and neurons [1]. The second area of research is the signals involved in the migration of neurons from the germinal epithelium into the cortex. Neurons and glia produced in the neuroepithelium migrate along radial glial fibers through the subplate and into the cortical plate. They migrate past previously laid down neuronal layers to reach the Cajal-Retzius (CR) cells of the marginal cells [2]. Abnormal migration, neurogenesis, connecting with CR cells result in the disordered development of the cortex. Reelin is involved in the migration of neuronal precursor and stratification in the cortex [3]. CR cells are located in the molecular layer of the cortex and secrete the glycoprotein reelin. Reelin is a large extracellular matrix molecule and encoded by reelin gene. Apolipoprotein E receptor 2 (Apo ER2) and very-low-density lipoprotein reelin function as neuronal receptors for a secreted glycoprotein reelin during development [4].

Disabled 1 (Dab1) is a cytosolic protein that activates tyrosin kinases. The response of cortical plate cells to reelin requires the tyrosine kinase adaptor Dab1. Cyclin-dependent kinase 5 (Cdk5) and its activator p35 are necessary for the development of the cortical plate, probably at a later stage than reelin/Dab1. It has been shown that Dab1 functions downstream of Reelin in a tyrosin kinase signal transduction pathway that controls appropriate cell positioning in the developing brain [5]. Reelin sends directly or indirectly some message to migrating cortical plate neurones. The signal is then relayed within the target cell where it initiates a cascade of events, involving the Dab1 tyrosine kinase adapter, and eventually instructing post-migratory neurones to detach from their radial guides, assume a radial orientation, and form a dense, well-organised cortical plate. Later-generated neurones migrate over larger distances, which require a more intimate interaction with radial guiding fibres as well as more refined machinery for migration, particularly at the level of the cytoskeleton. Cdk5, its p35 activator, and presumably other cofactors would be required for this later migration [6].

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Several growth factors have been implicated in the processes of cortical cell migration, differentiation, proliferation and survival [7]. The central nervous system (CNS) of vertebrates originates from neuroepithelial cells located within the embryonic neural tube. Progenitor cells located within the germinal epithelium lining the dorsal portion of telencephalic vesicles give rise to the cerebral cortex. The important issues in neural stem cell studies are the investigation of signals and mechanisms that regulate neural stem or progenitor cell production and migration [8].

C-Met, a member of the tyrosine kinase superfamily, is the receptor for hepatocyte growth factor (HGF), also known as scatter factor (SF). The mature c-Met protein is a disulfide-linked heterodimer with 190 kDa molecular weight [9]. During embryonic development, c-Met is crucial for gastrulation, angiogenesis, myoblast migration and bone remodeling [10]. c-Met is a membrane receptor that is essential for embryogenesis, because *MET*$^{-/-}$ mice die *in utero* due to severe defects in placental development [11]. Activation of c-Met triggers mitogenesis and morphogenesis [12]. C-Met expressing cells enhance proliferation, morphogenesis and migration [13]. HGF is expressed by astrocytes, neurons, oligodendrocytes and microglial cells [14]. It has been shown that HGF plays a role in the tangential migration of interneurons from the ganglionic eminence to the cerebral cortex [15]. HGF is found in the cerebrospinal fluid (CSF) [16]. HGF and its receptor, c-Met, are expressed within the developing cerebral cortex [17]. Molecules in the CSF can enter brain tissue [18]. In this study we examined the role of c-Met in reelin and Dab1 expression in the cerebral cortex employing a specific neutralizing antibody. It was found that blockade of c-Met *in vivo* reduces reelin and Dab1expression in the cerebral cortex, suggesting that the receptor may normally regulates neural cell migration.

MATERIALS AND METHODS

Animals: Balb/c mice were used in this study. They were maintained on 12:12 h light–dark cycle beginning at 8.00 a.m. They were kept at a constant temperature in mouse boxes with unrestricted access to food and water. The colony was maintained through random pair mating. Timed mating was carried out by placing a male and female together in a box and checking for the presence of a vaginal plug. The presence of a plug was taken to indicate successful mating and the time was taken as gestational day zero (E0). All animal procedures were carried out in accordance with the Animals (Scientific Procedure) Act, 1986.

***In vivo* Growth Factor Treatment:** One-day mice received IgG (control) or anti-c-Met antibody (Abcam plc, Cambridge, UK; 2 μg/pups). The antibodies were administered intracranially via cisterna magnum. For intracisternal injection, the atlantooccipital membrane was punctured with Hamilton syringe (Hamilton, Reno, NV). The correctness of needle placement into the cisterna magnum was insured by the presence of CSF in the Hamilton syringe on aspiration before of injections. One day after injections, the pups were harvested after euthanasia by IP injection of an overdose of sodium pentobarbitone and the brains were removed and processed as described. In each of the experimental groups the number of animals investigated was n=20.

Cell Extract: Cerebral cortex were removed from the brain and chopped into tiny pieces and suspended in 0.5 ml of protein lysis buffer [150 mM NaCl, 1.0% NP40, 20 mM Tris (pH 7.5), 5 mM EDTA, and Complete Mini protease inhibitor cocktail (Roche Diagnostics Ltd., West Sussex, UK)] and then mechanically homogenized by sonication. After centrifugation, the protein extracts were recovered and stored at -70°C until they were analyzed.

Western Blotting: For Western blot, protein extracts were separated on 12.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Ltd. Hertford- shire, UK). The membranes were blocked with PBS containing 0.05% Tween 20 and 5% dry milk and probed either with monoclonal mouse anti-reelin antibody (G10; Abcam plc, Cambridge, UK; 1:200 dilution) or anti-Dab1 antibody (Abcam, plc, Cambridge, UK, 1:500 dilution) or a mouse monoclonal anti-β-tubulin antibody (Abcam plc, Cambridge, UK; 1:10,000 dilution) and then treated with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive protein was visualized using the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, UK). Densitometric analysis was performed by scanning immunoblots and quantitating protein bands using an image analyzer. In all experiments, a minimum of 20 animals were taken in order to calculate a mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Analysis of Relative Reelin Expression by Western Blotting: Western blot analysis was performed to evaluate relative reelin expression in the cerebral cortex extracts. A Western blot analysis using anti-reelin antibody as a probe confirmed the presence of reelin in

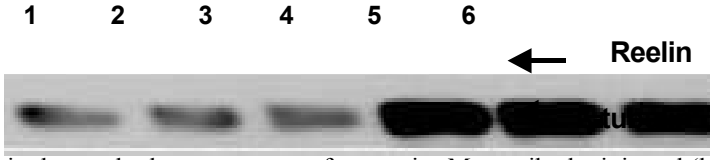

Fig. 1: Expression of eIF4E in the cerebral cortex extracts from anti-c-Met antibody injected (lanes 1 to 3) and control (lanes 4 to 6). ß-tubulin (50 kDa) expression was determined as a protein loading control. In each of the experimental groups the number of animals investigated was n = 20.

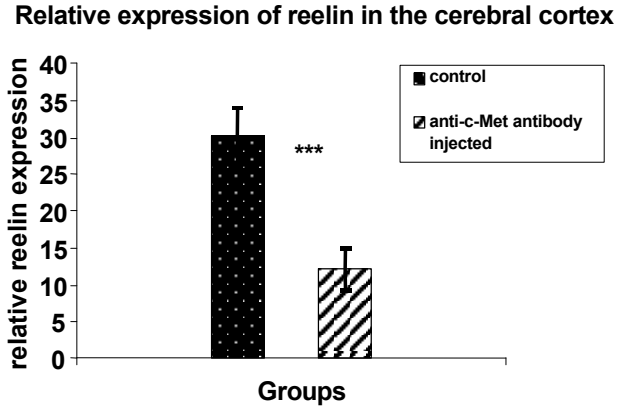

Fig. 2: Relative reelin expression. Signal intensities from control and anti-c-Met antibody injected immunoblotting experiments were determined by densitometric analysis. In each of the experimental groups the number of animals investigated was n = 20. Significant decrease in the reelin expression has been seen in anti-c-Met antibody injected cerebral cortex extracts when compared with control group. Significance values are shown as stars: 3 stars P <0.001.

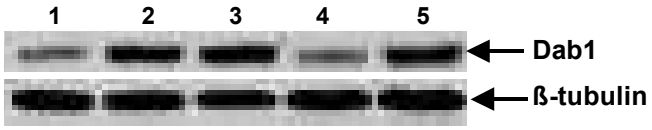

Fig. 3: Expression of Dab1 in the cerebral cortex extracts from anti-c-Met antibody injected (lanes 1and 4) and control (lanes 2, 3 and 5) mouse pups. ß-tubulin (50 kDa) expression was determined as a protein loading control. In each of the experimental groups the number of animals investigated was n = 20.

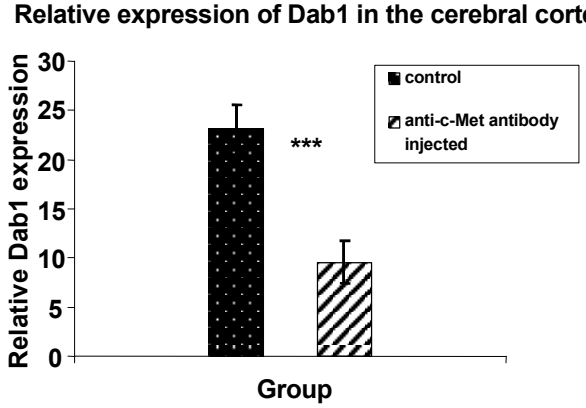

Fig. 4: Relative Dab1 expression. Signal intensities from control and anti-c-Met antibody injected immunoblotting experiments were determined by densitometric analysis. In each of the experimental groups the number of animals investigated was n = 20. Significant reduction in the Dab1 expression has been seen in anti-c-Met antibody injected cerebral cortex extracts when compared with control group. Significance values are shown as stars: 3 stars P <0.001.

the cerebral cortex extracts (Figure 1). An image analyzer was used to determine the intensities of the band in the respective lanes. Quantification of the Western blot bands from repeated experiments (n=20) showed that the expression of reelin was clearly decreased in the anti-c-Met-antibody-injected brain cerebral cortex compared with controls (Figures 2).

Analysis of Relative Dab1 Expression by Western Blotting: Western blot analysis was performed to evaluate relative Dab1 expression in the cerebral cortex extracts. A Western blot analysis using anti-Dab1 antibody as a probe confirmed the presence of Dab1 in the cerebral cortex extracts (Figure 3). An image analyzer was used to determine the intensities of the band in the respective lanes. Quantification of the Western blot bands from repeated experiments (n=20) showed that the expression of Dab1 was significantly decreased in the anti-c-Met-antibody-injected cerebral cortex ompared with controls (Figures 4).

The cerebral cortex develops from two lateral telencephalic vesicles by successive growth, cell proliferation and migration from the germinal epithelium. In an early stage of development, the pallium of the telencephalic vesicles consists of neuroepithelial cells. These cells originally produce pluripotent progenitors generating neuronal and glial precursors, which will give rise to the cells of cerebral cortex [19]. Neurons generated in the germinal epithelium migrate to their final locations in the cortex along radial glial cells that extend right across the wall of the neural tube. Reelin, a glycoprotein which is produced by Cajal Retzius (CR) cells in the marginal zone directs neuronal migration indirectly via the radial glial cells [20]. Neuronal cell migration along radial glial fascicles involves a complex molecular mechanism including cell recognition, membrane adhesion provided by cell adhesion molecules [21].

Dab1 is the reelin intercellular adaptor protein. Dab1 functions downstream of reelin in a tyrosin kinase signal transduction pathway. Decreased Dab1 expression in the anti-c-Met antibody injected extracts in this study may be due to decreased reelin expression, as Dab1 act on downstream of reelin [5].

Growth factors and their associated receptor protein tyrosine kinases can regulate a variety of neuronal functions such as neurite extension and migration [22]. HGF is a cytokine that, via the receptor c-Met, exhibits mitogenic and chemoattractive activities in neuronal cells [23]. HGF and c-Met are widely distributed in developing brain [24]. HGF has been shown to have motogenic effects on migrating cortical neurons [15].

Decrease in the reelin and Dab1 expression in the anti-c-Met antibody injected pups observed in our experiments may be due to blocking the c-Met by anti-c-Met antibody. As HGF/c-Met signaling pathway plays a role in the migration of neurons in the cerebral cortex [15] and c-Met and HGF are expressed within the developing cerebral cortex [17] , injection of anti-c-Met antibody blocks the endogenous c-Met activity and thus blocking HGF-c-Met signal.

This study using neutralizing antibody against c-Met suggests that HGF/c-Met may be important factor in neural cell migration by regulating reelin and its intracellular adaptor protein, Dab1. The antibody recognizes native protein, specifically blocks c-Met. The elucidation of the role of c-Met in the neural cell migration in this study adds to growing literature suggesting an important role of HGF/c-Met in this process. We have shown that injection of anti-c-Met-antibody decreased Reelin and Dab1 expression in the cerebral cortex. The results of this study could have been anticipated with some certainty given the findings of other studies that have investigated the role of HGF and c-Met in the neural cell migration. It is concluded that c-Met is essential for reelin and Dab1 expression in the cerebral cortex of the newborn mouse. Moreover, this method may be applied to the other factors and different CNS regions, allowing identification of molecules involved in neural cell migration.

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