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Laboratory Study

In vivo injection of fibroblast growth factor-2 into the cisterna magna induces glypican-6 expression in mouse brain tissue

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ABSTRACT

The proteoglycans (PGs) are multifunctional macromolecules composed of a core polypeptide and a variable number of glycosaminoglycan chains. In the nervous system, PGs regulate the structural organization of the extracellular matrix (ECM) and modulate growth factor activities and cell proliferation and migration. Most cortical neurons are generated from neural precursor cells that reside in the ventricular zone of the embryonic brain. The proliferation and differentiation of neural precursor cells are regulated by various growth and neurotrophic factors. Fibroblast growth factor-2 (FGF-2) is an important mitogen for cortical neural precursor cells, and glypicans regulate the action of FGF-2 on neural precursor cells. Glypican-6 is one of the most abundant ECM molecules in the brain. In this study the effects of FGF-2 on glypican-6 expression in brain tissue have been investigated. FGF-2 was injected into the cerebrospinal fluid (CSF) through the cisterna magna of mouse pups. Using Western blotting, it was shown that the expression of glypican-6 is increased in response to infusion of FGF-2 into the CSF. The injection of anti-FGF-2 antibody into the cisterna magna decreased glypican-6 expression in brain tissue. The results from this study suggest that glypican-6 is important in regulating FGF-2 activity during cerebral cortical development.

1. Introduction

The extracellular matrix (ECM) not only acts as a physical framework, but also exerts a profound effect on cell shape and behavior, including cell migration, proliferation and differentiation.[1] The major components of ECM are proteoglycans (PGs) and constitute up to 90% of the volume of the ECM.[2]

Evidence is accumulating to suggest that PGs are involved in the cell proliferation, neurite outgrowth and neural network formation in nervous tissue during the development of such tissue.[3,4] More than 25 different PGs have been identified in the brain. PGs are usually classified according to the molecular nature of glycosaminoglycan chains attached to their core protein. There are heparin sulfate proteoglycans (HSPGs), chondroitin sulfate proteoglycans (CSPGs), dermatan sulfate proteoglycans and keratin sulfate proteoglycans.[5,6] Glypican-6, a CSPG, is abundant within the central nervous system (CNS) ECM, and it interacts with a variety of other matrix components.[7,8]

The development of nervous tissues involves a diversity of interactions between neural cells and their environment. Many important interactions occur within the ECM. The interactions between the ECM and growth factors are critical for the development and regeneration of the nervous system.

Fibroblast growth factor-2 (FGF-2) is important in the control of cell growth, differentiation and embryogenesis.[9–13] FGF-2 also stimulates the division of neuronal progenitor cells from both adult and embryonic CNS.[14] Many of the PGs function as modulators of growth factors. The importance of FGF-2-heparin interaction has been demonstrated by the observation that heparin protects FGF-2 from denaturation and enzymatic degradation.[15] FGF-2 stimulates the short-term division of mesencephalic progenitors *in vitro*.[16] PGs are important in regulating the activity of FGF-2 by either interacting with its receptor or modifying its stability and functioning.[17] Inhibition of FGF signaling by SU5402 in *Xenopus* embryos phenocopies aspects of depleting glypican-4 function, such as increased apoptosis of forebrain progenitors.[18]

Glypicans are essential modulators of intercellular communication during embryogenesis. Glypican-4 regulates dorsoventral forebrain patterning by positive modulation of FGF signaling.[18] Glypican-6 transcripts are in all brain compartments.[19] Onset of neurogenesis also coincides with differential expression of glypican genes either in neural progenitors or in differentiating neurons.[8]

FGF-2 promotes *in vitro* proliferation and differentiation of neuronal progenitors, which led us to examine the *in vivo* effect of this growth factor when injected into the CSF of the developing chick

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brain. Molecules in the cerebrospinal fluid (CSF) can enter the brain, which indicates that there are spaces between the ependymal cells.[20] Upon secretion into the ventricles, peptides and growth factors are conveyed by CSF bulk flow to the various regions of the brain.[21] Here we show that the infusion of FGF-2 into the cisterna magna of mouse pups increases glypican-6 expression in the cerebral cortex.

2. Materials and methods

2.1. Animals

Balb/c mice were maintained on a 12 h light to 12 h dark cycle beginning at 8.00 am. They were kept at a constant temperature in mice boxes with unrestricted access to laboratory food and water. The colony was maintained through random pair mating. Timed mating was carried out by placing a male and female together and checking for the presence of a vaginal plug. The presence of a vaginal plug was taken as gestational day zero (E0) and the day of birth was designated postnatal day 0 (P0).

2.2. In vivo growth factor treatment

FGF-2 was administered intracranially via the cisterna magnum. A vehicle solution containing 0.4% trypan blue dye was injected to assess the blood-brain barrier. The newborn pups received immunoglobulin G (IgG) vehicle (control) or recombinant FGF-2 (Sigma-Aldrich, Dorset, UK) (5 μg/pup) or anti-FGF-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (5 μg/pup) at P0. One day after injection, the pups were harvested after euthanasia by intraperitoneal injection of an overdose of anesthetic (sodium pentobarbitone) and the brains were removed and processed as described. In each of the experimental groups the number of animals investigated was $n = 31$.

2.3. Cell extract

Frozen tissue samples (10 mg each) were chopped into tiny pieces and suspended in 0.5 mL of protein lysis buffer (150 mM NaCl, 1.0% NP40 buffer, 20 mM Tris [pH 7.5], 5 mM EDTA, and Complete Mini protease inhibitor cocktail [Roche Diagnostics, West Sussex, UK]) and then mechanically homogenized by sonication. After centrifugation, the protein extracts were recovered and stored at −20 °C until they were analyzed. The protein content was determined by the Bradford assay.

2.4. Western blotting

For Western blot analysis, protein extracts (50 μg/lane) were separated on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hertfordshire, UK). The membranes were blocked with phosphate buffered saline containing 0.05% Tween 20 and 5% dry milk and probed either with monoclonal mouse antiglypican antibody (Abcam plc, Cambridge, UK) (1:500 dilution) or a mouse monoclonal anti-β-tubulin antibody (Abcam plc) (1:10,000 dilution) and then treated with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactive protein was visualized using the Enhanced Chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis was performed by scanning immunoblots and quantifying protein bands using an image analyzer.

2.5. Antibodies and reagents

Avidin-biotin peroxidase complex and diaminobenzidine were purchased from Vector Laboratories (Peterborough, UK). The FGF-2 was purchased from Sigma-Aldrich. The IgG polyclonal antibody was purchased from Abcam plc. Anti-glypican and anti-β-tubulin antibodies were purchased from Abcam, and anti-FGF-2 antibody from Santa Cruz Biotechnology.

2.6. Statistical analysis

All data presented are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Student's t-test and only values with $p \leqslant 0.05$ were considered significant.

3. Results

3.1. Total protein concentration

The total protein concentration in the brain extracts from FGF-2 injected and control animals were determined by the Bio-Rad protein assay based on the Bradford dye mixture (Bio-Rad Laboratories, Hercules, VCA, USA). The total protein contents of controls, FGF-2 and anti-FGF-2 antibody injected mouse brain was 0.98 ± 0.13, 1.01 ± 0.18 and 0.97 ± 0.07 (g/L) respectively. No significant increase in the total protein concentration was seen in the FGF-2-injected brain samples compared with those from a control group of the same age ($p = 0.48$) (Fig. 1).

3.2. Analysis of glypican-6 western blotting

Western blot analysis was performed to quantitatively evaluate glypican-6 expression in the brain extracts. A Western blot analysis using antiglypican-6 antibody as a probe confirmed the presence of glypican-6 (Fig. 2). An image analyzer was used to determine the intensities of the bands in the respective lanes. Quantification of the Western blot bands from repeated experiments ($n = 31$) showed that the amount of glypican-6 was clearly increased in the FGF-2-injected brain extracts when compared with controls (Fig. 3). We also showed that the expression of glypican-6 is de-

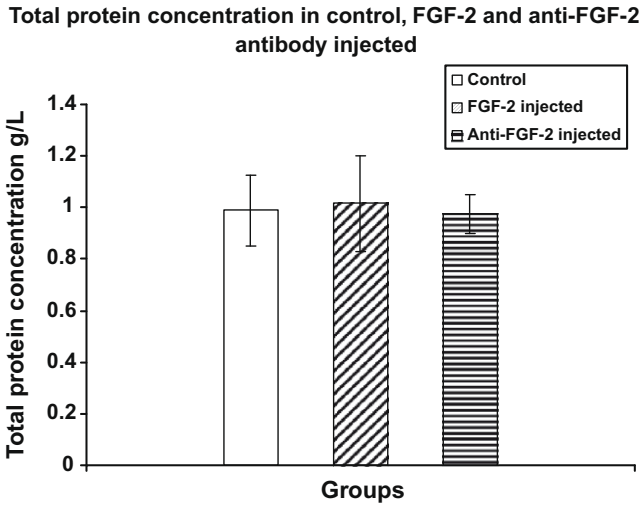

Fig. 1. Total protein concentration in the brain tissues from control (open bar), fibroblast growth factor-2 (FGF-2) (diagonal striped bar) and anti-FGF-2 (horizontal striped bar) antibody injected groups (g/L). No significant difference was seen in total protein concentration between the groups. In each of the experimental groups the number of animals investigated was $n = 31$.