Enhanced Immunoreactivity for Basic Fibroblast Growth Factor (FGF2) in the Adult Rat Spinal Cord in Experimental Syringomyelia

Shin MIYATAKE¹, Shin ONODERA¹, Toshiaki SANO², Shinsuke KATOH³, and Takaaki IKATA³

¹Department of Sports Medicine and Joint Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan
²Department of Human Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan
³Department of Orthopaedics, The University of Tokushima Graduate School, Tokushima, Japan

Synopsis
This study design is experimental study using a syringomyelia model. Fibroblast growth factor (FGF) 2 is an endogenous neurotrophic growth factor in the central nervous system (CNS). The aim of this study was to examine how the spatial and temporal expression of FGF2 in the rat spinal cord changes over the 20-week period following the induction of experimental syringomyelia in rats. Rats were subjected to intracisternal injection of kaolin, which causes syringomyelia. The rats were sacrificed at 0, 3, 7, 10, and 12 weeks, and the spinal cord was histologically examined. The localization of FGF-2 and glial fibrillary acidic protein (GFAP) was also examined by immunohistochemistry. In the normal, slight FGF2 immunoreactivity was observed in a few axons and myelin sheaths in the white matter of the spinal cord. Three weeks after injection, no syrinx formation and little vacuolar degeneration were seen in the white matter, while a mild increase in FGF2 immunoreactivity were in some axons and myelin sheaths. At 7, 10, and 20 weeks, the central canal and the syrinx formation were enlarged. These changes were followed by demyelination. The number of FGF2-positive axons and myelin sheaths increased over time, and this seemed to correlate positively with the progress of the vacuolar degenerative changes. These results showed a detailed expression pattern of FGF2 during the establishment process of experimental syringomyelia, and suggest that a role played by FGF2 in the pathophysiology of this disease.

Key words: syringomyelia; spinal cord; vacuolar degeneration; rat; basic fibroblast growth factor

Introduction
The pathogenesis of syringomyelia remains poorly understood. Currently, the most widely accepted hypothesis is based on the hydrodynamic mechanism proposed by Gardner [1], which was modified by Williams and colleagues [2, 3]. In this hypothesis, the lesion is considered to be hydrosyringomyelia that is caused by central canal dilation and resultant cavitation within the white matter.

The intracisternal administration of kaolin in dogs induces a progressive cavitary disease of the spinal cord [4]. In this experimental model and also in human syringomyelia, the distended central canal usually ruptures in the dorsal and lateral directions [2, 3, 5, 6], which exposes the gray and/or white matters to the syrinx fluid. The histological features of syringomyelia have been frequently recorded [2, 3, 7-9]. Light and transmission electron microscopic studies have revealed the prevalence of edema, which is considered to be one of the factors responsible for the axonal degeneration and demyelination [8, 10] within the neural tissues adjacent to the syrinx.

Cytokines and neurotrophic factors have been
reported to promote neural growth and to protect the spinal cord from ischemic, traumatic, and chemically-induced neural damage [11]. These factors, which are produced and secreted by glial and inflammatory cells, act primarily in a paracrine fashion [12].

Fibroblast growth factor 2 (FGF2), which is the prototype member of the family of heparin-binding growth factors [13], has been extensively studied in relation to its role in the pathophysiology of demyelination and remyelination [14-17]; however, the detailed spatial and temporal expression pattern of this growth factor in experimental animal models of demyelination still remains to be studied. In this study, we examined the detailed expression pattern of FGF2 in syringomyelia. To do this, we created communicating syringomyelia in rats by intracisternal injection of kaolin using the method of McLaurin et al. [4]. We then examined the spatial and temporal changes in FGF2 expression in the spinal cord tissue surrounding the syringomyelic cavity by immunohistochemical analysis.

Materials and methods

**Animals**

Male Wistar rats (n=23) weighing 350-400 g and aged 10-12 weeks were purchased from Charles River Laboratories (Kanagawa, Japan). This study was approved by the Institutional Animal Care and Use Committee of the School of Medicine, the University of Tokushima. The animals were maintained under normal conditions in the central laboratory animal facility throughout the study.

**Induction of experimental syringomyelia**

After anesthesia with an intraperitoneal injection of pentobarbital (Nembutal 0.1 ml/100 g, 60 mg/ml), each rat was fixed in a stereotaxic instrument (SR-5; Narishige Scientific instrument LAB). Experimental syringomyelia was then induced by injecting 0.2 ml of a 50% kaolin (15 μm or less in diameter, Wako Pure Chemical Industries, Osaka) suspension in physiological saline into the cisterna magna using the method of McLaurin et al. [4]. We then examined the spatial and temporal changes in FGF2 expression in the spinal cord tissue surrounding the syringomyelic cavity by immunohistochemical analysis.

**Histology and immunohistochemistry**

The sacrificed rats were fixed by transcardial perfusion with a 10% formalin solution (neutral pH) using a peristaltic pump (AP-2202, ADVANTEC TOYO). The cranial bone and spine containing the brain and spinal cord were extracted en bloc and immersed in a 10% formalin solution (neutral pH) for 1 week. Each of the medullary segments of the spinal cord from the first cervical spinal cord (C1) through to the fourth thoracic spinal cord (T4) was then separately embedded in paraffin. Serial 5-μm thick sections were prepared and subjected to hematoxylin-eosin (HE) and Kluver-Barrera (KB) staining. Immunohistochemical staining was performed by using an ABC kit (Vector Lab., Burlingam, CA, USA), which is based on the avidin-biotin-peroxidase complex (ABC) method. An anti-FGF2 rabbit polyclonal antibody (supplied by Kaken Pharmaceutical) and a rabbit polyclonal antibody raised against cow glial fibrillary acidic protein (GFAP:DAKOPATTS) served as the primary antibodies. After immunostaining, the nucleus was stained with hematoxylin. The maximum diameter of the central canal in each section of the spinal cord was then estimated for all rats.

We counted the number of FGF2-positive cells, cavities (30 μm or larger in diameter), and GFAP-positive cells in each sections from untreated rats to those sacrificed at 20 weeks. They were counted per one field at 40x magnification, which was repeated three times in different fields, and the average number of the three values was designated as the number of the section. Primary antibodies were replaced with normal rabbit immunoglobulin in control sections.

**Statistics**

Statistical analyses were performed with StatView (version J 5.0) using a personal computer. Significance was set at a P value of <0.05.

**Results**

Rats were injected in the the cisterna magna of their spinal cords with a solution of kaolin and then killed 3, 7, 10 or 20 weeks later. After sacrifice, each medullary segment from C1 to T4 of their spinal cords was fixed, sectioned and subjected to histological and immunohistochemical analysis.

**Histology**

Three weeks after kaolin injection, inflammatory granulation tissues adhering to the spinal cord was observed in the subarachnoidal space (Fig. 1a). An only slight demyelination but no syrinx formation were seen in the white matter of the spinal cord (Fig. 1b), and the central canal was not enlarged.
Figure 1. The spinal cord sections from rats sacrificed 3 and 7 weeks after kaolin injection were subjected to histological analysis. 

a: Anterior horn of the C5 spinal cord (3 weeks; HE, 100x). Some anterior horn cells show condensed nuclei that indicate an ischemic change.
b: The posterior funiculus of the C6 spinal cord shows little demyelination (3 weeks; KB, 200x). 
c: The anterior funiculus of the C7 spinal cord (7 weeks; KB, 40x). Higher magnification of (c) (KB, 100x). Several large vacuoles have fused to form the syrinx (arrow).
d: The central canal and posterior funiculus of the C2 spinal cord (7 weeks; HE, 40x). A slit-shaped syrinx in the posterior funiculus, enlargement of the central canal (cc), and discontinuity of the ependymocyte lining can be observed. 

e: The central canal of the C3 spinal cord shows polygonal enlargement (7 weeks; HE, 40x).

Figure 2. The spinal cord sections from rats sacrificed 10 and 20 weeks after kaolin injection were subjected to histological analysis. 

a: The C1 spinal cord (10 weeks; HE, 10x). Adhesive arachnoiditis extends all around the cord. 
b: Higher magnification of the posterior funiculus in (a) (KB, 100x). Extensive vacuolar degeneration is observed within the posterior funiculus. 
c: Higher magnification of the central canal in (a) shows occlusion (HE, 100x). 
d: The C4 spinal cord (20 weeks HE, 10x). Severe Adhesive arachnoiditis to the spinal cord is observed. 
e: Higher magnification of the anterior funiculus in (d) (KB, 40x). Moderate vacuolar degeneration is observed. 
f: Higher magnification of the anterior funiculus in (e) (KB, 100x).
At 7 weeks, exacerbation of the arachnoiditis, significant progression of the demyelination, syrinx formation, and porosity of the medulla of the anterior funiculus were observed (Fig. 1c, d). In addition, the formation of a slit-shaped syrinx in the posterior funiculus and the enlargement of the central canal were observed (Fig. 1e, f). At 10 weeks, further exacerbation of the adhesive arachnoiditis was observed along with more intense demyelination (i.e. vacuolar degeneration) in the white matter (Fig. 2a, b). The cavity of the central canal was enlarged in only one animal; in the other remaining animals, it was constricted or occluded by surrounding granulation tissues (Fig. 2c).

At 20 weeks, severe adhesion of the arachnoid to the spinal cord was observed (Fig. 2d). While demyelination similar to that seen at 10 weeks was observed in several rats, the demyelination in the remaining animals was marginal (Figs. 2e, f). In summary, of the rats examined 7, 10, and 20 weeks after kaolin injection, enlargement of the central canal was seen in 47% (8/17) and formation of the syrinx in the posterior or anterior funiculus was seen in 24% (4/17).

**Immunohistochemistry**

In untreated rats, very few axons and myelin sheaths in the white matter were positive for FGF2 (Fig. 3a, b). However, 3 weeks after kaolin injection, more were positive for FGF2, especially in the posterior funiculus (Fig. 3c, d). At 7 (Fig. 3e, f), 10 (Fig. 3g, h), and 20 (data not shown) weeks, there was a marked increase in the number of FGF2-positive axons and myelin sheaths relative to the number observed at 3 weeks. Rats that showed severe demyelination tended to have more FGF2-positive axons and myelin sheaths around the demyelinated areas. FGF2-positive axons and myelin sheaths were also abundantly present around the syrinx (Fig. 3i, j). With regard to the anterior, posterior and lateral funiculus, FGF2-positive cells were most abundantly present in the posterior funiculus. The number of cavities (30 μm or larger in diameter) in the white matter seemed to correlate positively with the number of FGF2-positive cells (Fig. 4, p<0.05).

We also examined the immunohistochemical localization of GFAP, a marker for astrocytes and some ependymal cells. In untreated rats, GFAP-positive cells were rarely seen in the gray matter, especially in the anterior horn (Figs. 5a, b). In contrast, after kaolin injection, there were more GFAP-positive cells, mainly in the white matter (Fig. 5c, e, g) and in the anterior horn (Fig. 5d, f, h). At 20 weeks after kaolin injection, the number of GFAP-positive cells slightly decreased compared to 10 weeks (data not shown). The GFAP-positive cells seemed to be mainly found in areas where FGF2-positive myelin sheaths were abundant, although their distributions did not always coincide (Fig. 6a, b). Statistically, the number of FGF2-positive cells correlated positively with the number of GFAP-positive cells (Fig. 7, p<0.05).

![Figure 4](image_url) **Figure 4.** The correlation between the number of cavities (30 μm or larger in diameter) and FGF2-positive cells, which were counted in each sections (40x magnification) of spinal cords from untreated rats and treated rats (sacrificed at 3, 7, 10, and 20 weeks).
Figure 3 The spinal cord sections from untreated and treated rats (3, 7 and 10 weeks) were immunostained for FGF2. a: Posterior funiculus of the C6 spinal cord of an untreated rat (40x). b: Higher magnification of (a) (400x). Only a few axons and myelin sheaths are positive for FGF2. c: Posterior funiculus of the C6 spinal cord 3 weeks after kaolin injection (40x). d: Higher magnification of (c) (400x). Axons and myelin sheaths are sparsely positive for FGF2. e: Posterior funiculus of the C6 spinal cord (7 weeks; 40x). f: Higher magnification of (e) (400x). Axons and myelin sheaths show strong immunoreactivity for FGF2, occasionally accompanied by an enlargement in size. g: Posterior funiculus of the C6 spinal cord (10 weeks; 40x). h: Higher magnification of (g) (100x). Axons and myelin sheaths show strong immunoreactivity for FGF2, occasionally accompanied by an enlargement in size and vacuolar degeneration. i: Posterior funiculus and syrinx of the C3 spinal cord (10 weeks; 100x). The syrinx, which has formed in the posterior funiculus, is surrounded by many vacuoles. j: Higher magnification of (i) (400x). Strong immunoreactivity for FGF2 is observed in non-vacuolated axons and myelin sheaths.
Figure 5  The spinal cord sections of the C1 spinal cord from untreated rats and treated rats (3, 7, 10, and 20 weeks) were immunostained for GFAP. a: An untreated rat (20x). In the gray matter, GFAP-positive cells are rarely observed. b: An untreated rat (100x). In the anterior horn, no GFAP-positive cells are observed. c: Three weeks after kaolin injection (100x). In the white matter, cells extending thin GFAP-positive processes are observed (arrow). d: Three weeks after kaolin injection (100x). In the anterior horn, GFAP-positive cell numbers are slightly increased. e: Seven weeks after kaolin injection (100x). In the white matter, GFAP-positive cells are increased in number and their processes are thicker. f: Seven weeks after kaolin injection (100x). In the anterior horn, GFAP-positive cells are markedly more numerous. g: Ten weeks after kaolin injection (100x). In the white matter, GFAP-positive cells are markedly more numerous and their processes are thicker and longer. h: Ten weeks after kaolin injection (100x). In the anterior horn, GFAP-positive cells are even more numerous compared with those at 7 weeks.

Figure 6  Immunostaining of the posterior funiculus and syrinx of the C3 spinal cord (10 weeks ). The sections were immunostained with either FGF2 (A, 200x) or GFAP (B, 200x). The GFAP-positive cells were distributed mainly in the areas where FGF2-positive myelin sheaths were abundant, although their distributions did not always coincide.
Discussion
In the present study, we used immunohistochemical analysis to investigate the expression and localization of FGF2 in rats during the development and progression of experimental syringomyelia. Our results indicate that intense FGF2 positivity was observed in the axons and myelin sheaths around vacuolar degeneration sites in the white matter and around the syrinx. After kaolin injection of rabbits that results in experimental hydrosyringomyelia, the following ultrastructural changes of the spinal cord surrounding the syrinx were recorded [1]: 1) the perivascular spaces around the syrinx and in the dorsal white matter were enlarged, 2) oligodendrocytes remained undamaged, and 3) remyelination was observed in some denuded axons, although it was incomplete in areas with severe edema. Although we did not examine the ultrastructural changes in the spinal cords of our rats, these neuropathological findings are largely consistent with ours.

Following axonal damage to the CNS, spontaneous regeneration does not appear to occur, [18] although several endogenous neurotrophic growth factors have been reported to promote regeneration, repair, and maintenance of neuronal integrity after injury [19]. FGF2 was expected as one such growth factor that exhibits potent neurotrophic activity in the CNS and play a role in the recovery of the CNS after injury [20]. However, Goddard et al. [15] reported that injection of FGF2 into the cerebrospinal fluid in rats resulted in a severe loss of myelin the caudal anterior medullary velum, suggesting a negative role of FGF2 in the demyelination. Armstrong et al. [16] and Murtie et al. [17] reported using FGF2 null mice that FGF2 deletions induce more frequent oligodendrocyte differentiation and improved remyelination in murine demyelination models. These suggested a role of FGF2 to inhibit oligodendrocyte lineage differentiation during myelination.

In untreated rats, only a few axons and myelin sheaths in the white matter showed some weak FGF2 staining, while none of the neuronal cells and glial cells in the gray matter were positive. In contrast, in the kaolin-injected rats, the remaining myelin sheaths and axons around the intense vacuolar degeneration that appeared in the white matter and around the syrinx were strongly positive for FGF2. Both the number of FGF2–positive axons/myelin sheaths and their immunoreactivity for FGF2 increased over time, and this correlated positively with the progress of the vacuolar degenerative changes in the white matter. Although it is impossible from this study to refer to the role of FGF2 played in the pathophysiology of syringomyelia, these observations at least might suggest that FGF2 expression in the axons and myelin sheaths may be related to the vacuolar degenerative changes in the white matter after the induction of experimental syringomyelia. Moreover, we found that at 10 weeks after kaolin injection, GFAP-positive cells were mainly distributed in areas around the syrinx, where bFGF-positive

![Figure 7](image.png)

**Figure 7** The correlation between the number of GFAP-positive cells and FGF2-positive cells, which were counted in each sections (40x magnification) of spinal cords from untreated rats and treated rats (sacrificed at 3, 7, 10, and 20 weeks).
myelin sheaths were abundant. This finding, which is consistent with the reports by Messersmith et al. [14], suggests that FGF2 may play a role in directly regulating oligodendrocyte lineage cell responses or may act through paracrine or autocrine effects in astrocytes, which are known to synthesize other growth factors and immunoregulatory molecules that influence oligodendrocyte lineage cells.

In conclusion, we observed enhanced immunoreactivity for FGF2 in the axons and myelin sheaths around the areas of vacuolar degeneration in the white matter and around the syrinx in rats with experimental syringomyelia. These findings might be instructive when referring to the detailed expression pattern of this growth factor after induction of syringomyelia.

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References

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Corresponding author:
Shin Miyatake, MD, Ph.D
Department of Sports Medicine and Joint Surgery,
Hokkaido University Graduate School of Medicine,
Sapporo 060-8638, Japan
Tel: +81-11-706-7211
Fax: +81-11-706-7822
E-mail: miyashin@med.hokudai.ac.jp