

## Neuronal Apoptosis Inhibitory Protein(NAIP) Gene Expression and Spinal Cord Development

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### Abstract

**Background/ Aims:** Apoptosis plays multiple roles for neuronal development and maintenance through several pathways which have complicate cross-linkage each other. Neuronal apoptosis inhibitory protein (NAIP) is believed to inhibit the normal process of apoptosis, which is the disintegration of single cells that results from programmed cell death, in motor neuron. Clinically NAIP gene has been discovered as a candidate gene for spinal muscular atrophy (SMA), a childhood genetic disorder characterized by motor neuron loss and progressive paralysis with muscular atrophy. This study attempted to elucidate the function and its role of NAIP during mouse spinal cord in pathogenesis of SMA. **Methods:** Female CD-1 mice at 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 16.5, 18.5, 20.5 and 22.5 of time pregnancy were obtained after mating. And the spinal cords from the mice of 1,2,5,20,22 days after birth were also used. The immunohistochemistry of NAIP was performed after NAIP antibody. This antibody was generated by gene immunization using NAIP cDNA and the recombinant NAIP was used for the booster injection. Rat NAIP cDNA fragment (1-488 bp in Fig. 2) was subcloned into the pET-28 C (Novagen, USA) and into the pcDNA-3 (Novagen, USA) utilizing the Bam HI and Hind III sites. Rabbits were first immunized with 300 g of pcDNA3-NAIP and 30 days later boosted twice with affinity purified pET-28C recombinant protein in incomplete Freund's adjuvant. Titers of anti-NAIP antibody were monitored by western blot analysis using SDS-PAGE purified recombinant NAIP. **Results:** Although all stage of murine spinal cord development appeared to express NAIP, pronounced elevation of NAIP expression was observed in the E9.5-14.5 E14, NAIP were detected in the neuroepithelium of neurotube. It was later localized in the dorsal root ganglia of E12.5 and E11.5 embryo as well as in the mantle layer of the spinal cord. Declining immunoreactivity of NAIP in intensity and extent was also observed after E16.5 in spinal cords, but still expression has been conserved to postnatal 22 days. NAIP were detected in the neuroepithelium of neurotube. This pattern was the same in E10.5 embryo with expression in the neurotube progressing more caudally. It was later localized in the dorsal root ganglia of E12.5 and E11.5 embryo as well as in the mantle layer of the spinal cord. **Conclusions:** This study provides an evidence that the protein of NAIP gene seem to have important roles during murine spinal cord development, suggesting anti-apoptotic role in SMA.

**Key words :** NAIP, Immunohistochemistry, Mouse spine development.

## 서 론

### 1. Introduction

SMA is the most common childhood hereditary neurodegenerative disorder which is characterized by early

degeneration of motor neurons in the spinal cord and brain stem 1. Recently, two candidate genes for SMA, survival motor neuron (SMN) and neuronal apoptosis inhibitory protein<sup>23</sup>, have been identified. There have been reports indicating the correlation between the neurons showing high level NAIP-immunoreactivity and those neuronal subpopulations<sup>33</sup> that have been reported to display histopathological alterations in severe forms of SMA<sup>41</sup>. An autosomal recessive disorder, spinal muscular atrophy (SMA), a clinically and genetically heterogeneous group of

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neuromuscular diseases, is a disorder of motor neurons characterized by degeneration of spinal cord anterior horn cells and muscular atrophy<sup>1)</sup>. This malady occurs on deletion of this gene and apparent unrestricted apoptosis. The manner it acts is unclear since NAIP does not interact with caspases-3 or-7.<sup>2)</sup> Whether the NAIP gene protein and other apoptosis associated proteins are directly involved in the initial stages of neuron degeneration and apoptosis, or acting downstream on the pathological pathway, has been difficult to determine. Therefore, this study attempted to investigate the role of NAIP during mouse spinal cord development to elucidate the apoptosis processes in pathogenesis of SMA.

## Material and methods

### Animals

Female CD-1 mice at 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 16.5, 18.5, 20.5 and 22.5 of time pregnancy were obtained after mating. And the spinal cords from the mice of 1,2,5,20,22 days after birth were also used. Mice were housed in a specific pathogen-free environment and all experiments were performed in accordance with the guidelines of the Korean Council on Animal Care. The uteri were collected in cold, RNase free, phosphate buffered saline and the embryos were dissected free of extraembryonic membranes in the same solution. Embryos were fixed in formaline for sectioning or fixed in 4% paraformaldehyde overnight at 4°C for whole mount for paraffin section.

### A. NAIP expression by immunohistochemistry

#### Tissue preparation

A halves of Embryos and spinal cords for the paraffin section for routine hematoxyline-eosin and immunohistochemical investigations were fixed by 10% phosphate-buffered formalin (PBF) for 1-2 days in 10% PBF. The fixed tissues were processed in an automatic

tissue processor and carefully embedded in the paraffin blocks

### Generation of NAIP antibody

A polyclonal rabbit antibody (made by Dr. BG Choe) was used to detect NAIP immunoreactivity. This antibody was generated by gene immunization using NAIP cDNA and the recombinant NAIP was used for the booster injection. Rat NAIP cDNA fragment (1-488 bp in Fig. 2) was subcloned into the pET-28 C (Novagen, USA) and into the pcDNA-3 (Novagen, USA) utilizing the *Bam* HI and *Hind* III sites. Rabbits were first immunized with 300 µg of pcDNA3-NAIP and 30 days later boosted twice with affinity purified pET-28C recombinant protein in incomplete Freund's adjuvant. Titers of anti-NAIP antibody were monitored by western blot analysis using SDS-PAGE purified recombinant NAIP.

### Immunohistochemistry of NAIP

Paraffin sections were deparaffinized in xylene, hydrated in a graded series of ethanol (100, 95, 80 and 70%) and PBS. Sections were incubated for 30 min in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. Then the tissue sections were washed in PBS and antigen retrieval was in two ways: with or without microwave irradiation (370 W, 10 min in 0.01 M citrate buffer, pH 6.0). After two PBS washes, the sections were incubated overnight at 4°C with the NAIP (diluted 1:200) primary antisera. Immunolabeling was detected using biotinylated universal immunoglobulins followed by visualization with a streptavidin peroxidase kit (DAKO LSAB kit). The sections were counter-stained in hematoxylin, and mounted with Canada Balsam.

## Results

### A. Expression of NAIP in the developing and postnatal spinal cords..

1) Expressions and Distribution of NAIP-immunoreactivity in the developing spinal cord

Generally, NAIP protein were detected in the cell cytoplasm at various stage of the developing spinal cord.

1. Early in development(E6.5-9.5), NAIP protein was present in the neuroepithelium of the spinal cord.
2. E 9.5-14.5: NAIP expression was observed in the neuroepithelium of the neural tube and spinal cord from E9.5 to E14.5. In E9.5 samples, NAIP were detected in the neuroepithelium of neurotube. This pattern was the same in E10.5 embryo with expression in the neurotube progressing more caudally. It was later localized in the dorsal root ganglia of E12.5 and E11.5 embryo as well as in the mantle layer of the spinal cord. Analysis of E12.5 and 13.5 embryos revealed the presence of NAIP in the mantle layer of the spinal cord. E13.5 and E14.5 embryo showed NAIP expressions along the spinal cord, in the mantle layer of the spinal cord of the lumbo-sacral region as well as in the dorsal root ganglia. Strong immunoreactivity was also seen around the central canal of the neural tube. The mantle layer of the embryonic spinal cord contains post-migratory and migrating young neurons as well as post mitotic young neurons and glioblasts destined to become the gray matter of mature spinal cord including motor neurons. (Fig.1-3.)
3. The expression of in the spinal cords after E16.5 embryo sections decreased notably relative to E14.5 embryo as well as as dorsal root ganglia



Fig.1. In E10.5 shows immunoreactivity for NAIP in dorsal root ganglia (→) and mantle layer(←). (ABC,×100).



Fig. 2. In E12.5, the immunoreactivity for NAIP shows stronger than E10.5. (ABC, x100) (→ : dorsal root ganglia, :← developing spinal cord).



Fig. 3. The spinal cord of E20 shows immunoreactivity for NAIP mainly in the gray matter. (ABC, ×40).

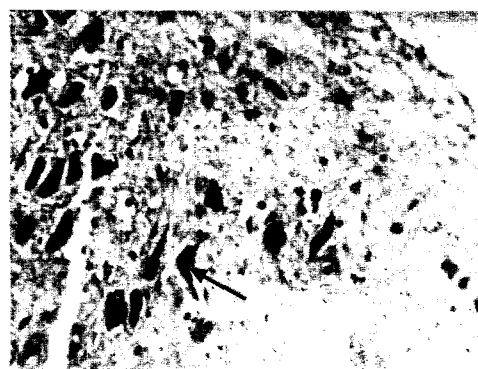


Fig.4. The motor neuron cells (←) show moderate degree of immunoreactivity for NAIP in postnatal 22 days. (ABC, ×200).

2) Expressions and Distribution of NAIP-immunoreactivity in Postnatal spinal cords

Motor neurons and other neurons in the spinal cord of postnatal 1-22 days of mice appeared to express low to focal moderate level of NAIP, which was lesser extent

and lesser intensity than E9.5-14.5embryo. Distinct difference of immunoreactivity according to postnatal ages did not show. The most prominently immunologically stained cells in ventral horn were very large neurons suggesting motor neurons. However, small neurons, presumably interneurons and sensory neurons revealed weaker immunoreactivity than presumably motor neuron. Numerous fiber staining was also weakly detectable within white matter of the spinal cord (Fig.4).

## Discussion

The recent report showed that immunohistochemical staining demonstrated that NAIP is strongly expressed in anterior horn and motor cortex neurons of normal brains, and is also located in human fetal neurons and in adult choroid plexus cells. Therefore, NAIP may be important in motor neurons.<sup>5)</sup> And there is a report that NAIP expression showed only E9.4-14.5 in the murine brain and spinal cord development.<sup>5,6)</sup> Ingram-Crooks<sup>6)</sup> reported after E16.5 showed no expression of NAIP. However, authors results showed weak or decreased expression of NAIP after E16.5. Further evaluation of this subject would be needed to clarify the expression period of NAIP during murine development.

The antenatal onset of many cases of type I SMA suggests an early functional motor neuron deficit, possibly combined with actual cellular attrition. This postulated early motor neuron loss has led to animal model involving a pathologic dysregulation of the motor neuron apoptosis which occurs in normal development.<sup>7-9)</sup> In this study E1.9-14.5 in the murine development shows the remarkable expression of NAIP.

During normal human development, motor neuron loss occurs between 11 weeks and 25 gestation with the greatest decline occurring between 12 and 16 weeks<sup>10)</sup>, a time which is analogous to E14 in the mouse, the period when the greatest murine motor neuron loss is observed

10,11).

Therefore this result means NAIP -remarkable expressing period is compatible with those of the extension of spinal cord of which the mantle layer of the embryonic spinal cord contains post-migratory and migrating young neurons as well as post mitotic young neurons and glioblasts destined to become the gray matter of mature spinal cord including motor neurons. And after E14.5 embryo, the decline and stand still expression of NAIP means that the NAIP need the maintenance of neurons in spinal cords. This phenomenon continues to perinatal spinal cord<sup>12)</sup>. The antenatal onset of many cases of type I SMA suggests an early functional motor neuron deficit, possibly combined with actual cellular attrition. This postulated early motor neuron loss has led to animal model involving a pathologic dysregulation of the motor neuron apoptosis which occurs in normal development.<sup>5,6,12)</sup>

A number of issues arise when assessing a potential role for NAIP in SMA. In addition to motor neurons, attrition of dorsal root ganglia is also in SMA. NAIP protein showed a uniform expression in the dorsal root ganglia of E11.5-E13.5 mouse embryo in this current study, indicating NAIP is also expressed in the peripheral nervous system. Low NAIP may render these cells dysfunctional and more prone to death. In human development of spinal cord, 9 Week-embryo showed the thickening of mantle layer; 12-15w-embryo, spinal cord extends to fetus; 10-11w-embryo, identification of neurons in caudal segment; 16-19w: identification of neurons in anterior and lateral horn. 20-23w: myelination of ventral root; 24-27w: dorsal root ganglia myelination<sup>12)</sup>. Regarding the corresponding of murine developmental age to humans, the expression of NAIP appears to be decreased after myelination in this study. Given that this time, this result shows the greatest expression of NAIP protein expression in the mouse, it is possible that loss or dysfunction of the SMN depleted motor neurons in SMA fetuses at this time exacerbated in the absence of NAIP. The expression of NAIP expression in embryonic murine spinal cord tissues

documented here supports the possibility of such a model consistent with a role for NAIP as a true SMA modifier<sup>12)</sup>. There have been reported NAIP overexpression impaired NGF-induced neurite outgrowth. Unregulation of cellular differentiation and/or caspase suppression may contribute to motoneuron dysfunction and cell death in spinal muscular atrophy where NAIP is mutated<sup>11,12)</sup>.

And NAIP may play a key role in conferring resistance to ischemic damage and that treatments that elevate neuronal levels of this antiapoptotic protein may have utility in the treatment of stroke.<sup>6,12)</sup>

These reports seem to be compatible with the expression pattern in this study which peak in E9.5-14.5 and decline from 16.5 embryo and standstill to the postnatal 22 days is. In this study the tissue distribution of murine neuronal apoptosis inhibitory protein during embryogenesis. NAIP protein is present in the developing spinal cord of E9.5-E14.5. The observation of NAIP in the mouse spinal cord between E9.5 and E14.5 is consistent with a role for NAIP in modifying spinal muscular atrophy.

If motor neuron attrition occurs in the second and third trimester of gestation in SMA, the observation of NAIP transcription in the mouse spinal cord between E9.5-E14.5 is consistent with a role for NAIP in modifying disorder.

Any report about the relationship between NAIP and myelination has not demonstrated yet, hence the further study would be needed for the verification of this result.

## Conculsion

This study provides an evidence that expression of protein of NAIP gene seem to have important roles during murine spinal cord development, especially in E9.5-14.5.

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## 국문요약

**배경:** 세포고사(apoptosis)에 관여하는 유전자들은 신경계의 발생과 유지 과정에서 여러 경로를 통해 복잡한 상호연관을 가지며 다양한 역할을 하며, 이들의 기능이나 발현의 조절이 제대로 이루어 지지 못하는 경우는 다양한 임상 증상이나 질병을 야기할 수 있다.

척수성 근위축증(spinal muscular atrophy:SMA)은 세포고사로 인한 척수 운동 세포의 소실과 근위축증을 동반한 진행성 마비를 특징으로 하는 유전성 질환으로, 임상적 치료나 예방을 위해서는 원인 유전자들에 대한 연구가 필요하다. 최근 항세포고사 기능을 가진 것으로 알려진 세포자연사 억제 단백질(neuronal apoptosis inhibitory protein:NAIP) 유전자의 돌연변이가 SMA의 세포고사와 관련이 있다는 보고가 있으나, 아직 유전자 기능에 대해서 논란이 많다. 더욱이 친-고사 작용 기전과 조절에 대해서는 명확히 정의된 바가 없으며, 발생기 동안(development)의 역할에 대해서도 거의 알려진 바가 없다.

**연구목적:** 생쥐의 발생과 출생후 초기 시기에 NAIP 발현을 경시적으로 조사하여, 발생시기에 따른 발현의 변화와 기능을 조사하여 척수성 근위축증 환자의 원인 유전자를 규명하고, 조기 진단과 유전자 치료 및 예방에 이용하고자 하였다.

**재료 및 방법:** 생쥐를 교배후 임신 7일부터 22일까지 매일 각각 10마리씩의 자궁을 적출후 배아(embryo)를 꺼내어 5마리의 것은 중성 포르말린에 고정후 파라핀에 포매하여 절편한 후 헤마톡실린-에오진 염색과 유전자 클로닝후 제작된 NAIP 항체를 이용하여 면역 염색을 시행하였다. 출생후 초기 유전자 발현과 비교하기 위하여 대조군으로 생후 1, 5, 10, 20, 22일째의 생쥐의 척수를 이용하였다.

**결과:** NAIP는 생쥐의 모든 발생 시기 동안에 발현되었지만, 특히 E9.5-14.5동안에 척수로 분화할 외투층(mantle layer)과 뒤뿌리 신경절(dorsal root ganglia)에서 강한 발현을 보였으며, E16.5시기 이후에서는 NAIP의 발현 강도가 다소 감소하였으나, 발현은 계속 지속되어, 운동신경세포(motor neuron)으로의 분화가 뚜렷이 관찰되는 생후 22일째의 척수와 뒤뿌리 신경절에서도 발현

을 보였다. 특히 생후 20, 22일째는 운동 신경 세포의 세포질에서 중등도의 발현을 보였으며, 간신경세포(interneuron)과 회백질(white matter)에서도 경도의 발현을 보였다.

**결론:** NAIP 유전자는 척수 발생과 분화 및 유지에 중요한 역할을 할 것으로 추정되며, 특히 NAIP의 발현 변화는 인체에서 SMA의 발생 결정 시기와 거의 일치하므로 이 시기에 NAIP 유전자에 돌연변이등의 변화가 생기면 SMA가 발생될 수 있을 것 생각되어, 임상적으로 SMA의 태내 조기 진단과 유전자 치료의 표적에 이용될 수 있을 것으로 생각된다.

**중심단어:** 세포자연사억제단백질(NAIP), 생쥐 발생, 척수성근위축증