Correlation between spina bifida manifesta in fetal rats and c-Jun N-terminal kinase signaling★

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Abstract

Fetal rat models with neural tube defects were established by injection with retinoic acid at 10 days after conception. The immunofluorescence assay and western blot analysis showed that the number of caspase-3 positive cells in myeloid tissues for spina bifida manifesta was increased. There was also increased phosphorylation of c-Jun N-terminal kinase, a member of the mitogen activated protein kinase family. The c-Jun N-terminal kinase phosphorylation level was positively correlated with caspase-3 expression in myeloid tissues for spina bifida manifesta. Experimental findings indicate that abnormal apoptosis is involved in retinoic acid-induced dominant spina bifida formation in fetal rats, and may be associated with the c-Jun N-terminal kinase signal transduction pathway.

Key Words

retinoic acid; neural tube defects; myeloid tissues; caspase-3; apoptotic kinase; c-Jun N-terminal kinase; mitogen-activated protein kinase; neural development; regeneration; neural regeneration

Research Highlights

(1) Neural tube defects are congenital embryonic diseases that involve many genes. However, the underlying mechanisms remain unclear. This study aims to investigate the correlation between caspase expression and c-Jun N-terminal kinase phosphorylation level in myeloid tissues of fetal rats with spina bifida manifesta.
(2) Experimental findings indicate that, the number of caspase-3 positive cells in myeloid tissues for spina bifida manifesta was increased, and the level of c-Jun N-terminal kinase phosphorylation in myeloid tissues for spina bifida manifesta was also increased. The c-Jun N-terminal kinase phosphorylation level was positively correlated with caspase-3 expression in myeloid tissues for spina bifida manifesta.

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INTRODUCTION

The incidence of neural tube defects ranges from 0.5/1 000 to 14/1 000 live births[1-2]. The most common types of neural tube defects are spina bifida, where the spinal cord does not close completely, and anencephaly, where the cranial regions of the brain do not develop. Spina bifida and anencephaly are the most frequent and the most severe forms of neural tube defects affecting about 1 in 2 000 live births worldwide[3]. The neural tube closes about 28 days after conception[4-5]. Neural tube closure involves cell migration, proliferation and apoptosis[6-7].

Mitogen-activated protein kinases are evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within cells. There are three known major mitogen-activated protein kinase cascades: the extracellular signal-regulated protein kinase cascade, the c-Jun NH$_2$-terminal kinase cascade, and the p38-mitogen-activated protein kinase cascade. Mitogen-activated protein kinase subgroups are classified based on the response and substrate specificity, among which c-Jun N-terminal kinase and p38-mitogen-activated protein kinase cascades, activated by proinflammatory and stress stimuli, are associated with inflammation and apoptosis[8-12]. However, the function of c-Jun N-terminal kinase in neural tube defects remains unclear.

This study was aimed to observe the correlation between caspase-3 and phosphorylated c-Jun N-terminal kinase in rats with spina bifida.

RESULTS

Spina bifida induced by retinoic acid

Retinoic acid may induce abnormalities including fetal death, fetal absorption and spina bifida manifesta (Figure 1) at 12, 15, 17 and 20 days after conception, respectively.

Other abnormalities such as cephalocele, spina bifida occulta, anorectal malformation, no tail, short tail, ring tail, acromphalus, chilopalatognathus and foot deformities were also observed in the experimental group. Incidences of spina bifida manifesta ranged from 41.8% to 42.9% were noted at different time points, with no significant statistical difference ($P > 0.05$; Table 1).

Caspase-3 expression increased in myeloid tissues of rats in spina bifida manifesta group

As indicated by immunofluorescence assay, more caspase-3 expressing cells in myeloid tissues were observed in the spina bifida manifesta group compared with the control group at 15, 17 and 20 days after conception ($P < 0.05$; Figures 2, 3).

Phosphorylation of c-Jun N-terminal kinase increased in myeloid tissues of rats in spina bifida manifesta group at various time points after conception

Western blot analysis was performed to investigate the phosphorylation of c-Jun N-terminal kinase at various time points after conception in the spina bifida manifesta group and control group. Results indicated that, higher levels of c-Jun N-terminal kinase phosphorylation was observed in myeloid tissues of the spina bifida manifesta group compared with that of the control group at 12, 15, 17 and 20 days after conception ($P < 0.05$; Figure 4).

Positive correlation between c-Jun N-terminal kinase phosphorylation and caspase-3 expression in myeloid tissues of fetal rats with spina bifida manifesta

Spearman's correlation analysis results showed that the level of c-Jun N-terminal kinase phosphorylation was positively correlated with the caspase-3 expression in myeloid tissues of fetal rats with spina bifida manifesta ($r = 0.783$, $P < 0.05$).
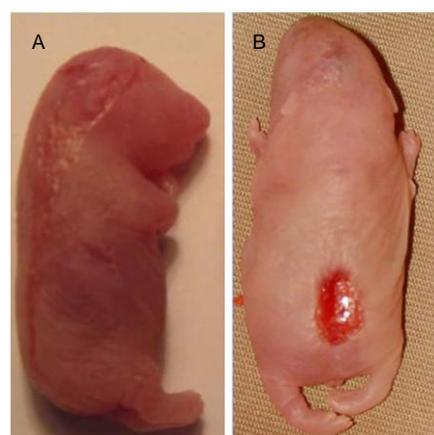
Figure 1 Rats showed neural tube defects after retinoic acid induction.

(A) Normal fetal rat. (B) Spina bifida manifesta rat.

Days after conception	Fetal rats (*n*)	Survived rats (*n*)	Spina bifida manifesta (*n*)	Incidence of spina bifida manifesta (%)	Survival rate (%)
12	55	50	23	42	91
15	35	32	15	43	91
17	38	34	16	42	90
20	41	37	17	42	90

Table 1 Incidence of spina bifida manifesta induced by retinoic acid at various time points after conception

Incidence of spina bifida manifesta (%) = number of spina bifida manifesta rats/number of fetal rats × 100%; Survival rate (%) = number of survival rats/number of fetal rats × 100%.
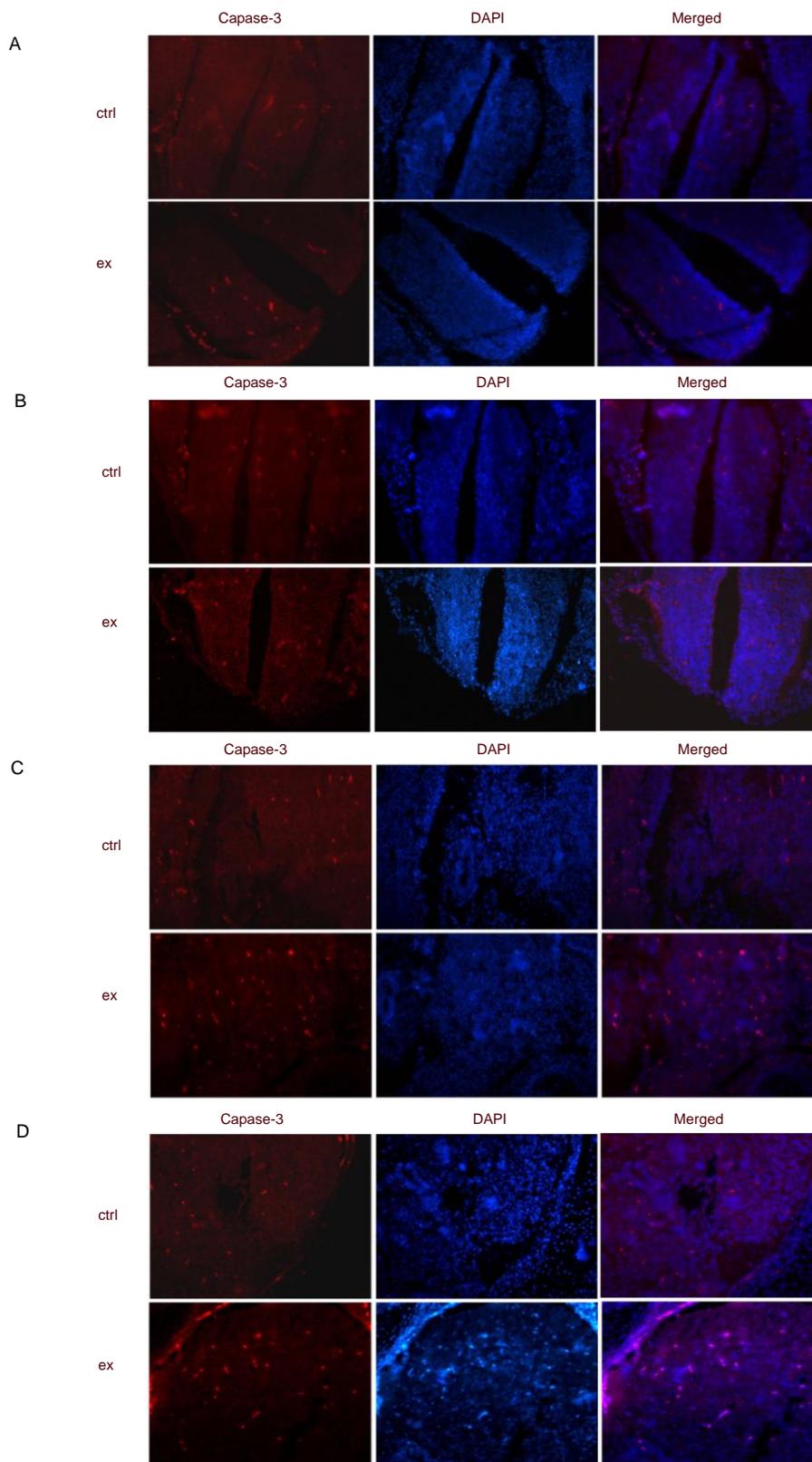
Figure 2   Expression of caspase-3 in myeloid tissues of fetal rats with spina bifida manifesta (immunofluorescence staining, × 200).

(A–D) 12, 15, 17, 20 days after conception. Caspase-3 staining was carried out on the myeloid tissues cryosections using rabbit polyclonal anti-caspase-3 antibody followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG. Counterstaining was performed with DAPI. Red (TRITC) represents positive capase-3 cells. Blue (DAPI) represents cell nuclei. The slips were visualized with an immunofluorescence microscopy. ex: Spina bifida manifesta group; ctrl: control group.
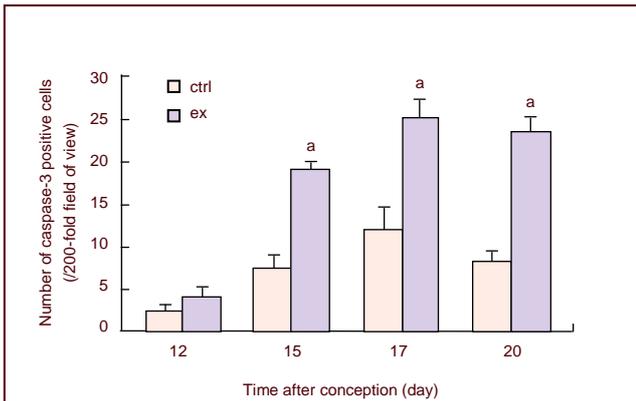
Figure 3 Number of caspase-3-positive cells in myeloid tissues of fetal rats in spina bifida manifesta group and control group.

Data were expressed as mean ± SD. There were 23, 15, 16 and 17 rats in spina bifida manifesta group, and each 10 rats in control group were detected at 12, 15, 17 and 20 days respectively. [a]$P < 0.05$, *vs*. control group (Student's *t*-test). ex: Spina bifida manifesta group; ctrl: control group.
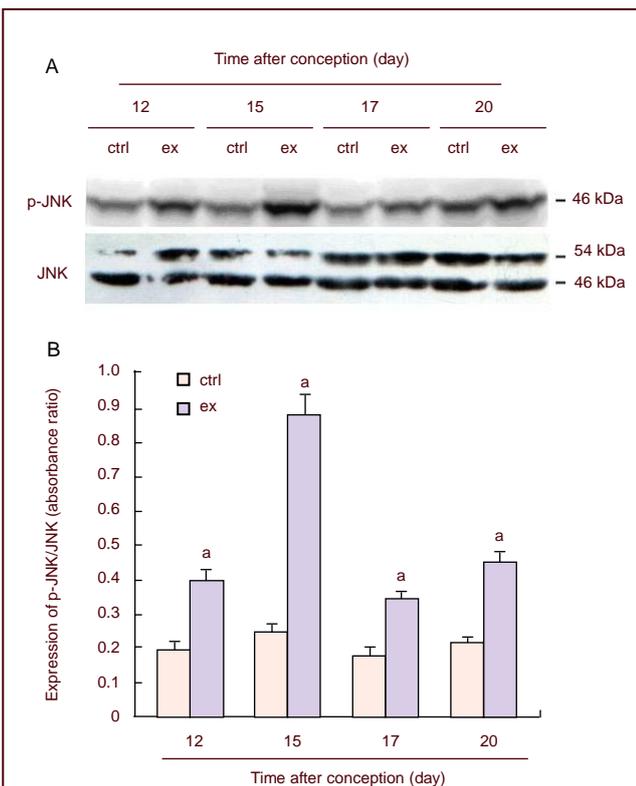
Figure 4 Levels of c-Jun N-terminal kinase phosphorylation in myeloid tissues in fetal rats in spina bifida manifesta group and control group.

Data were expressed as mean ± SD. There were 23, 15, 16 and 17 rats in spina bifida manifesta group, and each 10 rats in control group were detected at 12, 15, 17 and 20 days respectively. [a]$P < 0.05$, *vs*. control group (Student's *t*-test). ex: Spina bifida manifesta group; ctrl: control group.

DISCUSSION

Neural tube defect, a congenital malformation caused by abnormal neural tube closing during early embryonic development, is the leading cause of fetal perinatal death. Three major types of neural tube defect, including anencephalia, cephalocele and spinal bifida are most frequently reported[13-15]. Although several studies have indicated that apoptosis and cell proliferation were associated with neural tube defects[19-20], the mechanism is still unclear. In this study, rat models with neural tube defects were induced with retinoic acid to investigate the expression of caspase-3 and phosphorylation of c-Jun N-terminal kinase during the genesis of the neural tube.

Mitogen-activated protein kinase plays an important role in cell differentiation, proliferation and apoptosis. Many studies showed that c-Jun N-terminal kinase/mitogen-activated protein kinase was strongly associated with cell apoptosis through activation of caspase-3 and caspase-9[21-23]. Caspase, a special cysteine-aspartic protease, is involved in the signal pathways of cell apoptosis. Caspase-3 plays a pivotal role in the execution-phase of apoptosis. In previous reports, delayed apoptotic morphologies including chromatic agglutination, cell shrinkage, mitochondrial swelling and formation of apoptotic body were induced in caspase-3^{-/-} rats[24-25]. Several mitogen-activated protein kinase subgroups including c-Jun N-terminal kinases and p38s were reported to be associated with cell apoptosis[26-27]. Previous studies have indicated that neural tube defects were associated with cell apoptosis[28-31], however, its potential mechanism was still unclear. In this study, the expression of caspase-3 increased on day 15 after conception in the control group, and reached the maximum on day 17. In the experimental group, the same pattern was observed. However, significant differences in the expression levels of caspase-3 were observed in both groups. In addition, the phosphorylation of c-Jun N-terminal kinase reached a maximum on day 15 after conception in the experimental group. Statistical analysis indicated that the difference was significant in both groups.

Retinoic acid with a molecular formula of $C_{20}H_{28}O_2$ is the acid form of vitamin A. It is commonly used for inducing rat models with neural tube defects[32]. In a previous study, neural tube defects in rats were induced with retinoic acid on day 11 after conception[33]. However, its mechanism was not well defined. Neural tube formation is a dynamic process during which the lateral edges of

the neural plate first elevate, then bend towards each other, and finally fuse along the dorsal midline to close the neural tube. Cell migration, proliferation and apoptosis play vital roles during this dynamic process[34-38]. In this study, neural tube defects were induced in rats with retinoic acid on day 10 after conception. Then the animals were sacrificed on days 12, 15, 17 and 20, respectively to observe the potential abnormities. According to our results, abnormalities such as cephalocele, spina bifida occulta, anorectal malformation, no tail, short tail, ring tail, acromphalus, chilopalatognathus and foot deformities were also observed at each time point. In addition, an incidence of neural tube defect ranging from 41.8% to 42.9% was observed, which is consistent with the previous reports[32-38].

In this paper, caspase-3 and c-Jun N-terminal kinase phosphorylation were activated during the formation of neural tube defects. Further studies are needed to define the mechanism underlying the activation of these two factors.

MATERIALS AND METHODS

Design
A randomized, controlled, animal experiment.

Time and setting
The experiments were performed at the Major Laboratory of Health Ministry for Congenital Malformation Research, Shengjing Hospital of China Medical University, China from September 2008 to April 2011.

Materials
A total of 80 outbred Wistar rats, aged 10–12 weeks, specific pathogen free level, at a ratio of female:male=1:1.250, weighing 300 g, were purchased from Animal Center of China Medical University (License No. SCXK (Liao) 2003-0009). The protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[39].

Methods
Rat models with neural tube defects induced by retinoic acid
The appearance of vaginal plugs for the female rats in the morning after mating was timed as the embryonic day 0. On day 10 after conception, 32 pregnant Wistar rats were induced with retinoic acid (Sigma-Aldrich (Shanghai) Trading Co., Ltd., Shanghai, China) 40 mg/mL, dissolved in olive oil (Shanghai MLC Business Consulting Co., Ltd., Shanghai, China) *via* injection by stomach duct at a dose of 135 mg/kg[32]. Pregnant rats were anesthetized with sodium pentobarbital (60 mg/kg; Sigma), in randomly chosen groups of eight and fetal rats were born by cesarean section at 12, 15, 17 and 20 days. Control pregnant rats received an equal volume of olive oil on day 10.

Caspase-3 expression in myeloid tissues of fetal rats detected by immunofluorescence assay
Fetal rat spinal cord tissue slides (cryosections were prepared at 10 μm thickness) were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% Triton X-100 for 10 minutes, blocked with 3% bovine serum albumin, and incubated with rabbit anti-caspase-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 for 2 hours at room temperature. Then slices were incubated with TRITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at 1:500 for 1 hour at room temperature. Counterstaining was performed with 4',6-diamidino-2-phenylindole at room temperature for 5 minutes. Mounting was performed with glycerol after washing with PBS for 5 minutes. The slides were visualized by immunofluorescence microscopy at 200 × magnification (Olympus BX51, Tokyo, Japan). Caspase-3 positive cells in myeloid tissues of the experimental and control groups were counted from one of every six sequential sections and 10 sections were counted in each myeloid tissue. Results were represented by the mean values.

Phosphorylation of c-Jun N-terminal kinase in myeloid tissues of fetal rats detected by western blot analysis
The spinal cord tissues of fetal rats were washed twice with ice-cold PBS and lysed. The samples were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to the polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a semi-dry transfer cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The polyvinylidene difluoride membrane was blocked with 5% non-fat milk and incubated with rabbit anti-rat c-Jun N-terminal kinase monoclonal antibody (Cell Signaling Technology Inc., Beverly, MA, USA) at 1:100, or rabbit anti-rat p-c-Jun N-terminal kinase monoclonal antibody (Cell Signaling Technology Inc.) at 1:100 for overnight at 4°C. The blots were incubated with a horseradish peroxidase-conjugated goat-anti-rabbit IgG (Santa Cruz Biotechnology) at 1:1 000 for 1 hour at room temperature. Immunoreactive bands were visualized by

chemiluminescence. To further analyze the phosphorylation in each group, averaged absorbance analysis was performed with Glyco Band-Scan software (PROZYME®, San Leandro, CA, USA).

Statistical analysis

Measurement data were expressed as mean ± SD and analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA). Intergroup differences in the mean value were compared using one-way analysis of variance and Student's *t*-test. Correlation between c-Jun N-terminal kinase and caspase-3 was analyzed with Spearman's rank correlation analysis. A value of $P < 0.05$ was considered statistically significant.

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(Edited by Liu HY, Chen TY/Yang Y/Song LP)