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RESEARCH ARTICLE

Analysis of the Effects of Inhibitor and Activator Systems (Smad's Proteins) of TGF-βs on Chick Neural Tube Closure [1]

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Summary

The families of TGF-βs and Smads proteins that controls its intracellular signaling pathways are known to play a role in early neurulation. The aim of this study is to demonstrate distribution of TGF-βs (1, 2, 3) and Smads (1/2/3, 6, 7) proteins as a system in different hours of neural tube development of chick embryos. The SPF eggs were incubated at 37.8±2°C for 24th, 30th, 48th, 72nd h. After that, embryos were examined using immunohistochemistry and western blotting techniques. To the results, TGF-βs immunoreactivities (particularly TGF-β3) at the 24th, 30th and 48th h of chick development (during neural tube closure) were determined and decreased at the 72nd h (after neural tube closure), but expressions of TGF-βs were detected in all stage of embryos in western blotting. While Smad 1/2/3 immunoreactivitiy and expression was less than that of the Smad 6 and 7 at the 24th, it was increased at the 30th h. Smads proteins immunoreactivities were decresead at the 72nd h. In conclusion, the members of TGF-βs are play a role in chick neural tube closure, the secretions of TGF-βs are controlled different Smad proteins. In addition, immunoblotting results showed that TGF-βs and Smads proteins were effective in the development of all tissues and organs of the embryos.

Keywords: *TGF-βs, Smad's, Neural tube, Chick embryo*

Tavuk Nöral Tüp Kapanmasında TGF-βs İnhibitör ve Aktivatör Sisteminin (Smad's Proteinleri) Etkilerinin Araştırılması

Özet

TGF-β ve onun hücre içi sinyal iletim yolunu kontrol eden Smad protein ailesinin, erken nörülasyonda rol oynadığı bilinmektedir. Bu çalışmanın amacı, tavuk embriyosunda nöral tüp gelişiminin farklı saatlerinde bir sistem olarak TGF-β1,2,3, Smad 1/2/3, 6 and 7 proteinlerinin dağılımını göstermektir. Bunun için, SPF yumurtaları, 24, 30, 48, 72. saatlerde 37.8±2°C'de inkübe edildi ve sonrasında embriyolar, immunohistokimyasal ve immünoblotting yöntemleriyle incelendi. Elde edilen sonuçlara göre, tavuk gelişiminin 24, 30 ve 48. saatlerinde (nöral tüp kapanması sırasında) TGF-βs immünreaktivitelerinin (özellikle TGF-β3) var olduğu ve 72. saatte (nöral tüp kapanmasından sonra) azaldığı saptanırken western blotting yöntemi ile TGF-βs sentezinin embriyoların tüm dönemlerinde var olduğu gösterildi. Smad 1/2/3 immunreaktivitesi ve sentezi ise 24. saatte Smad 6 and 7' ninkinden daha az iken 30. saatte daha yüksekti. Sonuç olarak, TGF-β ailesinin, tavuk nöral tüp gelişiminde rol oynadığı ve salınımının farklı Smad proteinleri tarafından kontrol edildiği düşünülmektedir. Bununla birlikte, immünoblotting sonuçlar, TGF-βs ve Smads proteinlerinin embriyonun tüm doku ve organlarının gelişiminde etkili olduğunu göstermektedir.

Anahtar sözcükler: *TGF-βs, Smad's, Nöral tüp, Tavuk embriyosu*

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INTRODUCTION

Neural tube closure is accomplished by a complex morphogenetic program requiring precisely choreographed cellular proliferation, differentiation, adhesion and migration [1]. Inductive signals come from the notochord and mesoderm, after this induction the formation of the neural layer from the involved ectoderm region was triggered. During differentiation and formation of neural layer, different molecules such as Noggin, Chordin and Follistatin, Hedgehog Group, Wingless (Wnt) Group, Fibroblast Growth Factors (FGFs), Transforming Growth Factor-β (TGF-β) Superfamily have been detected [2,3].

The Transforming Growth Factor-β (TGF-β) superfamily members is a large family of cytokines that includes the TGF-βs, activins, bone morphogenetic proteins (BMP) and others [4]. TGF-β superfamily signaling is which then stimulate downstream Smad proteins to localize from the cytoplasm to the nucleus [5]. The Smad proteins are play a central role in the transduction of receptor signals to target genes in the nucleus [6]. According to structural and functional properties of the respective proteins, the Smad family can be divided into three distinct subgroups: receptor-regulated Smads (R-Smad; 1, 2, 3, 5, 8), common-partner Smads (Co-Smads; Smad 4) and inhibitory Smads (I-Smads; 6, 7), each of which plays a distinct role in the pathway. The signaling pathway of TGF-βs is controlling both Smad 1/2/3 which is activator, and Smad 6 and Smad 7, which are inhibitor, proteins [7,8].

TGF-βs family are multifunctional growth factors that are involved in cell cycle control, differentiation, migration, formation of extracellular matrix, regulation of early development, maintenance of neuronal life, immuno-supression and neuronal protection [4,9,10]. The central and peripheral nervous system functions of TGF-βs family which has the 3 described isoforms of TGF-β1, β2 and β3 has been studied and their expressions were observed in glial and neural cells of mice, rat and chicken [11-13].

During the embryonic development of the central nervous system, there are numerous studies on the effect and role of TGF-β superfamily members (especially molecules of BMP, noggin, etc.) that play an inductive role during the formation and differentiation of the neural tube [4,14,15]. However, studies evaluating both the effects of TGF-βs (β1, β2, β3) and effects of Smad proteins are insufficient.

In our study, we aim to evaluate of TGF-β1, β2, β3, Smad 1/2/3, 6 and 7 proteins in during 24th, 30th, 48th and 72nd h of neural tube development stage of chick embryos using both immunohistochemistry and immuno-blotting techniques.

MATERIAL and METHODS

Embryos Collection and Fixation

Sixty white Leghorn type specific pathogen free (SPF) eggs were obtained from the Institute of Veterinary Control and Research, Bornova, Izmir, Turkey. The eggs were incubated at 37.8± 2°C for 24th, 30th, 48th, 72nd h to let them develop to stage 8, 10, 13, 20 respectively, according to Hamburger&Hamilton classification [16]. After that, embryos were fixed with in 10% (v/v) formalin solution for 24 h for immunohistological studies or were homogenized on ice in extraction buffer for immuno-blotting studies.

Immunohistochemical Analysis

Sections (5 µm) were dewaxed at 60°C overnight and then in xylene for 30 min. After soaking in a decreasing series of ethanol, followed by a wash in tap water for 5 min, sections were washed with distilled water. They were then treated with 2% trypsin (ab970, Apcam, Cambridge, UK) in 50 milimolar (mM) Tris buffer (pH 7.5) at 37°C for 15 min and washed with phosphate-buffered saline (PBS). Sections were delineated with an Elite Pap pen (DBS, Pleasanton, CA, USA) and incubated in 3% H_2O_2 solution for 15 min to inhibit endogenous peroxidase activity. They were washed three times for 5 min each with PBS and incubated with primary anti-bodies against anti-TGF-β1, anti-TGF-β2, anti-TGF-β3, anti-Smad 1/2/3, anti-Smad 6 and anti-Smad 7 (respectively, sc-146, sc-90, sc-82, sc-7960, sc-13048, sc-11392, Santa Cruz, California, USA) were incubated for 18 h at +4°C. After washing with PBS, the secondary antibody biotinylated goat IgG anti-mouse (sc2005, Santa Cruz) (for anti-Smad 1/2/3) or anti-rabbit (sc2004, Santa Cruz) (for the others) was applied for 30 min, followed by three washes in PBS. The streptavidin-peroxidase complex (supplied ready to use by Zymed) was added for 30 min and washed 3 times with PBS. Samples were stained with 1-diaminobenzidine (DAB) with hydrogen peroxide (Histostain-Plus Kits, Zymed, San Francisco, CA) for 5 min to visualize immuno-labeling, and after rinsing with distilled water were counterstained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). The sections were dehydrated with 80% and 95% alcohol and immersed in xylene and covered with mounting media (01730 Surgipath, Cambridge, UK). The negative controls were performed for each of the antibodies and the labeling in all cases was negative. All sections were evaluated using a light microscope (Olympus BX40, Tokyo, Japan). Immunolabelling intensity was graded on the following scale: negative (-), weak (+), moderate (++), and strong (+++).

Statistical Analysis

Statistical analyses were performed using GraphPad Instat v3.01 software for Windows. Data groups were evaluated using ANOVA. ANOVAs were followed by multiple range tests when appropriate [17]. Differences at P<0.05 were considered significant

Immunoprecipitation and Western Blotting Analysis

The embryos (10 embryos for each group) that incubated at 37.8±2°C for 24th, 30th, 48th, 72nd h were collected and homogenized on ice in extraction buffer (40 µl/embryo) containing 20 mM HEPES (pH 7.6) (H-3375, Sigma, St. Louis, MO, USA), 20% Gliserol (G-5516, Sigma, St. Louis, MO, USA), 1.5 Mm $MgCl_2$ (63063, Fluka, St. Louis, MO, USA), 500 mM NaCl (S-7653, Sigma, St. Louis, MO, USA), 0.2 mM EDTA (E-5134, Sigma, St. Louis, MO, USA), 0.1% Triton X-100 (T-8532, Sigma, St. Louis, MO, USA), Protease inhibitors (EDTA-free, 1873850, Roche, Mannheim, Germany), 2 mM $Na_4P_2O_7$ (P-8010, Sigma, St. Louis, MO, USA), 1 mM $NaVO_3$ (S-6393, Sigma, St. Louis, MO, USA), 10 mM NaF (S-1504, Sigma, St. Louis, MO, USA) and 2.5 ng/ml Calculin A (C-5552, Sigma, St. Louis, MO, USA) for 30 min at 4°C. These lysates were centrifuged at 14.000 rpm for 10 min at 4°C. Supernatants were precleared with Protein A (Pansorbin A) (507858, Calbiochem Darmstadt, Germany) for 1.5 h at 4°C and centrifuged at 14.000 rpm for 5 min at 4°C. The samples were divided in 6 tubes (an equal amount: 550 µl) and then each samples were incubated overnight adding the primary antibodies separately (anti-TGF-β1, TGF-β2, TGF-β3, Smad1/2/3, Smad 6 and Smad 7) in each tube at 4°C with shaking. Antigen-antibody complexes were precipitated with adding the Pansorbin A in each tube and they were incubated for 1 hour at 4°C. The samples were then centrifuged at 14.000 rpm for 5 min at 4°C and super-natants were discarded. SDS gel electrophoresis sample buffer were added and boiled 2 min at ~96°C. The samples were fractionated using SDS-PAGE gels (18%- 6%). Proteins were then transferred on to nitrocellulose membrane (N-8392-5EA, Sigma, St. Louis, MO, USA) for 1 h. The membranes were blocked in 2% dry defated milk powder in PBS for 1 h at room temperature and they were incubated with primary antibodies overnight at 4°C with shaking. The membranes were then washed with PBS and incubated with HRP-conjugated anti-mouse (sc2005, Santa Cruz, CA, USA) for anti-Smad 1/2/3 or anti-rabbit (sc2004, Santa Cruz, CA, USA) for the others as a secondary antibody for 2 h. After washing 3 times with PBS, bound antibodies were visualized with DAB chromogen.

RESULTS

Immunohistochemical Results

In 24th h of embryos, TGF-β3 immunoreactivity (+++) which was more than TGF-β1 (++) and TGF-β2 (++) immunoreactivities (P<0.01), especially in mesenchymal and neuroectoderm cells was detected *(Fig. 1)*. Smad 1/2/3 immunoreactivitiy (+/-) was less and weaker than that of the Smad 6 (++) (P<0.001). Smad 6 immuno-reactivity was more than that of the Smad 7 (+) in 24th h of embryos (P<0.001) *(Fig. 1)*.

In 30th h of embryos, although TGF-β1 immuno-reactivity (++/+++) was similar to that of the 24th h (P>0.05). TGF-β2 immunoreactivity was (+) less and weaker than that of the 24th h of embryos (P<0.001). TGF-β3 immunoreactivity was (+++) strongly positive in this stage of development *(Fig. 1)*. Smad 1/2/3 immuno-reactivitiy which was (-/+) very weak in the 24th h, increased at the 30th h to (++/+++) moderately and partially strongly positive (P<0.001). Smad 6 immuno-reactivitiy was (+) less and weaker than that at the 24th h of embryos (P<0.01). Smad 7 immunoreactivitiy was weak (+) in 30th h of embryos *(Fig. 1)*.

In 48th h of embryos, TGF-β3 immunoreactivity (+++) which was similar to those of the 24th and 30th h of embryos (P>0.05), was more than TGF-β1 (++) and TGF-β2 (++) immunoreactivities (P<0.05). TGF-β2 immuno-reactivity was (++) moderate and more than that of the 30th h (P<0.001) *(Fig. 1)*. Smad 1/2/3 immunoreactivitiy (++) was weaker than that of the 30th h (P<0.05). Smad 6 immunoreactivitiy (++) was more than that of the 30th h of embryos (P<0.001). Smad 7 immunoreactivitiy (+) was similar to those of the 24th and 30th h of embryos (P>0.05) *(Fig. 1)*.

In 72nd h of embryos, immunoreactivities of TGF-β1 (+),TGF-β2 (+/-), TGF-β3 (-) were seen to have decreased when compared to the distributions at the 24th, 30th and the 48th h (P<0.001) *(Fig. 1)*. Immunoreactivities of the three Smad proteins were also decreased and partially negative at the 72nd h of embryos. Smad 1/2/3 immuno-reactivitiy (-) was seen to have decreased when compared to the distributions at the 30th and 48th h (P<0.001), however, it was similar to that of the 24th h (P>0.05). Smad 6 immunoreactivitiy was (-/+) less and weaker than those of the 24th and 48th h (P<0.001). Smad 7 immuno-reactivitiy was (-) negative and weaker than those of the 24th, 30th and 48th h (P<0.01) *(Fig. 1)*. Apart from the neuro-ectoderm, at the 48th and 72nd h, Smad 6 and Smad 7 immunoreactivities were also positive in mesenchymal cells *(Fig. 1)*. Results of immunohistochemical analysis of chick embryos at the 24th, 30th, 48th, 72nd h of developmental stages have been summarized in *Table 1*.

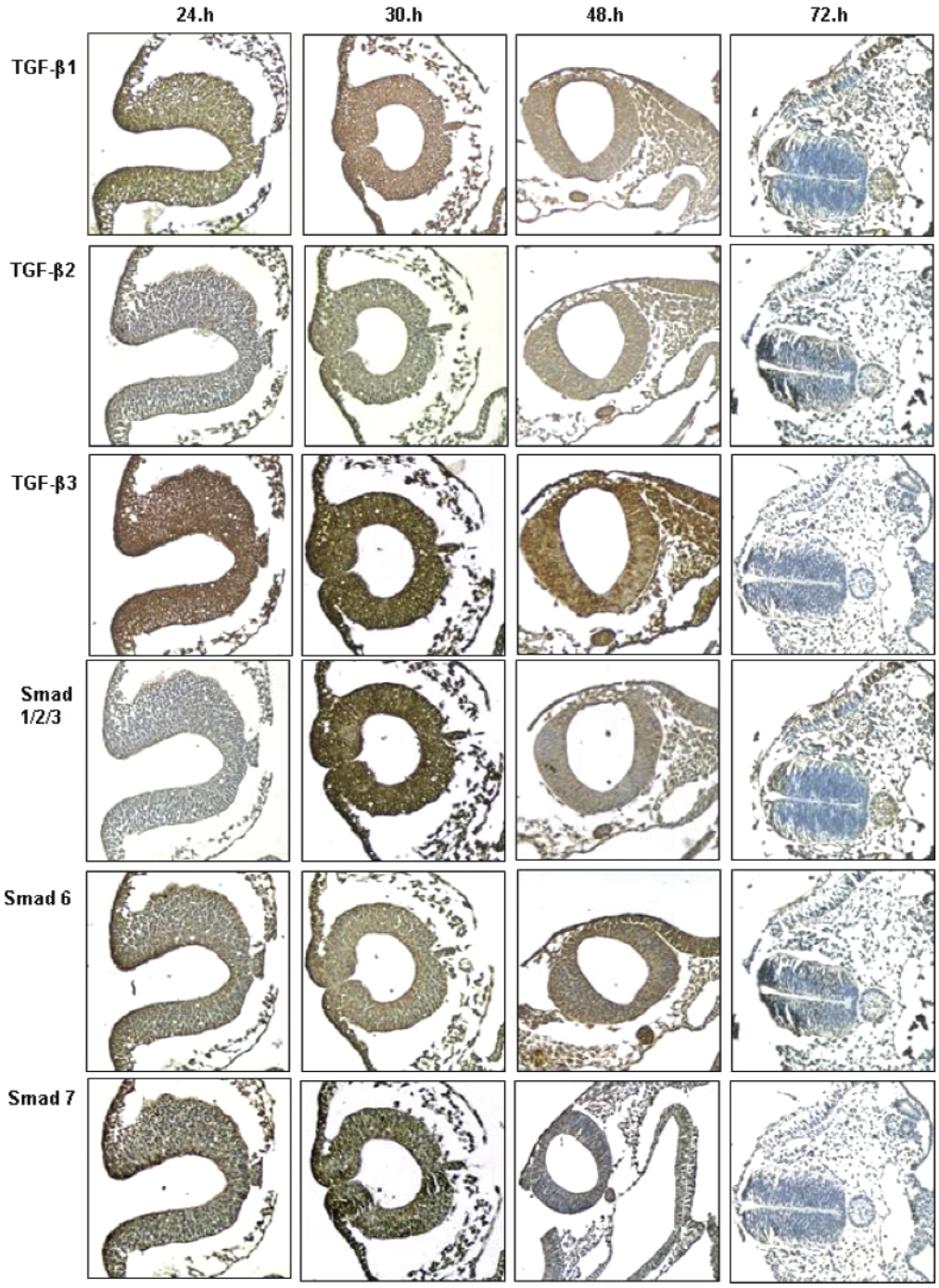

Fig 1. Immunoreactivities of proteins in sections from chick embryos at the 24th, 30th, 48th, 72nd hours of developmental stages (X200)

Şekil 1. Tavuk embriyolarının 24, 30, 48, 72. saatlerdeki gelişim döneminde alınan kesitlerdeki proteinlerin immünreaktiviteleri

Western Blotting Results

Similar to the immunohistochemical results, the expression of TGF-β3 was more than TGF-β1 and TGF-β2 expression *(Fig. 2)*. In addition, Smad 1/2/3 expression was less than Smad 6 and Smad 7 expressions at the 24th h of development *(Fig. 2)*. In the 30th hour of embryo, while expression of TGF-β3 was continue, it was lower than that of the 24th h. TGF-β1 and TGF-β2 expressions were still less than TGF-β3 *(Fig. 2)*. Smad1/2/3 expression at the 30th h was increased when compared to the 24th h expression, however expressions of both Smad 6 and Smad 7 were more detectable than Smad 1/2/3 *(Fig. 2)*. In the 48th h of embryo, the expressions of TGF-β1 and TGF-β2 were similar but the expression of TGF-β3 was decreased *(Fig. 2)*. However, TGF-β3 expression at the 48th h was still detectable. When the expressions of Smad proteins at the 48th h were detectable, Smad 6 expression was more than others *(Fig. 2)*. In the 72nd h of embryo, TGF-β3 expression was more than TGF-β1 and TGF-β2 *(Fig. 2)*. The expression of Smad proteins were continued at the 72nd h and Smad 7 expression was more *(Fig. 2)*.

Table 1. Immunolabelling intensity of proteins in sections from chick embryos at the 24th, 30th, 48th, 72nd hours of developmental stages

Tablo 1. Tavuk embriyolarının 24, 30, 48, 72. saatlerdeki gelişim döneminde alınan kesitlerdeki proteinlerin immün-işaretleme yoğunlukları

Antibody	Immunolabelling Intensity			
	24th	30th	48th	72th
TGF-β1	++	++/+++	++	+
TGF-β2	++	+	++	-/+
TGF-β3	+++	++	+++	-
Smad 1/2/3	-/+	++/+++	++	-
Smad 6	++	+	++	-/+
Smad 7	+	+	+	-

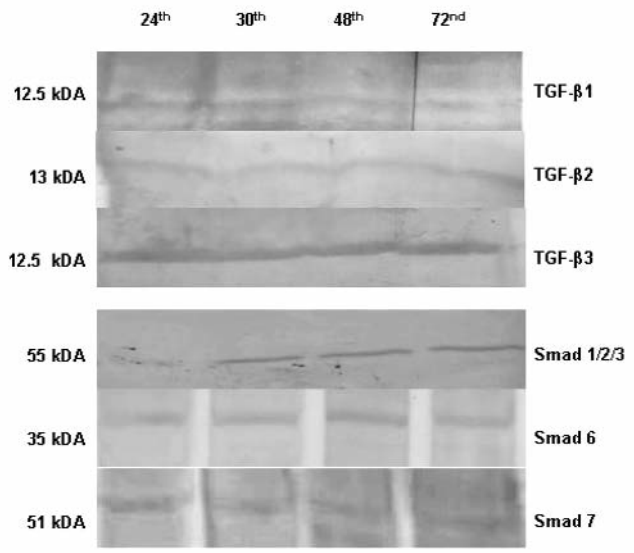

Fig 2. Protein bands obtained by using western blotting analysis of chick embryos at the 24th, 30th, 48th, 72nd hours of developmental stages

Şekil 2. Tavuk embriyolarının 24, 30, 48, 72. saatlerdeki gelişim döneminde Western Blotting analizi ile elde edilen protein bantları

DISCUSSION

Both TGF-β superfamily and Smads proteins are important in the development and functioning of central and peripheral nervous system in mouse and chicken embryos [2,11]. Despite the presence of several studies on the role and effect of molecules of TGF-β superfamily such as BMP, noggin, etc. during the formation and differentiation of the neural tube, a few studies exist on the distribution of TGF-βs (TGF-β1, TGF-β2, TGF-β3) family members [9,18-21].

In our study, we showed the distribution of TGF-βs family (TGF-β1, β2, β3) and the activator (Smad 1/2/3), inhibitor (Smad 6, 7) Smads protein family in chick embryos. The immunoreactivity and expression of TGF-β3 were detected in all stage of embryos, and it was more abundant protein when compared to the other TGF-βs proteins. In addition Smad 1/2/3 proteins were observed lowest amount when compared to the other Smads proteins. The induction of ventral and dorsal ectoderm is controlled by different mechanisms in the early embryonic development. In mammals, as Smad 1, 5, 8 are activated by bone morphogenetic protein (BMP) receptors, the activation of Smad 2 and Smad 3 occurs by the activation of TGF-β, activin receptors [22,23]. Thus, our study suggested that during the development of neuroectoderm from ectoderm at the 24th h, particularly as a result of the expression of TGF-β3, the development of neural tube could induced and the development of neuroectoderm may triggered as a result of BMP inhibition by weak Smad 1/2/3 and increased Smad 6 expression. However, it is known that TGF-βs activates the expressions of Smad 6 and Smad 7, which are the inhibitor proteins in the TGF-β/Smad signal pathway with the negative feedback mechanism [24,25]. Therefore, the increased TGF-βs (particularly TGF-β3) expressions may induced the development of neuroectoderm and this expression may controlled increase expression of Smad 6 and Smad 7.

At the 30th h, the period of neural tube closure, it has been shown by both immunohistochemical and immuno-blotting methods that the expression of TGF-βs and Smads proteins were continued. While strong immunoreactivity of TGF-β3 was detected that stage, a decrease of TGF-β3 expression was observed in the immunoblotting results. The observation of an increased immunoreactivity and consistent with the expression of Smad 1/2/3 may occured due to BMP inhibition at the 24th h in which neural tube differentiates may have disappeared at the 30th h. Therefore, at the 30th h, due to activation of both epidermal and neural induction, Smad 1/2/3 expression was increased. The both immunoreactivity and expressions of both Smad 6 and 7 proteins were not significantly different; it was considered that their effect may be relatively less. The balance between TGF-β superfamily and Smad proteins during the embryonic development has an important role in the development, and differentiation or inhibition of various cells or tissues [7,21].

At the 48th h, in which the neural tube was closed, the continuous effect of TGF-βs proteins, especially TGF-β3, may controlled neural tube closure at the 48th h of development. The presence of Smad 6 and 7 at the 48th h was supported that the continuous synthesis of these proteins may need for the organization of the signal pathway in the development of the neural tube.

At the 72nd h of development, the immunoreactivities of TGF-βs and Smads proteins in the neural ectoderm and neural tube areas were not observed any more because of the completion of neural tube development at the 72nd h. This has led us to consider that after neural tube closure, they were synthesized in these areas and therefore, they were responsible for the events during closure. The results of immunoblotting using the whole embryo showed that the synthesis of TGF-βs proteins, which was effective in the development of all tissues and organs of the embryo, has continued. Smads proteins were used not only by TGF-βs, but by the other members of TGF-β superfamily as well during the embryonic development, and showed that Smad proteins were as important as TGF-βs [11,20,26]. Therefore inconsistency of immunohistochemical and western blotting results may be clarify.

The development and closure of neural tube are under the control of various mechanisms. It is important to determine which molecules and their distributions playing roles in the closure of neural tube in order to prevent and explain the defects in the closure, which may occur as a result of deficiencies or disorders in the mechanisms or the release of molecules playing roles during closure. Our results were consistent with those of Pelton et al.[20] in developing mouse showing that although all three TGF-β immunoreactivity in embryos decreased at the 72nd h, Therefore, we thought that TGF-βs family, especially TGF-β3, may play a role in the early period of neurulation (24th, 30th, 48th). However, the evidence for Smad protein release which controls the synthesis of TGF-βs family supported that the development and closure of neural tube were under the control of different TGF-β and Smad molecules. The evidence from our study that different TGF-β and Smad proteins were released at different stages may be a reference for examples of neural tube closure defects. The examination of protein distribution in patients with neural tube defects may help explain their role in these defects.

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