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TGF-βs and SMADs Activities at the Site of Failed Neural Tube in the Human Embryos

İnsan Embriyolarında Nöral Tüp Kapanma Kusur Bölgesinde TGF-β ve SMAD'ların Aktivitesi

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ABSTRACT

AIM: Transforming growth factor β (TGF-β) and Smads control intracellular signaling pathways in neurulation. Although previously reported similar experimental animal studies, the aim of this human study is to investigate the expression of TGF-β (1,2,3) and Smads (1,2,3,6,7) in aborted human fetuses with myeloschisis.

MATERIAL and METHODS: Twelve human fetuses with neural tube defect were obtained. They were stained with antibodies against TGF-β1, TGF-β2, TGF-β3, Smad (1,2,3), Smad 6 and Smad 7 using the indirect immunohistochemical technique.

RESULTS: We noted mild immune reactivity of TGF-β1 and TGF-β2 in the open neural plate, motor neurons and surrounding tissue. Strong immune reactivity of TGF-β3 was shown in only open neural plate and surrounding tissue. Immunoreactivity of all Smads noted negative except Smad7.

CONCLUSION: These results suggested at the site where the neural tube failed to close, TGF-β 1,2 and Smads 1,2,3,6 do not continue their activity and decrease with internal timing of embryonic development. Additionally ectodermal layers are considered by embryo as "not closed wound" and TGF-β3 activity may be an effort to repair the failed closure.

KEYWORDS: Human fetus, Neural tube defect, TGF-βs, Smads

ÖZ

AMAÇ: Transforming growth factor beta (TGF-β) ve Smad'lar nörülasyonda hücre içi sinyal yolaklarını kontrol ederler. Daha once yayınlamış benzer deneysel hayvan çalışmaları olmasına rağmen bu insan çalışmasının amacı, medikal abortus yaptırılmış myeloşizisli insan fetuslerinde TGF-β (1,2,3) ve Smad'ların (1,2,3,6,7) ekspresyonunu incelemektir.

YÖNTEM ve GEREÇLER: On iki tane nöral tüp kapanma kusurlu insan fetusu elde edildi. Örnekler, TGF-β1, TGF-β2, TGF-β3, Smad (1,2,3), Smad6 and Smad7 antikorları ile indirekt immünohistokimya tekniği kullanılarak boyandı.

BULGULAR: Açık nöral plak, motor nöronlar ve çevre dokularda TGF-β1 ve TGF-β2' ye ait hafif immün reaktivite saptandı. TGF-β3' e ait güçlü immün reaktivite sadece açık nöral plakta ve çevre dokusunda saptandı. Smad7 hariç tüm smad'ların immün reaktivitesi negatif saptandı.

SONUÇ: Bu sonuçlar nöral tüp kapanmasının başarısız olduğu bölgede TGF-β1,2 ve Smad 1,2,3 ve 6'nın aktivitesine devam etmediği ve embriyonik yaşla birlikte azaldığını düşündürdü. Buna ek olarak, ektodermal katlar embriyo tarafından "kapanmamış bir yara" olarak kabul edildiği ve TGF-β3 aktivitesinin bu kapanma kusurunun tamiri için gösterilen bir çaba olabileceği düşünüldü.

ANAHTAR SÖZCÜKLER: İnsan fetusu, Neural tüp defekti, TGF-βs, Smad

INTRODUCTION

Neural tube closure is accomplished by a complex morphogenetic program requiring precisely choreographed cellular proliferation, differentiation, adhesion and migration (22, 28). Neurulation proceeds in a series of steps by which the neural plate is shaped elongated and bent to form a tube that extends through the entire length of the anterior-posterior axis (31). There are a number of molecular factors such as noggin, chordin and follistatin, hedgehog group, wingless (Wnt) group, fibroblast growth factor (Fibroblast Growth Factor - FGFs), transforming growth factor-β (TGF-β) superfamily, retinoic acid, folic acid that regulate neural tube morphogen-

esis and lack of these factors can cause neural tube closure defects (NTD) (2, 4, 16, 21, 42).

In the neural tube, especially dorsal part, this coordination is achieved by the combined action of multiple ligands of both the Wnt and TGF-β families, and their effectors, such as the basic loop-helix-loop (bHLH) proteins. TGF-β signaling acting through the BMP receptors is necessary for the generation of several dorsal interneuron types. Other TGF-β ligands expressed in the dorsal neural tube interact with the activin receptors, which signal via a different set of Smad proteins than bone morphogenetic proteins (BMPs) (34).

The TGF-β superfamily members are a large family of cytokines that includes the TGF-βs, activins, BMPs and the others (1, 3, 5). Members of the TGF-β superfamily mediate a wide range of biological activities, including cell proliferation, differentiation, migration, apoptosis, angiogenesis, hematopoiesis, extracelluler matrix formation, and induction of homeobox genes, suggesting that TGF-β signaling is important in pattern formation during embryogenesis. It known that TGF-βs family members have 3 described isoforms (TGF-β1, TGF-β2, TGF-β3) in neural tissue whereby only TGF- β2 and TGF- β3 are expressed in the developing nervous system (1, 7, 9, 11, 15, 26, 36, 37).

TGF-β signal is transmitted through the phosphorylation of Smad proteins from cell membrane to nucleus. ExtracellularTGF-β signals are transduced via membrane-bound TGF-βreceptor serine/threonine kinase, which phosphorylate intracellular Smads (8,13). On the other hand a major limitation to successful myelin regeneration arises from negative regulatory pathways that operate in the demyelinating environment, such as BMP, Wnt and Notch signaling. BMPs, members of the TGF-β family, bind to heteromeric complexes of BMP type I (mainly BMPR-Ia or b) and type II (e.g. BMPR-II) also serine/threonine kinase receptors and activate downstream gene expression including oligodendrocyte differentiation inhibitors Id2 and Id4 mainly through BMP receptor-activated Smads (Smad1,5,8) (17, 19, 25, 39).

Smad proteins are signal transducer and transcriptional modulators. They are classified into 3 subgroups according to structural and functional characteristics. The first group consists of receptor- regulated Smads (R- Smads; Smad 1,2,3,5,8). The second group contains the common mediator Smads (Co- Smads; Smad4). The third class comprises the inhibitory Smads (I- Smads; Smad6,7) (8, 13).

Smad1 is a receptor regulated Smad (R- Smad) and it's activated by BMP type 1 receptor kinase. Smad2 mediates the signal of the TGF-β. This protein is recruited to the TGF-β receptors through its interaction with the Smad anchor for the receptor activation protein. Like other Smads, Smad2 plays a role in the transmission of extracellular signals from ligands of the TGF-β superfamily of the growth factors into the cell nucleus. Phosphorylated form of Smad2 is than able to form a complex with Smad4. These complexes accumulate in the cell nucleus, where they are directly participating in the regulation of gene expression, which is a target for Smad-specific E3 ubiquitin ligases, such as SMURF1 and SMURF2 and undergoes ubiquitination and proteasome-mediated degradation (8, 23).

Component of the heterotrimeric Smad2/Smad3-Smad4 complex forms in the nucleus and is required for the TGF-mediated signaling. Promotes binding of the Smad2/Smad4/FAST-1 complex to DNA and provides an activation function required for 1 or Smad2 to stimulate transcription. Smad4 binds to R-Smads, such as Smad1 or Smad2 and facilitates the translocation of the heteromeric complex into the nucleus. Smad4 may form heterotrimeric, heterohexameric or heterodimeric complexes with R-Smads. In the nucleus the heteromeric complex binds promoters and interacts with transcriptional activators. Smad3/ Smad4 complexes can directly bind the SBE (Smad-binding DNA element), which is a four-base-pair sequence 5'-GTCT-3' or the complement 5'-AGAC-3'(12). Many TGF-β ligands use this pathway and subsequently Smad4 is involved in many cell functions such as differentiation, apoptosis, gastrulation, embryonic development and the cell cycle (13, 23).

Smad7 a member of the I-Smads, inhibits TGF-β signaling by preventing formation of Smad2/ Smad4 complexes which initiate the TGF-β signaling. It interacts with activated TGF-β type 1 receptor therefore block the association, phosphorylation and activation of Smad2. By occupying type 1 receptor for activin and BMP, it also plays a role in negative feedback of these pathways (13, 14, 18, 20, 33, 40).

There are numerous experimental studies on the effect and role of TGF-β and Smads on the embryonic development however studies evaluating both TGF-β and Smads in human embryo with neural tube closure defects are still insufficient.

In this study, TGF-βs (1,2,3) and Smads (1,2,3,6,7) immune reactivity in neural tube defects of aborted human fetal samples was evaluated.

MATERIAL and METHODS

Tissue Collection and Fixation

Prepared and stored 12 neural tube defected aborted human fetuses' paraffin blocks by Selcuki et al. in a previous study (29) were used for histochemical and immunohistochemical staining of TGF-βs and Smads.

Samples from 12 (5 male and 7 female) aborted human fetuses with NTD were obtained from the department of pathology, Tepecik Women Health Care Hospital, Izmir. The fetuses ranged from 12 to 24 weeks of gestation mean 17.1 and median was 17 weeks.

All extracted defective fetal neural tissue samples were fixed in 10% formalin solution. These samples were then washed with tap water and soaked in a series of 50%, 60%, 70%, 80%, and 90% ethanol for 30 minutes and then in 95% and 100% ethanol for one hour. Then they were held in a solution of

100% ethanol and xylene that had a 1/1 ratio for 30 minutes, and embedded in paraffin and held at 60˚C for one hour to make paraffin blocks (29).

5 µm thickness serial transverse sections were taken by using the Rotary Microtome (RM 2135i Leica) from the blocks and prepared for both histochemical and immunohistochemical staining.

Histochemical Observations

Sections dewaxed at less than 60°C overnight were immersed in xylene for one hour and then rehydrated through a graded series of ethanol (95%, 80%, 70%, and 60%) for 2 minutes in each concentration and they were then washed in tap water. Sections were stained with either Hematoxylin (01562E, Surgipath, Bretton, Peter Borough, Cambridgeshire) - Eosin (01602E, Surgipath, Bretton, Peter Borough, Cambridgeshire) (HE) or Periodic Acids Schiff (PAS, 38016SS4, Surgipath, Bretton, Peter Borough, Cambridgeshire) according to its routine protocols. Slides were mounted using entellan (UN1866, Merck) and covered with glass cover slips prior to viewing, and photographed under the light microscope (Olympus BX-40, Tokyo, Japan).

Immunohistochemistry

After deparaffination at 60°C overnight, sections were held in xylene for one hour. Followed washing with serial concentrations of ethanol (95%, 80%, 70%, and 60%, 2 minutes for each), the sections were washed with distilled water and phosphate buffered saline (PBS) for 10 minutes. Then they were held in 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37 °C for 15 minutes and underwent three 5-minutes washes in PBS. The around of sections were drawn with a immunohistochemistry pen (Dako S-2002, Carpinteria, CA, USA) and incubated in 3% hydrogen peroxidase for 15 minutes to inhibit the endogenous peroxidase activity in the room conditions. They were then washed with phosphate buffered saline (PBS, P-4417, Sigma, St. Louis, USA) and were then treated with a blocking solution (A Blocking solution 85-9043, Zymed) for one hour.

After removal of the blocking solution from the tissue, primary antibodies [anti-TGF-β1 (sc-146, Santa Cruz, California, USA), anti-TGF-β2 (sc-90, Santa Cruz, California, USA), anti-TGF-β3 (sc-82, Santa Cruz, California, USA), anti- SMAD1/2/3 (sc-7960, Santa Cruz, California, USA), anti-SMAD6 (sc-13048, Santa Cruz, California, USA) and anti- SMAD7 (sc-11392, Santa Cruz, California, USA) of 1/100 concentrations were incubated for 18 hours at +4 ºC. The secondary antibody biotinylated goat IgG, anti-mouse IgG (sc2005, Santa Cruz) (to anti-SMAD1/2/3) or anti-rabbit (sc2004, Santa Cruz) was applied for 30 minutes, followed by triple washes in PBS. The streptavidin–peroxidase complex (supplied ready to use by Zymed) was added for 30 minutes and washed three times with PBS. Samples were stained with diaminobenzidine (DAB, 1718096, Roche) for 10 minutes to determine the visibility of the formed immunohistochemical reaction. Slides were washed again two times with deionized water for 10 minutes each. Counter-stained performed with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) than dehydrated and cleared with xylene for 30 minutes and they covered with entellan (16125, Surgipath). The slides were examined under a BX 40 microscope (Olympus, Tokyo, Japan). The presence of a brown precipitate indicated positivity for the primary antibody. The negative immunolabelling controls received the same treatment, with rabbit IgG or mouse IgG instead of the primary antibody.

Serial sections were examined and immunolabelling patterns were compared. Three observers who were blinded to the clinical information of the samples independently evaluated the immunolabelling scores. Immunolabelling intensity was graded as negative (-), mild (+), moderate (++) and strong (+++).

RESULTS

We obtained positive Periodic Acids Schiff (PAS) staining, basal lamina was seen both closed and none closed neural plate. Epandymal cells, neurons and mesenchymal cells were also seen around the open neural plate. Mild (+) TGF-β1 (Figure 1A) and TGF-β2 immune reactivity (Figure 1B) were observed in both around the neural plate and the motor neurons. On the other hand strong TGF-β3 (+++) staining was detected in neural plate especially in mesenchymal and neuroectodermal cells. Immune reactivity of TGF-β3 on the motor neurons was negative (-) (Figure 1C). In addition at the motor neurons and mesenchymal cells around the neural plate remained open the immune reactivity of Smad1/2/3 (Figure 2A), and Smad6 (Figure 2B) were negative (-). However, mild (+) Smad7 immune labeling was detected in failed neural tissue to close (Figure 2C).

Staining intensity of TGF-βs and Smads in neural plate, ectodermal cells, mesenchymal cells and motor neurons were given in Table I.

DISCUSSION

Neurulation is the first and very important process that prepares the main frame of the central nervous system. While this procedure prepares the main frame it further has

Table I: Staining Intensity of TGF-βs and Smads

	Neural plate	E. Cells/M. Cells	Motor Neuron
PAS	++	++	++
TGF-β1	+	+	+
TGF-β2	+	+	+
TGF-β3	+++	+++	–
Smad 1	-	-	-
Smad 2	-	-	-
Smad 3	-	-	-
Smad 6	-	-	-
Smad 7	+	+	+

E. Cells: *Ectodermal cells.*
M. Cells: *Mesenchymal cells.*

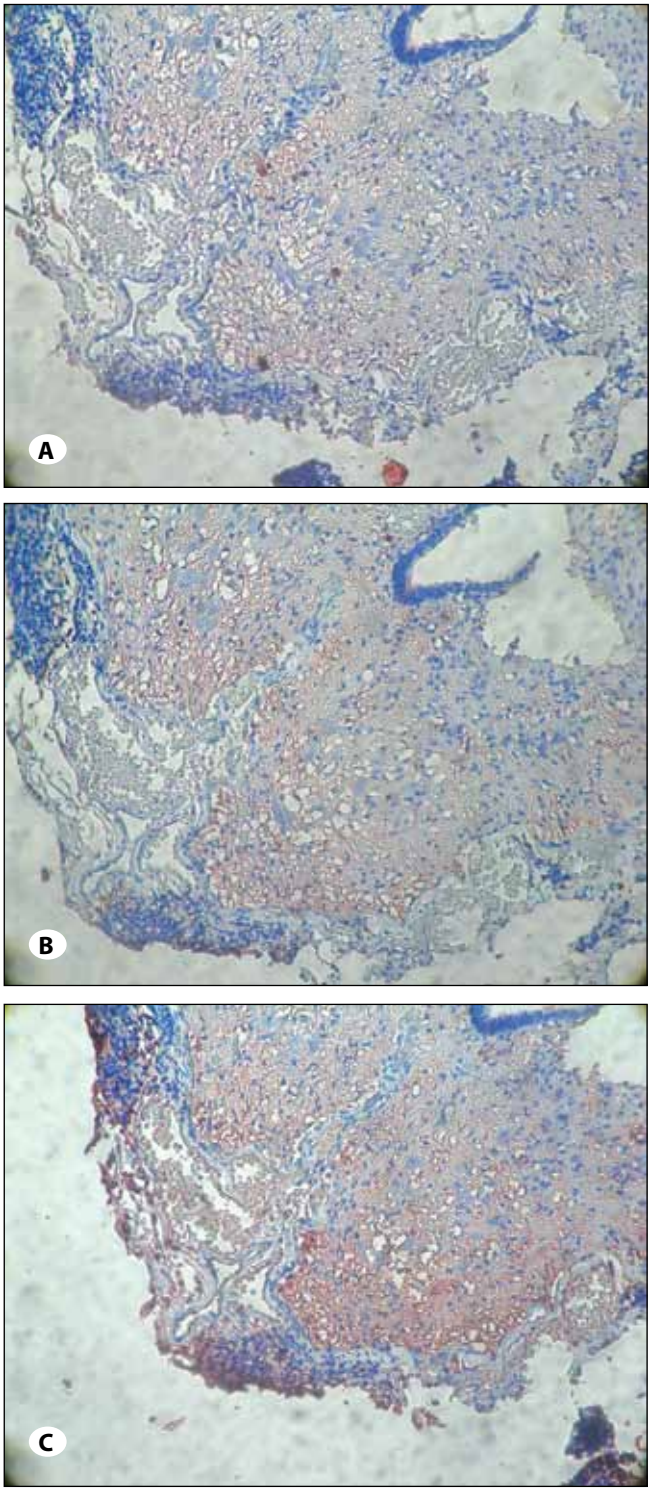

Figure 1: Mild (+) immune reactivity of TGF-β1 **(A)** and TGF-β2 **(B)**, intensive (+++)immune reactivity of TGF-β3 **(C)** on human samples with NTD x200.

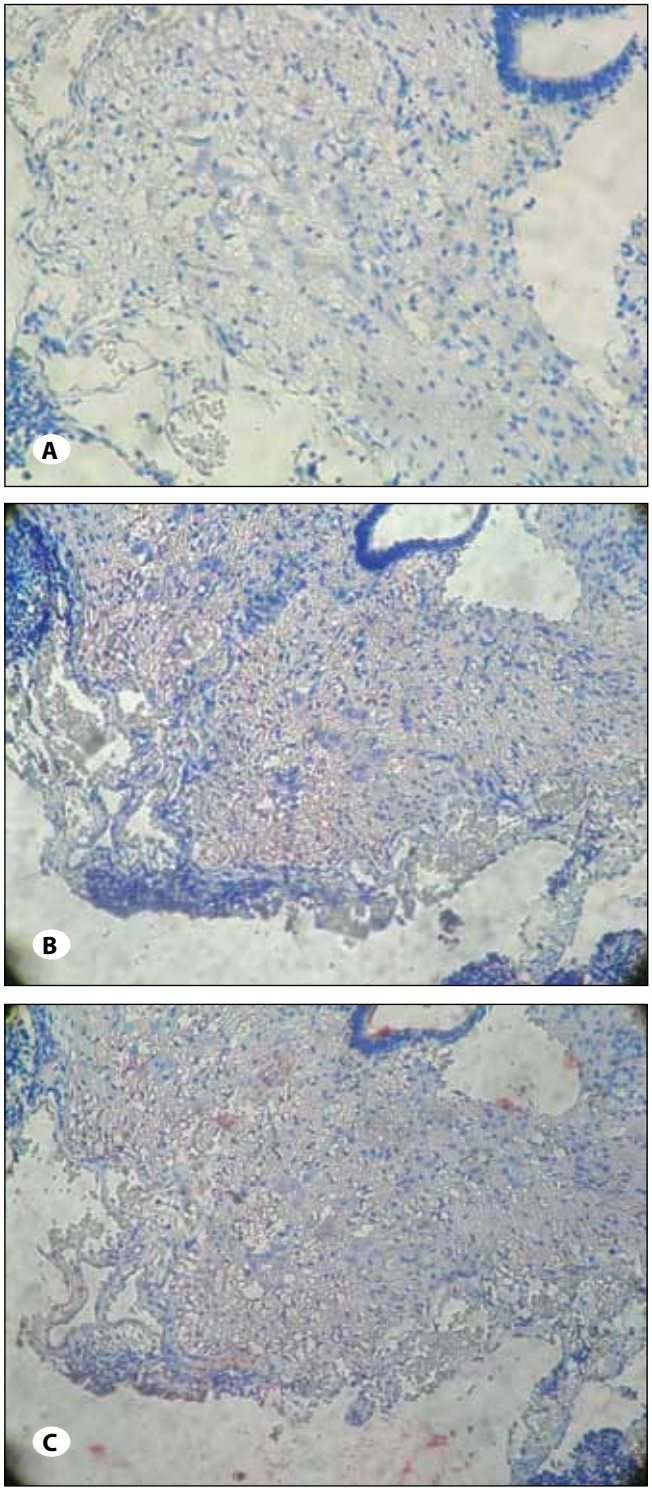

Figure 2: Negative (-) immune reactivity of Smad1,2,3 **(A)** and Smad6 **(B)** on human samples with NTD x200. **C)** Mild (+) immune reactivity of Smad7 on human samples with NTD x200.

four very important developmental steps. The failure of each of them causes neural tube defects of different kinds. These steps are respectively named, formation, shaping, bending of the neural plate, and the closure of the neural groove. Inductive signals and resultant molecules play the major role in the formation of the neural layer (2, 4, 6, 16, 21, 28, 31, 41).

It was believed that neurological functional loss was a consequence of the non-fusion of neural folds at the midline; recent investigations show that neural tissue continues its maturation in animal models. In our study we obtained positive PAS staining that means neural tissue and surrounding tissues continue its development and maturation despite the aberrant morphology at the side of NTD. PAS staining is mainly used to detect polysaccharides such as glycogen and mucosubstance and it is typically found connective tissue and basal laminae.

During neurulation, members of the TGF family, several bone morphogenetic proteins, secreted from the ectoderm overlaying the neural tube and from the roof plate, with the morphogen sonic hedgehog (Shh) secreted from the notochord and from the floor plate, are the major extracellular signaling pathways that lead to the generation of distinct classes of neural progenitors at specific dorsoventral locations within the spinal cord (10).

In the study performed by Flanders et al., each TGF-beta isoforms were localized immunohistochemically by specific antibodies raised to peptides corresponding to unique sequences in the respective TGF-β proteins in the nervous system of the 12-18 day mouse embryo. This study reports that TGF-β2 and TGF-β3 may play a role in regulation of neuronal migration and differentiation (9). Recent published article reports that in the chick embryos TGF-β1 and TGF-β2 activities on the neural tube have been decreased with embryonic stage (35). While in the first 48 hours, TGF-β1 and TGF-β2 activities detected moderate, weakening of activity of them were observed in the late stages. In fact strong immune reactivity of TGF-β3 in early stage, reported missing in 72nd hour embryos (35). Additionally class III beta tubulin; a neuron specific molecule is a major indicator of neural development and maturation (30). In the study of Selcuki et al., demonstrated positive immune reactivity of class III beta tubulin, this was the same sample group of our study. It was showed the completion of neural maturation (29, 30). Although TGF-β3 closely associated with cell differentiation and development, immune reactivity of TGF-β3 in motor neurons were not obtained in our study. Therefore we suggested that absence of immune reactivity of TGF-β3 in motor neurons may be the result of completed neuronal differentiation and maturation. On the other hand, absence of TGF-β3 is cause of failure of epithelial cells in both sides of the developing palate to fuse. Association of cleft palate defects with "off the midline defects" is known. According to the above data, opposite to our expectation, TGF-β3 had strongly positive immune reactivity in our study. TGF-β3 is believed to regulate molecules involved in cellular adhesion and extracellular matrix formation during the process of the wound healing. TGF-β3 controls this process by regulating the movements of epidermal and dermal cells in injured skin. We noticed strong staining of TGF-β3 in neural plate and surrounding epithelial and connective tissues. This result suggests that opened neural tube is not a result of absence of TGF-β3, on the contrary this may considered as the efforts of the organism to repair the failed neurulation like wound healing.

Pelton et al., revealed the immunohistochemical localization of TGF-β1, 2 and 3 in all embryonal tissues at the early period mouse embryo (24). They showed that in the CNS, TGF-β2 and β3 were found intensively, but that there was little TGF-β1. They also reported that intensive TGF-β2 was found in the meninges, and little TGF-β1 was observed in the CNS, and that TGF-β3 was found more intensively in the choroid plexus, brain tissue and the spinal cord (24). Despite the low TGF-β2 and TGF-β3 immunoreactivity of 72nd hrs chick embryo in the neural tube structure, the immunoreactivity has been found to be excessive in early embryonic structures such as the notochord, floor plate and the dorsal aorta. It has also been shown that TGF-β organizes the neural crest cell migration and supports the survival of neurons (1, 15, 37, 38). We obtained mild TGF-β1 and TGF-β2 staining both around the neural plate and the motor neurons. Although all these above-mentioned studies were experimental animal models, our immunohistochemical results in the human embryos with neural tube closure defects support them.

Smad proteins are the main TGF-β/BMP-receptor substrates that transduce signals. Formation of central and peripheral nervous system related cellular responses of the TGF-β family occurs by mediation of Smad proteins that are involved in intracellular signal conduction. Disruption of the TGF-β/Smad signal conduction path, and excessive or insufficient secretion of Smad proteins have been shown to cause diseases as a result of a defect in nervous system development or inability to function. Therefore understanding of TGF-β/Smad signal conduction mechanism is important for comprehension of this developmental pathway (11, 19, 23, 27, 32).We noted that Smad 1,2,3 and Smad6 was negative. Smad2,3 transduces TGF-β/activin responses, and 1,5,8 lie on the BMP pathway. Receptor-mediated phosphorylation of R-Smads increases their affinity for a common Smad (Smad4), an essential component for the assembly of transcriptional complexes and for the generation of specific Smad responses. Smad3 mRNA expression in discrete progenitor domains of the developing neural tube whereas differentiated neurons showed no expression (10). Consequently absence of Smad3 staining in our study may a comprehensible result in neural tissue. However like many others Smad3 is involved in cell signaling and modulates signals of activin and TGFβ's in developing embryonic tissue. At this point mild TGF-β1 and TGF-β2 immune reactivity and negative Smad3 activity is concordant because Smad3 is a receptor regulated Smad (R-Smad) therewithal. While Smad6 protein is only inhibits BMP signaling pathway, Smad7 inhibits both TGF-β and

BMP signaling pathways. In human samples, the activation of Smad7, not Smad6, may be triggered after inhibition of the TGF-β3 expression rather than BMP expression. In our suggestion, to limit the expression of exaggerated TGF-β3, observing positive activity of Smad7-that the natural inhibitor (behaviors)-exceed normal.

CONCLUSION

There are numerous experimental animal studies on the effect and role of TGF-β superfamily members or Smads. However, human studies evaluating both of TGF-βs and Smad proteins in aborted neural tube defected fetuses are very rare.

In our study the mild immune reactivity of TGF-β1 and TGF-β2 was detected in both the neural plate site and the motor neurons, the strong immune reactivity of TGF-β3 was found in neural plate, especially surrounding epithelial and connective tissue unlike the motor neurons. Our results suggest that migration and differentiation of neural cells conclude at the related time of embryonic development, so we observed mild immune reactivity of TGF-β1and β2 on the neural fold. Additionally, high values of TGF-β3 activity suggested, that the neural tube failed to close, as well as the ectodermal layer are considered by embryo as a wound and this strong TGF-β3 activity may be an effort for healing. The high effectiveness of the positive smad7 seems to cause down regulation of exaggerated TGF-β3 activity. On the other hand, Smad7 activity seems to be suppressing other Smads activities.

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