Int. J. Dev. Biol. 42: 817-820 (1998)

Short Contribution

Immunohistochemical localization of TGF-β type II receptor and TGF-β3 during palatogenesis *in vivo* and *in vitro*

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ABSTRACT The disappearance of medial edge epithelium (MEE) is a critical event for palate fusion. TGF-β3 is one factor participating in the regulation of this process. To investigate the nature of ligand-receptor interactions *in vivo* between TGF-β3 and the type II TGF-β receptor (TβR-II), we compared the expression pattern of the receptor with TGF-β3. Immunohistochemical analysis of the mouse fetus from E12 to E15 showed that expression of TβR-II in the palate began at E13 when the palatal shelves were in a vertical orientation. TβR-II was localized in the epithelial cells. This epithelium-favored distribution remained during palatal shelf elevation, the medial edge epithelial adherence, and midline epithelial seam disruption. After palate fusion and mesenchyme confluence, weak expression of TβR-II was present in the mesenchyme. To verify the possibility that TGF-β3 and TβR-II expression coincide, immunohistochemistry was used to localize them both in serial sections. The distribution pattern of TGF-β3 was also epithelium-limited in the palate from E13 to E15, and the spatial localization was correlated with the expression of TβR-II. Immunohistochemical localization of TβR-II and TGF-β3 in palatal shelves in organ culture had patterns that were consistent with the *in vivo* results. These results suggest that TGF-β3 exerts its developmental role through TβR-II in an autocrine fashion. The expression of both TGF-β3 and TβR-II was below the detectable level in the mesenchyme following MEE disruption, suggesting that the TGF-β3 signal might not be required once the MEE has completed phenotypic transformation/migration.

KEY WORDS: *immunohistochemistry, TβR-II, TGF-β3, medial edge epithelium, palatogenesis*

The disappearance of medial edge epithelium (MEE) is a critical event for palate fusion. The MEE that remain viable may either migrate to join the oral and nasal surface epithelia or transform to a mesenchymal phenotype (for review, Shuler, 1995). Previous studies have provided evidence that the *TGF-β* family, especially *TGF-β3*, is important in the regulation of this process (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1991; Brunet *et al.*, 1995; Kaartinen *et al.*, 1995,1997; Proetzel *et al.*, 1995; Sun *et al.*, 1998).

TGF-β family members initiate their cellular action by binding to specific cell surface proteins. Three major types of TGF-β receptors (TβR) have been identified by receptor affinity labeling assays (for review, Massagué *et al.*, 1990). Both type I (TβR-I) and type II (TβR-II) receptors are transmembrane serine/threonine kinases indispensable for TGF-β signaling. Type III (TβR-III) is a membrane protein lacking a cytoplasmic protein kinase domain. TGF-β ligand first binds TβR-II, which triggers heterodimerization with TβR-I. Following heterodimerization, the TβR-II serine/threonine kinase transphosphorylates the TβR-I, resulting in propagation of the phosphorylation signal to downstream substrates. Thus, it appears the expression and signaling by the TβR-II is crucial to the integrity of the TGF-β signal transduction pathway (for review, Heldin *et al.*, 1997).

A correlation of the expression of the receptor and ligand by immunohistochemistry can provide insight into the target tissue and addresses the question of whether TGF-β exerts its actions in an autocrine/paracrine fashion. To address directly the nature of ligand-receptor interactions *in vivo* between TGF-β3 and TβR-II during palatogenesis, we compared the expression pattern of the receptor with that of TGF-β3 by immunohistochemical analysis.

Abbreviations used in this paper: MEE, medial edge epithelium; TGF-β, transforming growth factor-beta; TβR, TGF-β receptor; TβR-I, TGF-β receptor type I; TβR-II, TGF-β receptor type II; TβR-III, TGF-β receptor type III.

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0214-6282/97/$10.00

Printed in Spain

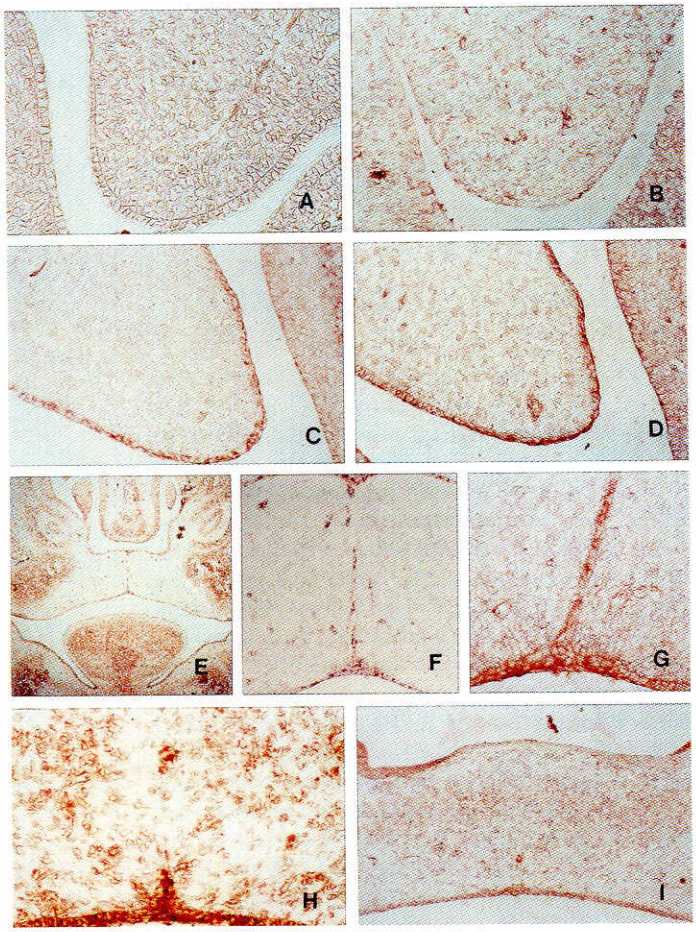

Fig. 1. Immunolocalization of TβR-II (A,C,E,F and H) and TGF-β3 (B,D,G and I) in palate tissues *in vivo*. (**A** *and* **B**) *E12, the palatal shelf has grown into the oral cavity. The distribution of TβR-II (A) or TGF-β3 (B) is not detectable in palatal epithelium and mesenchyme.* (**C** *and* **D**) *E13, the palatal shelf is in a vertical orientation along the lateral side of the tongue. The distribution of TβR-II (C) or TGF-β3 (D) is localized in the epithelial component of the shelf, but not in palatal mesenchyme.* (**E,F** *and* **G**) *E14.5, the midline seam is disrupted as the progress of palate fusion. The distribution of TβR-II (E and F) or TGF-β3 (G) is discontinuous in the midline, but persists in oral and nasal epithelium.* (**H** *and* **I**) *E15, after palate fusion and mesenchyme confluence, the distribution of TβR-II (H) predominates in epithelium although appears in mesenchyme. The distribution of TGF-β3 (I) is weaker in both epithelium and mesenchyme compared with that of TβR-II.*

Immunolocalization of endogenous TβR-II in palate tissue and comparison with that of TGF-β3

At E12 when the palatal shelves have first grown into the oral cavity as extensions of the maxillary processes, immunolocalization of TβR-II was not detectable in either palatal epithelium or mesenchyme (Fig. 1A). The expression of TβR-II appeared at E13 when the palatal shelves were in a vertical position along the lateral sides of the tongue. TβR-II, however, was immunolocalized only in the epithelial component of the shelf, not in palatal mesenchyme (Fig. 1C). This epithelium-favored expression pattern remained constant as the palatal shelves elevated, the medial edges of the two palatal shelves contacted in the midline, and a single layer of MEE cells were present in the midline (E14-E14.5). The distribution of TβR-II was only in oral epithelium, nasal epithelium and the midline epithelial seam, not in the underlying mesenchyme. As development continued, the midline seam was disrupted and the distribution of TβR-II in the midline became discontinuous (Fig. 1F). At E15 after palatal fusion and mesenchymal confluence weak expression of TβR-II was also present in the mesenchyme in addition to the continuous localization in the oral and nasal epithelium (Fig. 1H).

In general, the distribution of TGF-β3 in palate tissue was similar to the pattern observed for TβR-II with expression limited to the epithelium covering the palatal shelves and absent from the underlying mesenchyme. The expression of TGF-β3 was not detectable until E13 (Fig. 1B and D). Immunolocalization of TGF-β3 was also limited to palatal epithelium at E13-E14.5 (Fig. 1D and G). Spatial localization of TGF-β3 was correlated with the expression of TβR-II at all developmental stages examined (Fig. 1A-D; 1F-I).

Immunolocalization of TβR-II/TGF-β3 in cultured palate tissue and comparison with the in vivo expression pattern

Based on previous experience, organ cultured palatal shelves were recovered after different time periods that match *in vivo* stages of palatal development. After 7 h in organ culture the medial edges of opposing palatal shelves remained apart, and the distribution of TβR-II was observed in the epithelium covering the shelves (Fig. 2A). As organ culture continued, the palatal shelves were adherent and a two-cell layer thick seam of epithelial cells was present in the midline of the palate (after 24-36 h of organ culture). The TβR-II immunostaining was restricted to the epithelial cells covering the palatal shelves and in the midline seam, but not observed in the palatal mesenchyme (Fig. 2C). The distribution of TβR-II remained epithelial specific as the midline MEE seam was reduced to a single layer of cells. Once the MEE midline seam became fragmented around 48 h of organ culture, the distribution of TβR-II lost its continuity at midline (Fig. 2E).

To verify the possibility that TGF-β3 and TβR-II expression also coincide under palatal shelf organ culture conditions, immunohistochemistry was used to localize TGF-β3 on tissue sections adjacent to those examined for TβR-II. The expression pattern of TGF-β3 showed remarkable similarity with that of TβR-II (Fig. 2B,D and F). The results from immunolocalization of TβR-II/TGF-β3 in organ cultured palatal tissues confirmed the limitations on the pattern of expression that were observed *in vivo*.

Based on *in situ* hybridization, *TGF-β3* mRNA was the first gene in the *TGF-β* family expressed in the palatal shelf at E13.5 (Fitzpatrick *et al.*, 1990). This is consistent with our data presented here showing that both *TGF-β3* and TβR-II began to express at this stage. TGF-β3 mRNA was defined only in MEE once palatal shelves contacted in the midline (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990), however, both TGF-β3 and TβR-II were immunolocalized in the entire palatal epithelium. Conflicting data from *in situ* hybridization and immunohistochemistry were also seen in previous studies. For example, TGF-β1 and TGF-β2 polypeptides were in palatal tissues detected earlier than their mRNA was identified (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1991). *TGF-β* gene expression appears to be localized to specific cells however diffusion of the growth factor may expand the range of affected tissue. Thus, correlating growth factor localization with its receptor is important to determine signaling events critical to palatogenesis.

The antibody specificity in this study was sufficient to assess the important role for TGF-β3 and TβR-II in palatal fusion. Negative results at E12 were contrasted with the positive data provided by E13 to E15. Both TGF-β3 and TβR-II also showed temporal and spatial distribution patterns in other tissues, such as tongue, in which the distributions were localized predominantly in tongue muscles (Fig. 1E).

Several conclusions can be drawn from the comparison of the sites of expression of TβR-II and TGF-β3 in the developing palate. First, the receptor localizes predominantly to epithelium including oral epithelium, nasal epithelium and the midline epithelial seam. TβR-II is lost in the midline coincident with the disappearance of the MEE, indicating that this epithelium may be the major target tissue. It is likely that TGF-β signaling through the type II receptor may play a role in the phenotypic transformation of MEE. Second, the receptor expression coincides with the expression of the ligand TGF-β3. Thus, co-localization strongly suggests that TGF-β3 exerts its activities in an autocrine fashion during palate development.

Studies on phenotypic transformation mediated by TGF-β3 in lipocytes to myofibroblast-like cells during hepatic fibrosis and endothelium to mesenchyme during formation of chicken endocardial cushion tissue also suggested that it transduced signal in an autocrine fashion via TβR-II (Potts *et al.*, 1991; Nakajima *et al.*, 1994,1997; Brown *et al.*, 1996; Demirci *et al.*, 1996; Ramsdell and Markwald, 1997).

The correlation between TGF-β3 and TβR-II does not rule out the possibility that TGF-β1 or TGF-β2 also transduce signal through TGF-β receptors. In contrast, we believe that the achievement of palate fusion is in part the outcome of the collaboration of all three TGF-β isoforms (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1991; Gehris and Greene, 1992; Brunet *et al.*, 1993). The expression of *TGF-β1* and *TGF-β2*, however, seems a later event than *TGF-β3* in this process (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). The exact relationship between *TGF-β3* and the two other isoforms requires examination.

All members of the TGF-β family are synthesized as a large precursor protein, and secreted as latent complexes, which can not interact with TGF-β receptors. To exert its actions, TGF-β must be liberated from this complex to yield a mature carboxyterminal unit of 112 amino acids (for review, Munger *et al.*, 1997). The antibody used in the present study corresponds to the C-terminal domain of the precursor form. A previous study which showed exogenous TGF-β3 accelerating the fusion process under culture condition (Brunet *et al.*, 1993) provides evidence suggesting a role of mature TGF-β3 in palatal fusion. It is likely that exogenous TGF-β3 bypasses the time required for the cell to process latent TGF-β3, which has been suggested in endocardial cells (Ramsdell and Markwald, 1997).

Epithelial-mesenchymal transformation is a temporal multi-step process involving overlapping changes of both morphology and gene expression. The mechanism which mediates the epithelium-mesenchymal transformation may be initiated by the mesenchyme generating inductive signals to overlying epithelium (Ferguson and Honig, 1984; Nakajima *et al.*, 1997; Ramsdell and Markwald, 1997). We found in this study the presence of TβR-II and TGF-β3 precursor throughout the entire epithelium of the palatal shelf from E13 to E15, while only MEE cells undergo epithelial-mesenchymal transformation. It is possible that the specific inductive molecules reside in the mesenchyme underlying the MEE, which trigger the functional activity of TGF-β3 in the targeted MEE cells.

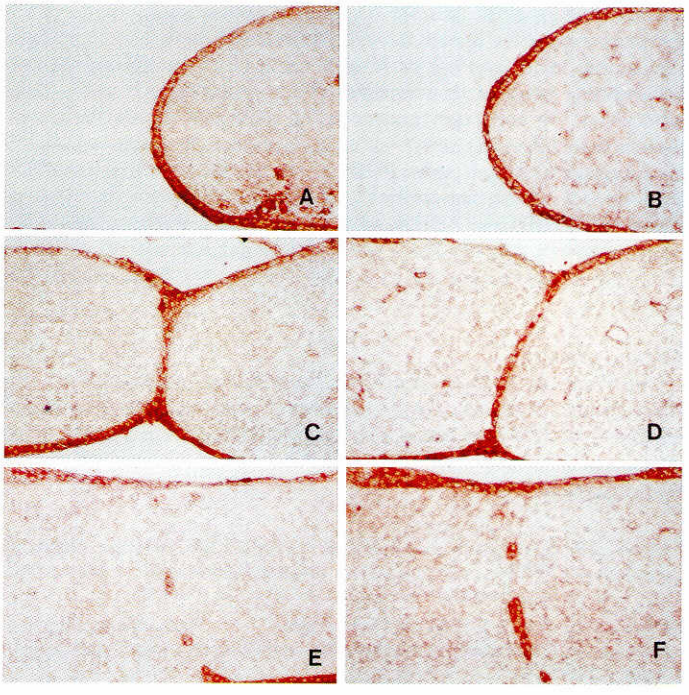

Fig. 2. Immunolocalization of TβR-II (A,C and E) and TGF-β3 (B,D and F) in palate tissues in culture. (A *and* **B)** *E13+7h, the medial edges from opposing palatal shelves remain apart. The distribution of TβR-II (A) or TGF-β3 (B) predominates in entire epithelium.* **(C** *and* **D)** E13+36h, *a two-layer-MEE seam is present in the midline of palate. The distribution of TβR-II (C) or TGF-β3 (D) is restricted to MEE and the epithelium covering the palate tissue, but not in mesenchyme.* **(E** *and* **F)** *E13+48h, the midline seam becomes fragmented. The distribution of TβR-II (E) or TGF-β3 (F) loses its continuity at midline, however, persists in oral epithelium.*

Experimental Procedures

Timed pregnant Swiss-Webster mice were sacrificed from E12 to E15. The fetal heads were fixed immediately in 4% paraformaldehyde-PBS at 4°C, followed by routine procedures for embedding in paraffin. The coronal sections (5 μm) were mounted in serial order on poly-L-lysine coated slides. The tissues were examined by immunohistochemistry to analyze the expression pattern of endogenous TβR-II and compare it with TGF-β3.

To more readily compare the expression levels between different developmental stages, each slide contained sections from all developmental stages, E12 to E15. To verify the possibility that TβR-II and TGF-β3 expression occur in the same cells, immunohistochemistry was used to localize both TβR-II and TGF-β3 in serial sections.

To compare the expression pattern of TβR-II/TGF-β3 during palatal shelf organ culture conditions with that of *in vivo*, palatal shelves were dissected from E13 fetal murine heads and placed in pairs on Millipore filters with their medial edges in contact. The palatal shelves were cultured at the air-medium interface in Grobstein organ culture dishes in BGJb medium (Gibco) at 37°C and a 5% CO_2/air atmosphere. The organ cultures were maintained for up to 48 h. The first specimens were collected at 7 h and the rest were continuously harvested every 12 h. The procedures of tissue preparation and the experimental strategy were the same as above.

The primary antibody (rabbit polyclonal IgG) against TβR-II or TGF-β3 (Santa Cruz Biotechnology, Inc.) was incubated on the tissue at a concentration of 2 μg/ml overnight at room temperature. The immunizing epitope of TβR-II corresponds to amino acids 550-565 mapping within the carboxy terminal domain of the precursor form of the human homologue and the antibody is mouse reactive. The immunizing epitope of TGF-β3 corre-

sponds to amino acids 350-375 mapping at the carboxy terminus of the precursor form of the human homolog (identical to corresponding mouse sequences). Incubation was followed by addition of a biotinylated secondary antibody. Streptavidin-peroxidase was then added to bind the biotin residues on the secondary antibody. The presence of peroxidase was revealed by addition of substrate-chromogen solution. Peroxidase catalyzed the substrate (hydrogen peroxide) and converted the chromogen (AEC) to a red deposit, which demonstrates the location of the antigen TβR-II or TGF-β3 (HISTOSTAIN™ BULK kit, from Zymed Laboratories Inc.). As a negative control, rabbit IgG was substituted for the primary antibody. Each of the experiments was repeated 3-4 times to show the consistency of the results.

Acknowledgments

We would like to thank Drs. Yihsin Liu and Wen Luo for their suggestions. We also thank Mr. Pablo Bringas and Valentino Santos for photographic work. This work was supported by NIDR grant DE-09165.

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Received: March 1998
Accepted for publication: April 1998