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Original Article

Immunodetection of the transforming growth factors ß1 and ß2 in the developing murine palate

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ABSTRACT The expression of some members of the TGFß family of genes in embryonic craniofacial tissue suggests a functional role for these molecules in orofacial development. In other systems, TGFß1 and TGFß2 have been shown to regulate cell proliferation and extracellular matrix metabolism, processes critical to normal development of the secondary palate. We have thus determined the amount and tissue distribution of both TGFß1 and TGFß2 in embryonic palatal tissue. Cellular extracts of murine embryonic palatal tissue from days 12, 13 and 14 of gestation were assayed for the presence of TGFß1 and TGFß2 by immunoprecipitation. Physiological levels, ranging from 0.05-20 ng/µg protein, of both growth factors were detected in all tissues examined. Immunostaining with antibodies directed against either TGFß1 or TGFß2 demonstrated the presence of these growth factors in palatal epithelium and mesenchyme early during palatal development (gestational day [GD] 12) a period characterized by tissue growth. On GDs 13 and 14, characterized by palate epithelial differentiation, immunostaining for both growth factors predominated in epithelial tissue. Immunostaining for TGFß1 and TGFß2 was also intense in mesenchyme surrounding tooth germs and in perichondrium. Chondrocytes and cartilage extracellular matrix did not stain for either TGFß1 or ß2. Combined with existing evidence for the presence of functional receptors for the transforming growth factor-ßs in the developing palate, these results provide rationale for studies designed to clarify a specific role for these signalling molecules in palate epithelial differentiation and/or epithelial-mesenchymal interactions.

KEY WORDS: *TGFß, growth factor, palate, embryo*

Introduction

Development of the embryonic palate is a complex process contingent on a multiplicity of events including growth, tissue differentiation, extracellular matrix production, epithelial-mesenchymal interactions, as well as a number of other molecular autocrine and paracrine interactions (Ferguson, 1988; Greene, 1989). The midfacial region in mammalian embryos develops largely from the first pharyngeal arch which contains mesenchymal cells derived from the cranial neural crest (Johnston, 1966; Noden, 1975). The secondary palate originates as bilateral extensions from the oral aspect of the maxillary processes. In mammals, the palatal processes make contact, fuse with one another and give rise to the secondary palate (roof of the oral cavity). As the medial edge epithelium (MEE) of the palate is removed by both cell death (Greene and Pratt, 1976) and epithelial-mesenchymal transformation (Fitchett and Hay, 1989), epithelia on the nasal aspect differentiates into pseudostratified ciliated columnar cells and oral epithelia becomes stratified squamous (Pratt and Greene, 1976). Previous experimental results suggest that control of these events involves regulatory molecules such as catecholamines, cyclic-AMP, prostaglandins and various growth factors (Pisano and Greene, 1986).

The TGFßs are representatives of a large family of polypeptides exhibiting sequence homology and a broad range of regulatory activities (Rizzino, 1988). First purified from human platelets and designated TGFß1 (Assoian *et al.*, 1983; Derynck *et al.*, 1985), it is now apparent that there are at least five genetically distinct forms of this growth factor (Mummery and van den Eijnden-van Raajj, 1990). TGFß2, originally isolated from bovine bone (Seyedin *et al.*, 1985, 1987) and porcine platelets (Cheifetz *et al.*, 1987), was shown to be identical to cartilage inducing factor implicating a role for this factor in chondrogenesis (Seyedin *et al.*, 1987). TGFß1 is synthesized as an inactive precursor which is proteolytically cleaved to release the C-terminal mature peptide. This then remains noncovalently associated with the amino-terminal peptide as an inactive complex which can be activated from the latent form to the biologically active peptide (Sporn *et al.*, 1987; Lyons *et al.*, 1989). From sequence analysis, TGFß2 appears to be processed to its mature form much like TGFß1 (Madisen *et al.*, 1988). TGFß2 shares

Abbreviations used in this paper: TGB-ß, transforming growth factor beta; GD, gestational day; EGF, epidermal growth factor; TGFα, transforming growth factor alpha; ELISA, enzyme linked immunosorbant assay; RIA, radioimmunoassay; ECM, extracellular matrix; PBS, phosphate buffered saline; BSA, bovine serum albumin.

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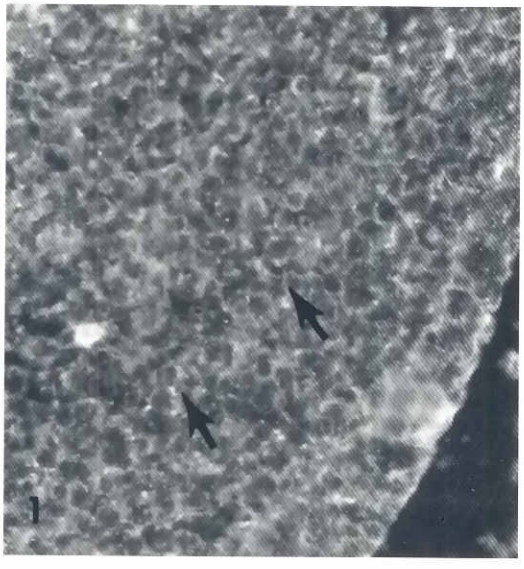
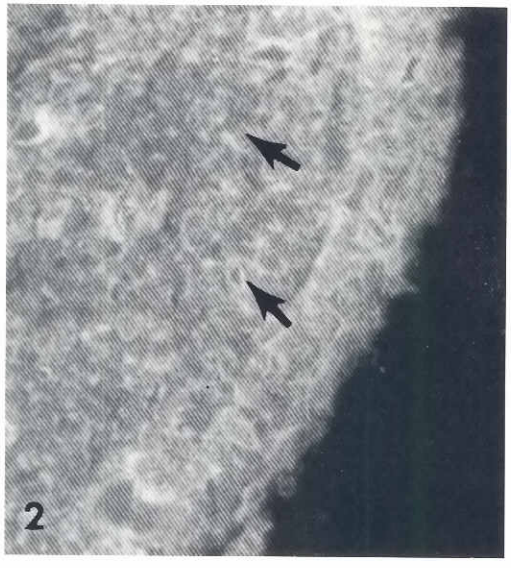




Figs. 1 and 2: Coronal sections of developing murine embryonic palatal tissue on day 12 of gestation. *These early palatal buds from the maxilla were immunostained for TGFß1* **(1)** *and TGFß2* **(2)** *by the indirect immunohistochemical method. In addition to pericellular staining for both TGFß1 and ß2 (arrows), TGFß exhibits distinct cytoplasmic staining in both epithelial and mesenchymal cells (Fig. 2). x80*

71% homology with the TGFß1 precursor and both TGFßs are potent modulators of cell differentiation and proliferation in many normal and transformed cell types (Sporn *et al.*, 1987; Rizzino, 1988).

All peptide growth factors can be considered as «peptide signalling molecules» as their activities encompass such diverse biological responses as growth stimulation and inhibition, cellular differentiation, and regulation of embryogenesis (Massague, 1987; Sporn *et al.*, 1987). Among the classical growth factors, only epidermal growth factor (EGF), has been extensively investigated as a possible regulatory molecule in palatal ontogenesis. Current evidence supporting the hypothesis that EGF, or a related peptide, transforming growth factor-α (TGF-α) modulates development of the secondary palate, has recently been reviewed (Pratt, 1987). Because of mounting evidence for the regulation of various developmental processes by TGF-ßs, these growth factors have also recently been described in developing orofacial tissue (Heine *et al.*, 1987; Fitzpatrick *et al.*, 1990).

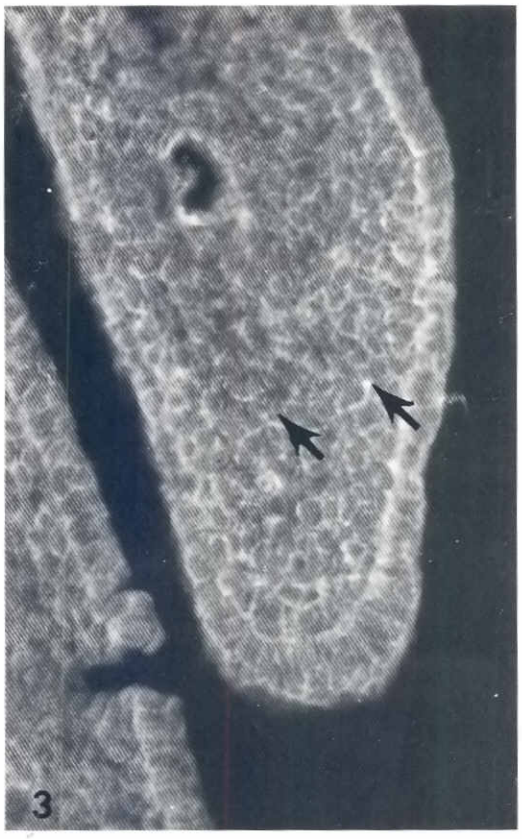
Evidence in support of the concept that TGFß1 represents an important mediator of embryonic development comes from observations that, often in synergy with other growth factors, TGFß1 can induce mesoderm (Kimelman and Kirschner, 1987; Weeks and Melton, 1987; Rosa *et al.*, 1988), control angiogenesis (Madri *et al.*, 1988), chondrogenesis (Seyedin *et al.*, 1985), bone formation (Robey *et al.*, 1987; Sandberg *et al.*, 1988) and the synthesis of extracellular matrix molecules (Varga *et al.*, 1987; Dean *et al.*, 1988; Redini *et al.*, 1988; Wrana *et al.*, 1988; Pelton *et al.*, 1989; D'Angelo and Greene, 1991). The transforming growth factor ß family thus exhibits multifunctional regulatory capacities and is documented as playing a significant role in embryogenesis.

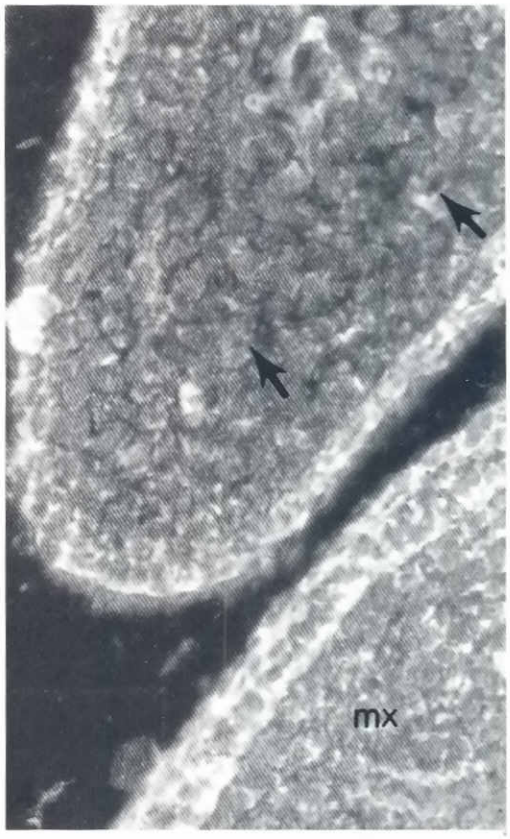
Although few biological differences have been determined for TGFß1 and ß2, there are several important distinctions. TGFß2 has been shown to be a more potent mesoderm inducing factor in *Xenopus laevis* during gastrulation (Rosa *et al.*, 1988) and TGFß1 a more potent growth inhibitor in some cells (Jennings *et al.*, 1988). In addition, mRNA encoding each of these molecules appears to be distributed uniquely in the embryo: TGFß1 to epithelial cells and TGFß2 to the mesenchymal component of tissues such as bone, cartilage, skin, and blood vessels (Lenhert and Akhurst, 1988; Pelton *et al.*, 1989). *In situ* localization of TGFß1 mRNA appears to change temporally throughout development in a way that is consistent with a role for the protein in mediating epithelial-mesenchymal interactions (Lenhert and Ankhurst, 1988; Miller *et al.*, 1989; Pelton *et al.*, 1989; Fitzpatrick *et al.*, 1990). TGFß1 protein has been immunolocalized in the developing mouse embryo particularly in the mesenchyme and most interestingly in areas critical for epithelial-mesenchymal interactions (Heine *et al.*, 1987). Furthermore, TGFß1 mRNA was localized in epithelia overlying mesenchyme in which the protein had been previously localized (Lenhert and Akhurst 1988, Akhurst *et al.*, 1990). The importance of epithelial-mesenchymal interactions has been established in the developing palate where, in a series of recombination studies, mesenchyme exhibited a regional control on the overlying epithelium (Ferguson and Honig, 1984). The tissue-specific localization of both the protein and TGFß mRNA suggests a role for both of these molecules in morphogenesis and tissue differentiation. Although the pattern of localization for the TGFßs in organs and tissues undergoing epithelial-mesenchymal interactions is suggestive of paracrine regulation, the precise nature of the way in which TGFß exerts its effects remains to be determined.

We have examined the presence and localization of TGFß1 and TGFß2 protein in the developing embryonic palate in order to better characterize these regulatory molecules in palatal morphogenesis. Our results, together with those of Fitzpatrick *et al.* (1990) and Pelton *et al.* (1990) wherein TGFß mRNAs were localized in the developing palate, suggest the importance of signalling molecules originating in palatal epithelium.

Results

In general, immunolocalization of TGFß1 and TGFß2 in palatal tissue on GD12, 13, and 14 demonstrated a similar pattern of distribution. On day 12 of gestation, when the palatal shelves have first grown into the oral cavity as extensions of the maxillary processes, there appeared to be a homogeneous distribution of





Figs. 3 and 4: GD13 palatal tissue immunostained for TGFß1 (3) and TGFß2 (4). *Immunostaining predominates in the epithelium, although distinct pericellular TGFß1 immunostaining persists in mesenchymal cells (arrows). Epithelium of the tongue (t) and maxilla (mx) exhibit pronounced epithelial staining for both TGFß1 and TGFß2. x80*

these growth factors in both palatal epithelium and mesenchyme (Figs. 1 and 2). As the shelves assume a vertical position alongside the tongue on day 13 of gestation, a marked reduction in immunostaining for these growth factors was seen in the mesenchyme while immunostaining in palatal epithelium remained prominent (Figs. 3 and 4). This temporal shift in immunostaining patterns was most dramatic on day 14 of gestation as homologous palatal shelves contact and form a midline epithelial seam (Figs. 5-7).

The staining was pericellular for both growth factors, although cytoplasmic staining in both epithelium and mesenchyme was more pronounced for TGFß2 (Fig. 2) than for TGFß1 (Fig. 1) on GD12. Epithelium of the tongue and maxilla also demonstrated pronounced epithelial immunostaining for TGFß1 and TGFß2 (Figs. 5-7). Notably, epithelium of the lateral surface of the tongue (Fig. 3) did not demonstrate the intensity of staining generally seen on the superior surface (Fig. 7). Endothelium (Fig. 5) and mesenchyme of the tongue (Fig. 7), particularly muscle cells, displayed intense immunostaining for TGFß1 and ß2. This latter observation agrees with TGFß staining patterns reported in the GD15 murine tongue by Heine *et al.* (1987). Distinct pericellular staining for both TGFß1 and TGFß2 was seen in nasal epithelium (Fig. 8). Cartilaginous regions (cranial base, nasal septum, Meckel's cartilage) exhibited positive staining for both TGFß1 and ß2 in the perichondrium while chondrocytes and cartilage extracellular matrix did not display the presence of immunoreactive material (Fig. 8).

Although immunostaining for both TGFß1 and TGFß2 predominated in epithelial tissue by day 13 of gestation, some mesenchymal cells remained intensely staining for these growth factors (Figs. 3 and 5). This may be explained by the heterogeneous nature of the mesenchymal tissue of the palate, which contains cells of similar developmental origin but with multiple differentiative potentials. One may speculate that some of these cells in the palate may be positive for the TGFßs because they represent mesenchymal cells primarily responsible for the deposition of extracellular matrix under the positive autocrine regulation of these growth factors (D'Angelo and Greene, 1991).

Our immunoprecipitation protocol allowed detection of TGFß1 and TGFß2 between 0.05 - 5 ng/µg total protein. Although exact values could not be determined, concentrations on each of gestational days 12-14 ranged between 0.05 and 0.5 ng/µg total protein. Values for TGFß2 were greater than those for TGFß1 on gestational days 13 and 14 and were calculated to be approximately 0.5 ng/µg total protein.

Results from these immunobinding experiments document that TGFß2 is present in embryonic palatal tissue at higher concentrations than TGFß1 on GDs 13 and 14. This is supported by our immunostaining studies wherein intensity of staining for TGFß2 was consistently greater than that seen for TGFß1. Although these latter observations must be cautiously interpreted in that differences in antibody affinities may contribute to differential staining intensities, the recent demonstration that expression of mRNA for TGFß1 and TGFß2 is greatest in palatal tissue on GD13.5 and 14.5 (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990) provides additional support for the premise that TGFßs are most abundant in embryonic palatal tissue during the gestational period characterized by tissue differentiation.

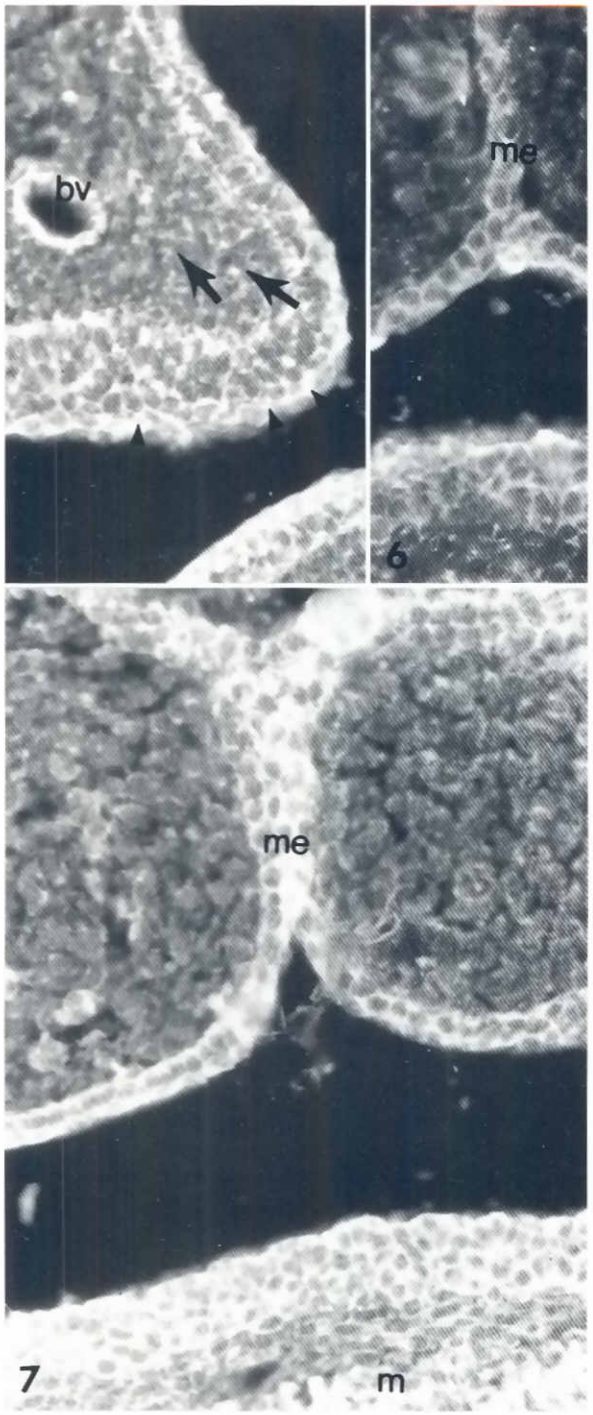


Fig. 5: GD14 palatal shelf that has elevated but not yet fused to the opposing shelf immunostained for TGFß2. *Some mesenchymal staining remains (arrows) but immunostaining predominates in epithelium (arrowheads). Endothelial-lined blood vessel (bv) immunoreactive for TGFß2. Note intense immunoreactivity of tongue epithelium (t). x80*

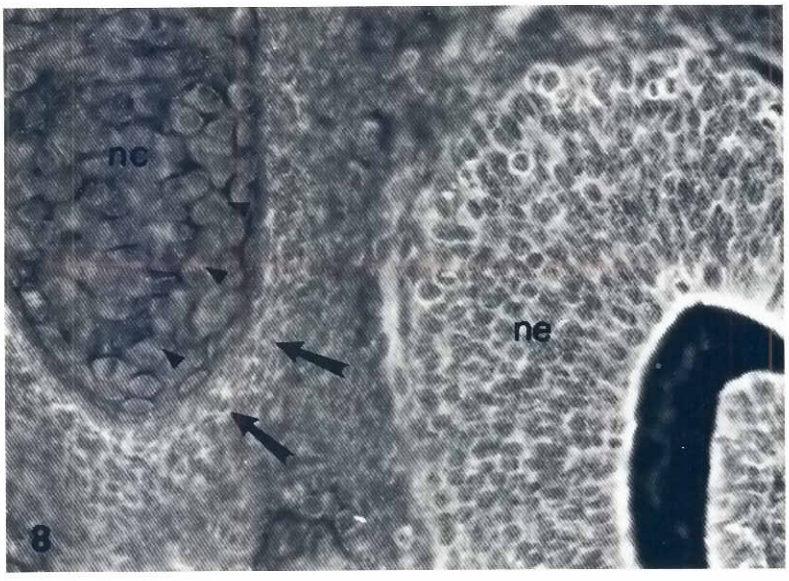
Figs. 6 and 7: GD14 palatal shelves that have fused to form a midline epithelial seam (me). *Immunostaining for TGFß1* **(6)** *and TGFß2* **(7)** *is seen primarily in epithelial cells. Notice the mesenchyme of the tongue, particularly the muscle cells (m), brightly immunostained for TGFß2. x80*

Discussion

Northern blot analysis of mRNA from the murine embryo has shown that TGFß1 and TGFß2 are expressed during embryogenesis in a wide range of tissues (Heine *et al.*, 1987; Miller *et al.*, 1989). *In situ* hybridization studies have further defined tissue and cell types in which these growth factor mRNAs are expressed (Lenhert and Akhurst, 1988; Pelton *et al.*, 1989). Recently, the temporal and spatial distribution of TGFß mRNAs in the developing palate has been reported (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). To characterize cellular and tissue distribution of TGFß polypeptides during palate development we have immunostained sections of the murine embryonic head, during the period of development of the secondary palate, with antibodies specific to TGFß1 and TGFß2. In addition, immunoprecipitation of cellular extracts of palatal tissue indicated relative amounts of these growth factors in developing palatal tissue.

The data presented here demonstrate the presence and shifting patterns of distribution of both TGFß1 and TGFß2 protein in embryonic palatal tissue. On GD 12 of palate development TGFß1 and TGFß2 protein was localized and evenly distributed in both epithelium and mesenchyme of palatal tissue. By GD13 and GD14 both TGFß1 and TGFß2 were most prominent in palatal epithelial cells. Most interesting is the pattern of epithelial distribution we have seen for TGFß1. This contrasts with a previous demonstration of TGFß1 in facial mesenchyme and other tissue of mesenchymal origin (Heine *et al*,. 1987; Aott, personal communication). A possible explanation for this apparent discrepancy may be the use of different antibodies in these two studies. In an earlier investigation of TGFß1 in the developing mouse embryo, the anti-CC (1-30) antibody was used (Ellingsworth *et al.*, 1986). This antibody was prepared against a synthetic peptide corresponding to the first 30 amino acids of the mature TGFß and was shown to immunoreact primarily with *extracellular* TGFß1 (Flanders *et al.*, 1989). In the studies presented here, the anti-LC (1-30) antibody, which recognizes *intracellular* TGFß1 was utilized. The epithelial staining we demonstrate correlates well with previous reports in the literature that staining with the anti-LC (1-30) is primarily intracellular in both the epithelium (*i.e.* intestinal mucosa, bronchi and renal distal tubule) and some mesenchymal cells (*i.e.* costal chondrocytes), while staining with the anti-CC (1-30) antibody is generally restricted to mesenchyme (Thompson *et al.*, 1989). Although anti-CC (1-30) does not recognize TGFß2 on western blots (Flanders *et al.*, 1989), it may crossreact with other TGFß peptides. It is possible that anti-LC (1-30) recognizes sites of TGFß1 biosynthesis while TGFß identified with anti-CC (1-30) recognizes the mature form of the growth factor. *In situ* localization of TGFß mRNAs in palatal tissue (Fitzpatrick *et al.*, 1990), as well as the observation that staining patterns for anti-LC (1-30) are similar to that seen with an antibody raised to a peptide corresponding to part of the TGFß1 precursor sequence (Flanders *et al.*, 1989), support this notion. Moreover, ELISA and RIA analyses indicate that these two antibodies recognize different epitopes of TGFß1 (Flanders *et al.*, 1989). Because extracellular staining patterns seen with anti-CC (1-30) resemble the distribution of fibronectin which has been demonstrated to be associated with TGFß (Fava and McClure, 1987), it is quite possible that extracellular TGFß1 is bound to matrix proteins and suggests a means by which this growth factor could exert its effects (see below).

That TGFß1 was not detected in tissue shown to transcribe



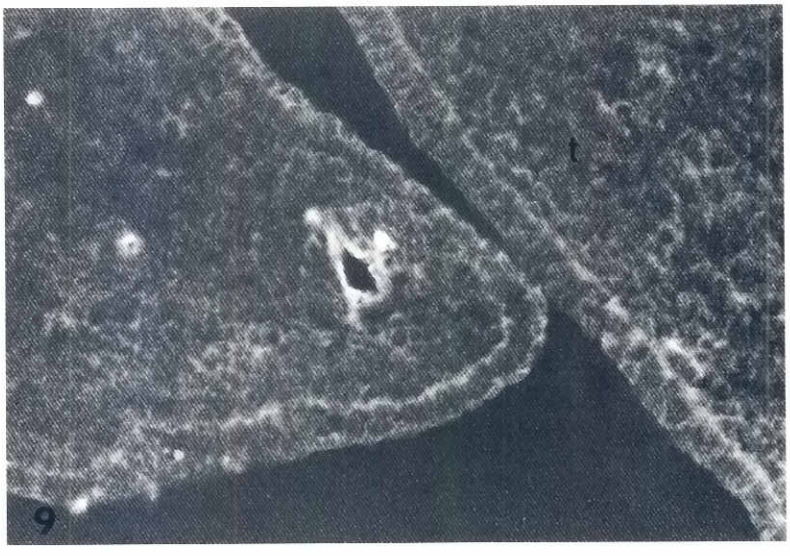


Fig. 8: GD14 nasal epithelium (ne) and nasal cartilage (nc) immunostained for TGFß2. *Nasal epithelium exhibited positive pericellular staining for ß1 as well as for ß2 (data not shown) and perichondrium of the nasal septum stained positively (arrows) while chondrocytes (arrowheads) and cartilage ECM remained negative. x80*

Fig. 9: GD13 palate control incubated with PBS containing 0.5% BSA in place of primary antibody.

TGFß1 mRNA (Heine *et al.*, 1987, Lenhert and Akhurst, 1988) can be explained by the fact that TGFß is secreted by virtually all cell types in an inactive form with a latency dependent on its ability to bind to a receptor (Wakefield *et al.*, 1988). Acidification or proteolysis activates TGFß1 *in vitro* (Lawrence *et al.*, 1985, Keski-Oja *et al.*, 1988) although the precise mechanism by which TGFß is activated *in vivo* remains uncertain. Specificity of action of TGFß may be determined by a cell or tissue's ability to activate latent TGFß (Sporn *et al.*, 1987). It can be speculated that during palatal development, regulation of medial edge epithelial differentiation may result from the ability of these cells to activate the latent form of TGFß1, possibly recognized by anti-LC (1-30), which affects, in a paracrine manner, cells in the underlying mesenchyme.

Distribution of the TGFß2 protein in embryonic craniofacial tissue has not been previously reported. We have demonstrated, using an antibody specific to TGFß2 and that does not crossreact with other TGFß peptides in ELISA and western blot assays, the distribution of this growth factor in developing orofacial tissue. In addition, we have localized this polypeptide in the epithelia of other developing tissue in the craniofacial region including the eye, nasal glands and tooth buds. These observations, in conjunction with recent *in situ* localization of TGFß mRNAs to these same cell populations (Fitzpatrick *et al.*, 1990), lend credence to a projected role for TGFßs in epithelial-mesenchymal interactions during palate development and also suggest both autocrine and paracrine modes of action as previously discussed (Heine *et al.*, 1987; Lenhert and Akhurst, 1988). Our data may also be interpreted as supporting a paracrine role for TGFß1 (Lenhert and Akhurst, 1988), wherein TGFß1, synthesized in epithelial tissue, exerts influence on the underlying mesenchyme. A similar model for TGFß2 has been suggested whereby this gene product, synthesized in the mesenchyme (Pelton *et al.*, 1989; Fitzpatrick *et al.*, 1990), exerts its effects on the overlying epithelium where we demonstrate the presence of this protein.

It is well documented that the epithelia of many developing organs are dependent upon tissue interactions with adjacent mesenchyme (Grobstein, 1967). In embryonic palatal tissue, epidermal growth factor (EGF) influences mouse palatal medial edge epithelial differentiation via action on the underlying mesenchyme (Tyler and Pratt, 1980; Yoneda and Pratt, 1981). It may be hypothesized that TGFß plays a similar role. TGFß can stimulate the synthesis of the extracellular matrix (ECM) molecules, fibronectin, type IV collagen and hyaluronic acid by palatal mesenchymal cells (Sharpe and Ferguson, 1988; D'Angelo and Greene 1991) while, in other systems,TGFß can stimulate epithelial cells to synthesize receptors for some of these molecules (Ignotz and Massague, 1987). Thus, dialogue between epithelium and mesenchyme in the embryonic palate may be under the control of TGFß1 and TGFß2 via modulation of the extracellular matrix and other growth factors. Indeed, TGFß can positively regulate its own expression in both normal and transformed cells (Van Obbhergghen-Schilling *et al.*, 1988) as well as modulate the expression of other genes including collagen, fibronectin and some receptors for cell adhesion molecules (Ignotz and Massague, 1986, 1987; Sharpe and Ferguson, 1988). More recently, the complex autoregulatory capacity of TGFß1, ß2 and ß3 mRNA has been documented (Bascom *et al.*, 1989). TGFß1 and TGFß2 were also shown to regulate the expression of other closely related TGFß genes indicating a complex pattern of auto- and heterologous regulation.

Treatment of a wide variety of cell and tissue types with TGF-ß results in the accumulation of extracellular matrix components both via increased *synthesis* (Ignotz and Massague, 1986) and decreased *degradation* (Edwards *et al.*, 1987; Lund *et al.*, 1987). These alterations could alter the architecture of the matrix subjacent to the epithelium and affect the overlying epithelium. Further support for this notion comes from demonstration of the ability of TGFß to alter the organization of the ECM and cell-cell junctional complexes (Merwin *et al.*, 1990). Because TGFß has been demonstrated to alter synthesis of ECM components in murine embryonic palate mesenchyme cells (Ferguson, 1988; D'Angelo and Greene, 1991), we suggest that one function of TGFß may be to regulate ECM metabolism by neural crest cells as they migrate into the branchial arches.

Several investigators have suggested a role for many growth factors in different facets of murine development (Mercola and Stiles, 1988; Hollenberg, 1989; Mummery and van den Eijnden-van Raajj, 1990). In many *in vitro* systems, TGFß modulates cellular responses to other growth factors (Massague, 1987; Fernandez-Pol *et al.*, 1989; Ranganathan and Getz, 1990) and the number of cell surface receptors for other growth factors (Gronwald *et al.*, 1989; Thompson, *et al.*, 1989). In palatal tissue, TGFß1 interacts with other growth factors to synergistically modulate glycosaminoglycan synthesis (D'Angelo and Greene 1991; Ferguson, personal communication). These observations, combined with the demonstration that TGFß can regulate various aspects of mandibular development (Slavkin, *et al.*, 1989) suggest a critical role for this paracrine/autocrine factor in craniofacial ontogeny.

Materials and Methods

Animals

Male and female ICR mice were housed in a facility with a 12 hour dark-light cycle. The animals were given Purina mouse chow and water *ad libitum*. Two nulliparous females were mated with one male overnight and the presence of a vaginal plug the following morning (Day 0 of gestation) was considered evidence of mating.

Antibodies

Polyclonal anti-LC(1-30) antibodies, recognizing TGFß1, were a generous gift from Dr. Kathleen Flanders (Laboratory of Chemoprevention, NCI, NIH). The anti-LC (1-30) antibody is specific for intracellular TGFß and was made in rabbits to a synthetic peptide representing the amino terminal 30 amino acids of TGFß as described by Flanders *et al.* (1989). TGFß2-specific neutralizing antibody was purchased from R&D Systems Inc., (Minneapolis, MN).

Immunohistochemistry

The distribution of TGFß1 and TGFß2 was determined by indirect immunohistochemical staining of paraffin-embedded coronal sections of murine embryonic heads from days 12, 13 and 14 of gestation. Pregnant female ICR mice were killed by cervical dislocation, embryos excised from the uterus and extraembryonic membranes, rinsed in Dulbecco's phosphate buffered saline (PBS). Embryos were decapitated and embryonic heads fixed in 1% acetic acid/absolute ethanol (v/v), dehydrated in serial dilutions of ethanol and embedded in paraffin. Coronal sections, 6µm thick, were cut and mounted onto albumin coated slides. Sections were then deparaffinized and immunostained.

For indirect immunohistochemical staining, sections were first incubated overnight (18 hr) at 4°C with primary antibody (TGFß1, 56µg/ml; TGFß2, 50µg/ml) in a humidified incubation chamber. Sections were then washed in PBS containing 0.5% BSA for 5 min, rinsed in dH_2O, dried and further incubated for 60 minutes at room temperature with appropriate affinity purified secondary antibody conjugated to fluorescein isothiocyanate (Cooper Biomedical) at a 1:40 dilution in PBS/0.5% BSA. Sections were then mounted in glycerol/PBS (9:1), pH 9.0, and viewed and photographed using a Nikon Optiphot microscope equipped with epifluorescence optics. Control sections were incubated with PBS containing 0.5% BSA in place of primary antibody.

Immunoprecipitation

Quantitation of growth factors in total palatal extracts was accomplished by an immunobinding technique modified from BRL's (BRL, Inc., Gaithersburg, MD) immunoselect kit. Palatal shelves were cleanly dissected from mouse embryos on days 12, 13 and 14 (plug day = day 0) of gestation. Tissue was minced in Ca^{+2}/Mg^{+2}-free phosphate buffered saline (CMF-PBS), rinsed and gently pelleted. An extracellular matrix fraction was prepared by extraction overnight at 4°C in in a Tris-HCL extraction buffer containing 1.0M urea. After centrifugation at 16,000 rpm for 1 hour the extract was dried, resuspended in distilled water, dialyzed against $NaHCO_3$ buffer, pH 9.6, and total protein determined by the method of Lowry (Lowry *et al.*, 1951). Nitrocellulose strips (pore size 0.45µm; Schleicher and Schuell, Inc., Keene, New Hampshire) were wet evenly with distilled water and allowed to air dry. Samples containing 20µg total protein in a total volume of 21µl $NaHCO_3$ buffer were applied to the nitrocellulose strips. TGFß1 and TGFß2 standards (5-.05 µg protein blotted) (R&D Systems, Minneapolis, MN) were similarly blotted onto nitrocellulose strips. Blots were incubated at room temperature overnight, fixed with 10% acetic acid/ 25% isopropanol for 15 min and blocked with 1% BSA in Tris buffered saline (TBS). Primary TGFß1 or TGFß2 antibodies (1µg/ml IgG) were brought up to volume in 0.05% Tween-20, were applied to blots and incubated overnight at 4°C. Controls were incubated with TBS buffer instead of primary antibody. Blots were then washed in TBS, incubated with a goat-anti rabbit IgG (whole molecule) peroxidase conjugate (Sigma Chemical, St. Louis, MO) at a working dilution of 1:1000 for 60 min at room temperature, and accumulation of insoluble substrate formed from the reaction of hydrogen peroxidase with 4-chloro-1-naphthol and hydrogen peroxide was visualized.

Acknowledgments

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