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Research Article

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Transforming Growth Factor β2 in Epithelial Differentiation of Developing Teeth and Odontogenic Tumors

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Abstract

Dysregulation of TGF β2, a modulator of cell growth and differentiation, can result in uncontrolled growth and tumor formation. Our comparative studies on the expression of TGF β2 mRNA and protein indicate that TGF β2 may primarily be a regulator of epithelial differentiation during tooth development (between 13 and 20 gestational wk) and tumorigenesis of odontogenic neoplasms. A paracrine mode of action for TGF β2 in early human tooth germ (cap/early bell stage) is suggested by location of mRNA in the mesenchyme surrounding the tooth germ, whereas protein is found in the epithelial dental lamina and enamel organ. During the late bell stage, TGF β2 gene expression shifted from the mesenchyme to the odontogenic epithelium and was colocalized with protein, suggesting an autocrine role for the terminal differentiation of ameloblasts. In odontogenic tumors of epithelial origin (ameloblastomas) and epithelial-ectomesencymal origin (ameloblastic fibromas), TGF β2 mRNA was mostly located in the mesenchymal tumor component and protein in the epithelial tumor component. Odontogenic ectomesenchymal tumors (myxomas) were not associated with TGF β2 mRNA and protein expression. The results imply that TGF β2 may play an important role in epithelial-mesenchymal interactions in human tooth morphogenesis and development of odontogenic tumors. (*J. Clin. Invest.* 1993. 91:1019–1027.) Key words: growth substances • immunocytochemistry • molecular probe techniques • neoplasms • odontogenesis

Introduction

The developing tooth and odontogenic tumors provide biological models for comparative studies of normal and pathological tissue interactions. Tooth morphogenesis involves differentiation of epithelial precursor cells to enamel-secreting ameloblasts and neural crest–derived mesenchymal cells to dentine-secreting odontoblasts through a sequence of epithelial-mesenchymal interactions (1–3). Odontogenic tumors are rare neoplasms of the jaw bones or soft tissues of tooth-bearing areas. They may originate from tissue remnants of the developing tooth or the tooth germ proper (4, 5). They form a histologically variable group of tumors, possibly reflecting different developmental stages in tooth formation. Odontogenic tumors are grouped according to their putative origins, into epithelial, epithelial-ectomesenchymal, and ectomesenchymal neoplasms (6). There is no information on the involvement of diffusible growth and differentiation factors during the development of human teeth. Such information is essential if their possible roles in the pathogenesis of odontogenic tumors are to be understood.

TGF β is a multifunctional growth factor that has several biological effects in vivo, including control of cell growth and differentiation, cell migration, and synthesis and degradation of extracellular matrix (ECM)[1] (7–9). There is increasing evidence that dysregulation of TGF β function is implicated in the genesis of diseases such as systemic sclerosis (10), intraocular fibrosis (11), and glomerulonephritis (12). Impaired TGF β signaling can also lead to tumor formation (13). TGF βs are members of a large growth and differentiation factor superfamily of structurally related genes (8). Five isoforms of TGF β have been described. TGF β1 (14), TGF β2 (15, 16), and TGF β3 (17, 18) have been found in human beings and various other mammals. TGF β4 (19) and TGF β5 (20) have only been found in chicken and *Xenopus laevis*, respectively. Other members of this multigene family include Müllerian inhibitory substance (21), members of the inhibin/activin subunit gene family (22), and members of the decapentaplegic-Vg-related gene family (23). Although TGF β1-3 affect cells in qualitatively similar ways (24, 25), each isoform may have unique developmental functions in vivo (26–33).

Recent studies on growth factor expression in rodent and bovine fetal teeth suggest that TGF β1-3 (28, 32, 34–39), and other TGF β family members (40, 41) are involved in tooth development. However, the role of TGF β family members during development of human teeth has not been explored. Previous studies have indicated that of the three TGF β isoforms, TGF β2 may particularly function as an inducible homeostatic regulator of epithelial growth and differentiation (26, 27, 30, 42). Therefore, we compared distributions of TGF β2 mRNA and polypeptide in developing human teeth and odontogenic tumors. The results of this comparison suggest that TGF β2 is primarily a regulator of epithelial differentiation for developing teeth and for odontogenic tumors.

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1. *Abbreviations used in this paper:* Ck, cytokeratin; ECM, extracellular matrix; FA, formamide sodium; gwk, gestational weeks; PFA, paraformaldehyde; RT-PCR, reverse transcription PCR.

Methods

Fetal tissues. Eight mandibles (13–20 gestational wk, [gwk]) were obtained in connection with legal abortions induced using prostaglandins. The study was approved by the Ethical Committee of the Helsinki Maternity Hospital. Gestational ages were estimated from the fetal foot lengths (43). Five mandibles (13th, 15th, 17th, 18th, and 20th gwk) were hemisected. Half were snap frozen in liquid nitrogen and stored at −70°C until used. Serial cryostatic sections (5 μm) were cut from each jaw sample and mounted on aminoalkylsilane-treated objective slides for in situ hybridization (44). The other half of the jaw was fixed in cold 4% paraformaldehyde (PFA), decalcified in 10% EDTA in neutral 10% formalin, and embedded in paraffin. Serial sections (5 μm) were cut on slides coated with poly-L-lysine (100 μg/ml, Sigma Chemical Co., St. Louis, MO) for immunostaining and histological staining using haematoxylin and eosin. All sections included three to five deciduous tooth germs at cap, bell, or apposition stages of tooth development, and surrounding tissues, including oral epithelium.

For RNA extraction, five deciduous tooth germs from three hemisected mandibles (16th, 17th, and 20th gwk) were dissected under a microscope from surrounding tissues, snap frozen in liquid nitrogen, and stored at −70°C. The remaining mandibles without tooth germs, as well as fetal kidney (20th gwk), and placenta (15th gwk) were used for control purposes.

Tumors. Eight benign odontogenic tumors were included in the study. Samples of fresh tumor specimens were obtained during surgery, frozen promptly in liquid nitrogen, and stored at −70°C until used for RNA extraction, in situ hybridization, or immunocytochemistry. The ameloblastomas included one granular cell and three plexiform variants found in two women (40 and 74 yr old) and two men (40 and 73 yr old). The two ameloblastic fibromas studied were from the maxillae of a 4-yr-old boy and a 14-yr-old girl. Two myxomas included were mandibular tumors from a 17-yr-old man and a 30-yr-old woman.

Probes. A 603-bp PstI-HindIII fragment spanning the preproregion of human TGF β2 from the λ-SUP-40 cDNA clone (nucleotides 250–852) (15) was subcloned into a pGEM-4Z ribovector (Promega Biotec, Madison, WI). For in situ hybridization and Northern analysis, cRNA probes were labeled with ^{35}S-α-UTP (1,000 Ci/mmol) and [^{32}P]α-UTP (1,000 Ci/mmol) by in vitro transcription using SP6 or T7 RNA polymerases (Promega Biotec). A 214-bp XhoI-NcoI genomic fragment of human cytokeratin (Ck) 19 subcloned into a Bluescript vector (Stratagene, Inc., La Jolla, CA) (45) was also used as a control for in situ hybridization to demonstrate the integrity of tissue sample mRNA. For Southern hybridization, linearized TGF β2 cDNA containing plasmid was labeled with [^{32}P]α-dCTP (3,000 Ci/mmol) (Amersham, Buckinghamshire, UK) by random priming using an oligolabeling kit (Pharmacia LKB, Uppsala, Sweden). Specificity of β-actin PCR products was controlled by a 3′-end–labeled internal 27-mer antisense oligoprobe 5′CTCGGGAGCCACACGCAGCTCATTGTA-3′ corresponding to nucleotides 312–338 in the previously described human β-actin cDNA (46).

RNA extraction and Northern analysis. Total cellular RNA was extracted by means of the guanidium isothiocyanate-cesium chloride method (47) and determined spectrophotometrically at 260 nm. It was stored at −70°C until use. For Northern analysis, RNA samples (15 μg) were denatured in glyoxal and dimethylsulfoxide, run in an 1.5% agarose gel, and transferred onto a filter (Hybond-N; Amersham) by capillary blotting (48). The filters were hybridized with [^{32}P]α-UTP–labeled TGF β2 cRNA, and were then washed and exposed to autoradiographic film as described previously (49). Under these conditions, our TGF β2 probe recognizes transcripts of 6.0 and 4.0 kb in human Tera 2 cells, consistent with results of previous studies by others (50).

Reverse transcription PCR (RT-PCR) and Southern blotting. For RT-PCR, 1 μg of each RNA sample was reverse transcribed as previously described (49). The synthetic oligonucleotide primers for human TGF β2 (15) and human β-actin (46) used in the PCR are detailed in Table I. The PCR was undertaken (GeneAmp DNA-Amplification Reagent Kit; Perkin Elmer Cetus, Norwalk, CT) following the manufacturer's protocol. The PCR reaction volume was 50 μl, containing 25 pmol of 3′- and 5′-end primers, 5 μl of 10 × PCR reaction buffer, 4 μl of dNTP mixture (0.2 μM final concentration for each deoxynucleotide), 37 μl of water, 1 μl of RT-reaction mixture, and 2.5 U of AmpliTaq enzyme. After addition of 50 μl mineral oil (Perkin Elmer), denaturation was performed at 94°C for 3 min. Reaction was amplified for 35 cycles (denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min 30 s) using a thermal reactor (Hybaid, Teddington, UK). 10-μl portions of PCR reaction products were size fractionated in 1.6% agarose gel and stained in ethidium bromide. HaeIII-digested φX 174RF DNA was used as a molecular size marker. Southern transfer was undertaken by capillary blotting the DNA to nylon membranes (Hybond-N; Amersham). The membranes were hybridized with [^{32}P]α-dCTP–labeled TGF β2-cDNA and β-actin oligoprobes, as described above. The specificity of the TGF β2 product was controlled by restriction enzyme analysis (RsaI).

In situ hybridization. In situ hybridization with ^{35}S-α-UTP–labeled TGF β2 and Ck 19 riboprobes was performed on cryostat sections as previously described (49, 51). Briefly, before hybridization, sections were fixed in 4% PFA/5 mM $MgCl_2$, rinsed in Tris-buffered saline, and proteinase-K (0.5 μg/ml) treated for 5 min. The reaction was stopped by means of glycine addition (0.1 M in PBS), postfixed in 4% PFA/5 mM $MgCl_2$, and rinsed in 50% formamide sodium (FA)/2 × standard saline citrate (SSC) at room temperature (1 × SSC is 0.15 M NaCl/15 mM trisodium citrate). Sections were then acetylated, rinsed again in 50% FA/2 × SSC, and prehybridized for 1–2 h before hybridization overnight in buffer containing 60% FA at 50°C. Posthybridization washes and RNAse treatment of the sections have been described in detail by Miettinen and Heikinheimo (49). The sections were subjected to autoradiography by dipping the slides into film emulsion (NTB-2; Eastman Kodak Co., Rochester, NY) and exposed for up to 6 wk at 4°C. After developing the slides (D-19 developer; Kodak), the sections were counterstained in Harris' haematoxylin and assessed using dark and light field microscopy.

Immunocytochemistry. A biotin-streptavidin detection system and affinity-purified rabbit antibodies were used for immunolocation of TGF β2 polypeptide. The antibodies were raised against a synthetic peptide corresponding to the first 29 NH_2-terminal amino acid residues of human TGF β2 (52). Immunostaining was performed using a staining kit (StrAviGen Multilink; BioGenex Laboratories, San Ramon, CA) with alkaline phosphatase. Antibodies were diluted in PBS containing 1% BSA. The working dilution of the TGF β2 antibodies, as

Table I. Sequences of TGF β2 and β-actin Amplification Primers; the Expected Lengths of PCR Products Are Shown

	5′-Sense primer	3′-Antisense primer	PCR product (numbering of nucleotides according to references)
TGF β2	5′-CCAGAAGACTATCCTGAGCC-3′	5′-AGATGTGGGGTCTTCCCACT-3′	682 bp (nucleotides 342–1023); (reference 15)
β-actin	5′-CCCAGGCACCAGGGCGTGAT-3′	5′-TCAAACATGATCTGGGTCAT-3′	263 bp (nucleotides 153–415); (reference 46)

determined by dilution series, was 1:30 corresponding to an IgG concentration of 16 μg/ml. The specificity of the immunoreaction was monitored by replacing the primary antibody with purified preimmune IgG fraction of the rabbit donating TGF β2 antibodies and normal rabbit serum provided with the kit.

Results

The presence of TGF β2 mRNA in developing human teeth and odontogenic tumors was first studied by Northern blot analysis. No TGF β2 transcripts were detected in 15 μg of total RNA from each specimen (data not shown). Subsequently, a more sensitive RT-PCR was applied. Amplification of the samples yielded a single band of expected size (682 bp) for TGF β2 in three out of three tooth germ samples and three out of three ameloblastomas. TGF β2 mRNA was not detected in three out of three mandibles without tooth germs, in one out of one ameloblastic fibroma, and in two out of two odontogenic myxomas (Fig. 1). In all tissues studied, hybridization with internal β-actin oligo gave a single band of 263 bp for each β-actin PCR product (Fig. 1). Control tissues, including placenta and fetal kidney, were positive for TGF β2 mRNA.

TGF β2 mRNAs in developing teeth. Early tooth development is characterized by sequential developmental stages. In the initial stage (bud stage, not studied) (from 6th gwk onwards), enamel organ is formed from the cells of the oral epithelium. The adjacent ectomesenchymal cells, condensing under enamel organ, form the dental papilla. When the epithelial bud has become concave in shape, the tooth bud is at the cap stage (Fig. 2 *A*). At this stage, in situ hybridization revealed that TGF β2 mRNA was concentrated into the dental follicle; i.e., in mesenchymal cells surrounding the dental organ (Fig. 2, *A* and *B*). Oral epithelium, dental lamina, enamel organ, dental papilla, and nondental connective tissue were devoid of TGF β2 hybridization signal.

As the tooth germ grows, it enters the bell stage (Fig. 2 *C*), during which the tooth crown is formed. The cells of the inner enamel epithelium will differentiate into ameloblasts, which later secrete enamel matrix. The cells of the dental papilla adjacent to the inner enamel epithelium develop into odontoblasts; i.e., into cells secreting dentin matrix. In the early and intermediate bell stages, TGF β2 transcripts were found in the dental follicle, in nondental mesenchyme around the dental lamina, and beneath the oral epithelium (Fig. 2, *C* and *D*). No specific hybridization signals were obtained in the tooth germ proper, more distant connective tissue and immature alveolar bone.

In the late bell stage, TGF β2 mRNA was no longer found in mesenchyme surrounding tooth germ. Epithelial elements of the enamel organ, dental lamina, and oral surface epithelium expressed TGF β2 mRNA. A strong TGF β2 hybridization signal was evident in the inner enamel epithelium (Fig. 2, *E* and *F*). A weaker signal was detected in the stellate reticulum, in the outer enamel epithelium, and in the stratum intermedium (Fig. 2, *G* and *H*). Alveolar bone, including osteoblasts and surrounding connective tissue, was devoid of TGF β2 mRNA. However, a strandlike accumulation of hybridization signals was noted in periosteal connective tissue at the periphery of the alveolar bone (data not shown).

When odontoblasts first start to secrete dentin matrix at the tip of the tooth cusp, tooth germ has reached the early apposition stage (Fig. 2 *I*). TGF β2 mRNA had at this stage switched from ameloblasts to odontoblasts (Fig. 2, *I* and *J*). The opposite situation was evident in areas where dentine matrix production had not yet started. The ameloblast cell layer exhibited TGF β2 mRNA but developing odontoblasts were negative (Fig. 2, *I* and *J*). The oral surface epithelium, dental lamina, stellate reticulum, and outer enamel epithelium exhibited positive hybridization signals. Only a background signal level was seen in alveolar bone and other mesenchymal tissues.

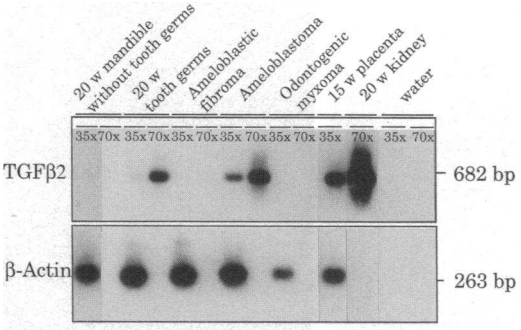

Figure 1. Expression of TGF β2 and β-actin mRNA in developing human teeth and odontogenic tumors. RT-PCR results after 35 cycles (*35×*) and 35 subsequent cycles (*70×*) of amplification are shown in fetal tooth germs (20 g wk) and odontogenic tumors using TGF β2 and β-actin primers. After Southern transfer and hybridization with a ^{32}P-labeled human TGF β2 cDNA, positive bands of the size expected for TGF β2 PCR products (682 bp) were seen in tooth germs, ameloblastoma, placenta, and kidney. Similarly, after hybridization with human β-actin oligo probe, a band of the correct size of 263 bp for β-actin was seen in all tissue samples (kidney not shown). Water instead of cDNA was used as a negative control.

At all developmental stages, the Ck 19 probe confirmed the integrity of the mRNA in odontogenic epithelium of the tissue samples studied (data not shown) (51). The TGF β2 sense probe gave only a background signal (Fig. 2, *K* and *L*).

TGF β2 mRNAs in odontogenic tumors. An ameloblastoma is composed of islands and nests of odontogenic epithelium in mature connective tissue stroma (Fig. 3, *A* and *C*). Peripheral cells of epithelial islands are typically cuboidal or columnar, resembling preameloblasts or ameloblasts. Central cells are histologically similar to those of stellate reticulum in the developing enamel organ.

In the granular cell ameloblastomas, large, roundish cells with granular cytoplasm in neoplastic epithelial follicles were prominent. No TGF β2 transcripts were detected in epithelial tumor cells. Connective tissue stroma between epithelial islands showed a weak hybridization signal for TGF β2 mRNA (Fig. 3, *A* and *B*).

Three of the ameloblastomas studied were plexiform variants, consisting of networks of sheets and strands of odontogenic epithelium. In all of these tumors, the stellate reticulum component was scanty, and there was little stromal tissue. One tumor, faintly solid, exhibited large copy numbers of TGF β2 transcripts in the stroma. The epithelial component was negative (Fig. 3, *C* and *D*). In the other two tumors, a weaker hybridization reaction for TGF β2 mRNA was detected in the stroma, which also exhibited considerable cystic degeneration. In these tumors, TGF β2 transcripts were also noted focally in some epithelial cells.

Both ameloblastic fibromas consisted of islands and strands of odontogenic epithelial tissue in an immature ectomesenchy-

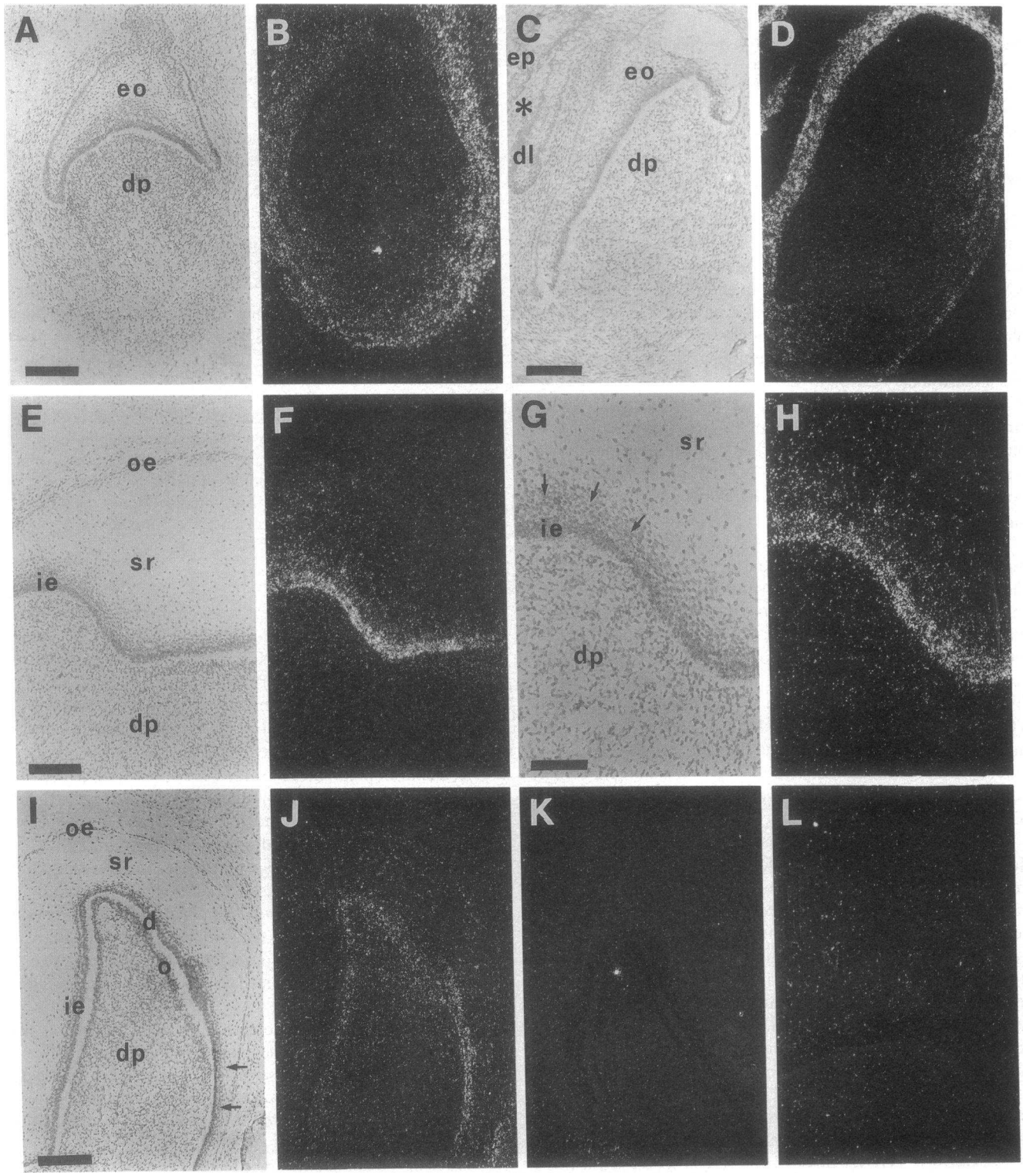

Figure 2. TGF β2 mRNA in developing teeth, detected using in situ hybridization with ^{35}S-labeled cRNA-probes. (*A* and *B*) At the cap stage (13 gwk), TGF β2 mRNA was concentrated in connective tissue surrounding the tooth germ. Dental papilla (*dp*) and epithelial elements of enamel organ (*eo*) were negative. (*C* and *D*) At the bell stage (13th gwk), and intensive hybridization signal was seen in connective tissue surrounding the tooth bud and dental lamina (*dl*). Oral epithelium (*ep*) was negative, but subepithelial connective tissue expressed TGF β2 mRNA (*). (*E* and *F*) At the late bell stage (20th gwk), TGF β2 mRNA expression was seen in the inner enamel epithelium (*ie*). The stellate reticulum (*sr*) and outer enamel epithelium (*oe*) were slightly positive. The dental papilla was negative. (*G* and *H*) Higher magnification shows more clearly the hybridization signal in the cells of the inner enamel epithelium. Note also that the cells of the stratum intermedium (*arrows*) and stellate reticulum are positive. (*I* and *J*) During the early apposition stage, TGF β2 mRNA expression switched from the cells of the inner enamel epithelium to odontoblasts (*o*) in the area where early dentin matrix (*d*) production took place. Note, however, that odontoblasts were negative, and the inner enamel epithelium remained positive in the apical area of tooth still devoid of dentin matrix production (*arrows*). The stellate reticulum and outer enamel epithelium were also weakly positive. (*K*) Dark-field illumination of a section corresponding to *J* and (*L*) corresponding to *H* revealed lack of specific signals when hybridized with sense probe. Exposure times: *A–D*, 6 wk; *E–L*, 3 wk. Bars: *A–F* and *I–K*, 200 μm; *G* and *H* and *L*, 100 μm.

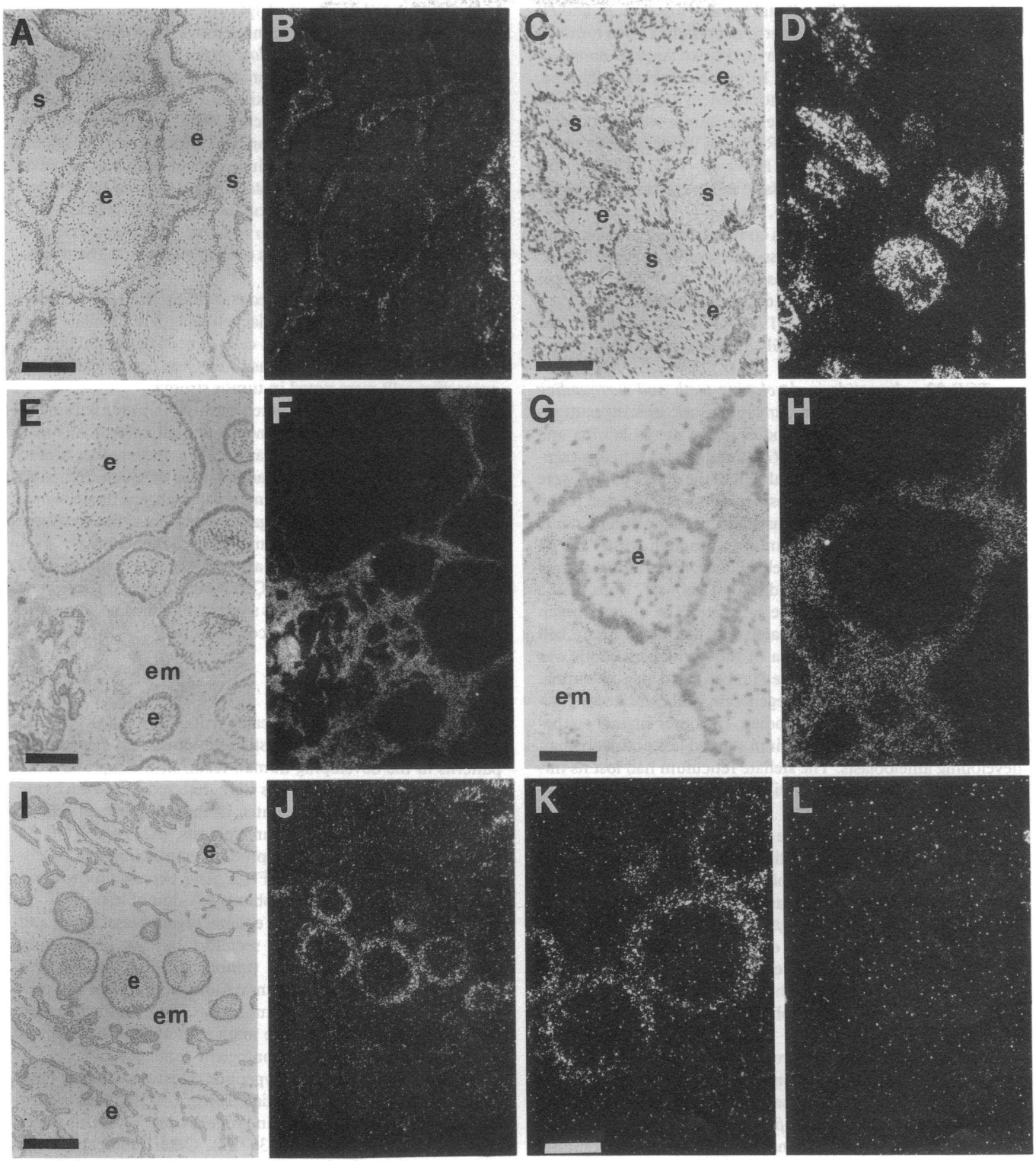

Figure 3. TGF β2 mRNA in ameloblastoma (*A–D*) and ameloblastic fibroma (*E–L*). (*A* and *B*) Epithelial tumor islands (*e*) in granular cell ameloblastoma were devoid of TGF β2 mRNA but a weak hybridization signal was seen in stroma (*s*). (*C* and *D*) A strong hybridization signal was detected for TGF β2 mRNA in the stroma of plexiform ameloblastoma. Neoplastic epithelial tissue was negative. (*E* and *F*) In one ameloblastic fibroma, hybridization signals were confined to the ectomesenchymal neoplastic tissue component (*em*). Epithelial islands were negative. (*G* and *H*) Higher magnification revealed hybridization granules in the ectomesenchymal tumor component. (*I* and *J*) In the other ameloblastic fibroma, TGF β2 mRNA was seen in the peripheral cells of only a few epithelial islands. Otherwise, epithelial nests were negative. Ectomesenchymal tissue in this field was negative. (*K*) Higher magnification of the previous field demonstrated more clearly the location of hybridization signals in the peripheral epithelial cells. (*L*) Control section for *K* hybridized with sense probe showed lack of hybridization signals. Exposure times: (*A–L*) 6 wk. Bars: (A, B, E, F, I, and J) 200 μm; (*C*, *D*, *K*, and *L*) 100 μm; (*G* and *H*) 50 μm.

mal component resembling dental papilla (Fig. 3, *E*, *G*, and *I*). Both tissue elements are considered neoplastic. In one tumor, a hyalinized zone of ectomesenchymal tissue around epithelial islands was faintly prominent. In one ameloblastic fibroma, TGF β2 mRNA was found in ectomesenchymal but not epithelial tissue (Fig. 3, *F* and *H*). The other tumor was partly negative, partly positive, TGF β2 mRNA was located mostly in the ectomesenchymal component. However, TGF β2 transcripts were detected in peripheral cells of a few epithelial islands in otherwise negative areas (Fig. 3, *J* and *K*).

Both odontogenic myxomas contained poorly collagenous, faintly acellular myxomatous tissue, resembling dental follicle. No odontogenic epithelium was seen. No TGF β2 transcripts were detected in these tumors (data not shown).

In all tumor samples, Ck 19 mRNA was located in the neoplastic epithelium. The TGF β2 sense probe gave only a background signal (Fig. 3 *L*).

TGF β2 polypeptide in developing teeth and surrounding tissues. Faintly positive granular cytoplasmic staining patterns were seen with TGF β2 antibodies in the dental lamina and enamel organ at the cap stage (data not shown). Ectomesenchymal and capillary endothelial cells of the dental papilla were negative. Occasional weakly positive cells were detected in the dental follicle, especially in areas adjacent to the outer enamel epithelium. A faintly positive immunoreaction with TGF β2 antibodies was seen in all epithelial components of the enamel organ at the early bell stage (data not shown). Cells of the dental papilla and follicle were negative. The enamel organ became even more immunoreactive at the intermediate bell stage. Successive dental lamina of the permanent tooth was slightly immunoreactive. At the late bell stage, dental lamina, which had already started to degenerate, still exhibited a positive reaction for TGF β2 antibodies. The outer enamel epithelium and the stratum intermedium stained less positively than developing ameloblasts. The stellate reticulum had lost its immunoreactivity. The dental papilla and dental follicle were negative.

Secretory odontoblasts were immunoreactive with TGF β2 antibodies at the initial apposition stage, when the first dentin matrix was secreted by odontoblasts at tip of the cusp (Fig. 4, *A* and *B*). Presecretory ameloblasts remained positive and the stellate reticulum remained negative. The outer enamel epithelium and stratum intermedium exhibited marked positive reactions. The dental papilla and dental follicle were mostly negative. However, some cells of the ectomesenchymal papilla at the cusp region, next to odontoblast layer, were weakly immunoreactive.

As the apposition stage proceeded, and a wider zone of dentin matrix was secreted, odontoblasts and adjacent ectomesenchymal cells in the coronal part of the dental papilla continued strongly positive for TGF β2 polypeptide. The remaining cells of the dental papilla were negative. Secretory ameloblasts, cells of the stratum intermedium, degenerating outer enamel epithelium, and degenerating dental lamina exhibited positive immunoreactions.

Oral surface epithelium, dental lamina (Fig. 4 *D*), muscle cells and osteoblasts (Fig. 4 *F*) exhibited positive staining reactions throughout the developmental span studied. Developing alveolar bone (Fig. 4 *F*) and surrounding connective tissue, including capillary endothelial cells, remained negative.

A constantly negative staining pattern was observed in all fetal mandibular specimens studied when the primary antibodies were replaced by purified IgG fraction from preimmune serum (Fig. 4 *C*) or nonimmune serum (Fig. 4 *E*).

TGF β2 polypeptide in odontogenic tumors. Various expression patterns for TGF β2, mostly located in tumor epithelium, were seen in the three plexiform ameloblastomas studied. A strong positive immunoreaction with occasional negative cells was found in epithelial islands of the solid ameloblastoma (Fig. 4 *G*). Tumor stroma, including capillaries, was negative. In cystic ameloblastomas, the staining pattern was more heterogenous. Staining intensities of the central stellate-like cells varied from strongly positive to negative. This was also true of ameloblast-like peripheral cells of epithelial nests. The stroma was mainly negative.

Faintly positive heterogeneous staining for TGF β2 polypeptide was seen in large granular cells (Fig. 4 *H*), stellate reticulum-like cells, and peripheral cells of neoplastic epithelial islands in granular cell ameloblastoma. Only occasional immunoreactive cells were noted in tumor stroma.

TGF β2 antibodies exhibited positive immunostaining in the epithelial component of one ameloblastic fibroma (Fig. 4 *I*). Ectomesenchymal tissue was negative. In the other tumor, more heterogenous immunoreaction areas were detected. Most immunoreactivity was found in epithelial tumor tissue, which exhibited both positive and negative areas. Focal positivity was seen also in the ectomesenchymal tumor component.

Both odontogenic myxomas were negative with TGF β2 antibodies (data not shown). Capillaries in tumor tissue were also negative. All tumor tissues studied remained negative with preimmune and nonimmune control sera.

Discussion

The study reported here indicates that TGF β2 mRNA and polypeptide exhibit characteristic temporospatial distribution patterns in the developing human teeth. In the cap and early bell stages, TGF β2 transcripts were detected in mesenchyme surrounding the tooth bud. Later, in the bell stage, all cells of enamel organ, especially preameloblasts, expressed TGF β2 mRNA. When dentin matrix production was initiated by differentiated odontoblasts, TGF β2 gene expression switched from the differentiating ameloblasts to odontoblasts. TGF β2 polypeptide was located in the enamel organ, presecretory and secretory ameloblasts, and in secretory but not presecretory odontoblasts. In odontogenic tumors, TGF β2 mRNA was mainly found in the mesenchymal tumor component and protein in the epithelial tumor component. These findings suggest that the epithelial cells are the main sites of TGF β2 effect in both developing teeth and odontogenic tumors.

Recent mRNA and polypeptide location studies have shown that all three mammalian TGF β1–3 isoforms are expressed in developing bovine and rodent teeth at various stages of development (28, 30, 32, 34–39). Other members of the TGF β multigene family so far reported in rodent tooth development include Vgr 1, bone morphogenetic protein (41), and the inhibin-activin βA subunit (40). The data reported here on TGF β2 and our unpublished observations on other members of the TGF β superfamily indicate that each gene has a distinct, partly overlapping mRNA and protein expression pattern during human tooth development, suggesting coordinated regulatory roles for several TGF βs.

The TGF β superfamily member most studied in rodent tooth development at mRNA and polypeptide level is TGF β1.

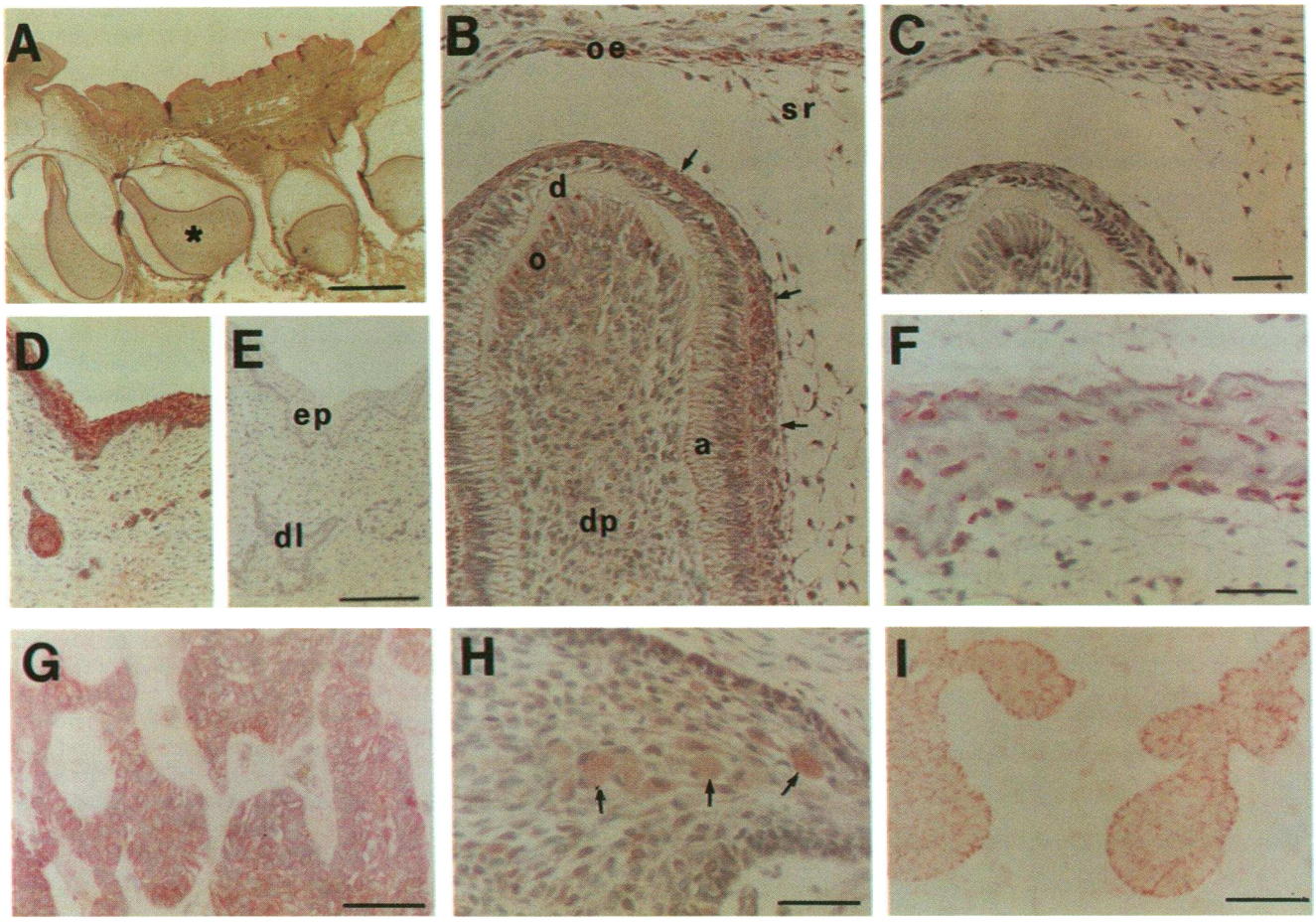

Figure 4. Immunolocation of TGF β2 polypeptide in developing teeth (*A–F*) and odontogenic tumors (*G–I*). Haematoxylin counter stain was used in all panels except *I*. (*A*) General view of histological section of frozen mandibular specimen (20th gwk) with two tooth buds in early apposition stage (*left*) and two in late bell stage (*right*). Higher magnifications of the bud in the center (*) are shown in *B* and *C*. (*B*) Positive immunoreaction with TGF β2 antibodies was evident at early apposition stage in ameloblasts (*a*), stratum intermedium (*arrows*), and outer enamel epithelium (*oe*). Odontoblasts (*o*), secreting dentin matrix (*d*), and adjacent ectomesenchymal cells at the tip of developing tooth also exhibited positive staining for TGF β2 polypeptide. Other cells of the dental papilla (dp) and of the stellate reticulum (sr) were negative. (*C*) No staining occurred in an adjacent serial section when a purified IgG fraction of the preimmune serum was used as the primary antibody. (*D*) Positive staining in epithelial cells of the oral epithelium (ep) and dental lamina (dl) with TGF β2 antibodies. (*E*) Control section stained with normal rabbit serum remained negative. (*F*) Osteoblasts in and around developing alveolar bone exhibited positive immunostaining with TGF β2 antibodies. (*G*) Neoplastic epithelial cells of plexiform ameloblastoma expressed TGF β2 polypeptide. (*H*) Positive immunoreaction for TGF β2 polypeptide was seen in neoplastic epithelial island of granular cell ameloblastoma. Central and peripheral cells, and granular cells (*arrows*) expressed TGF β2. (*I*) Epithelial islands of ameloblastic fibroma exhibited positive staining with TGF β2 antibodies. Mesenchymal component remained negative. No counterstaining. Bars: (*A*) 2,000 μm; (*B*, *C*, *F*, and *H*) 50 μm; (*D* and *E*) 200 μm; (*G* and *I*) 100 μm.

TGF β1 mRNA has been detected in the bud stage dental epithelium, cap stage epithelial cervical loop, and condensed dental mesenchyme, in developing ameloblasts at the bell stage, and in secretory odontoblasts (28, 30, 34, 35, 39). Little is known about expression of TGF β2 and 3 (28, 30). TGF β2 mRNA has been demonstrated in invaginating dental epithelium, in mesenchyme surrounding the early tooth bud, and in later stages in the odontoblast cell layer and the developing pulp. The studies concerned, however, covered only the bud and apposition stages of development. We also found TGF β2 mRNA in the mesenchyme surrounding the cap and early bell stage tooth germs, and transiently in presecretory ameloblasts in the late bell stage, when neural crest–derived mesenchymal cells are induced to differentiate into odontoblasts by overlying preameloblasts. Only after maturation of odontoblasts will preameloblasts differentiate into ameloblasts. TGF β2 mRNA was found to switch from preameloblasts to differentiated odontoblasts when hard tissue formation began. A similar transcriptional shift from the preameloblasts to odontoblasts has been reported for TGF β1 mRNA in developing murine teeth (39). This suggests a signaling role for TGF β1 and 2 during this critical differentiation stage.

Recently, TGF β1–3 polypeptides have been located using immunocytochemistry at different stages of tooth development (32, 36–38). Briefly, TGF β1 has been detected in stellate reticulum and dental papilla, and TGF β3 has been detected in ameloblasts and dental papilla. In the study reported here, TGF β2 polypeptide was found increasingly in preameloblasts from the cap stage onwards. Ameloblasts remained positive for TGF β2 polypeptide after dentin formation began, indicating a possible role of TGF β2 in their terminal differentiation. In contrast to our results, Pelton et al. (32) detected no TGF β2 in

murine preameloblasts or ameloblasts because different antigens were used in preparing the TGF β2 antibodies (32, 52) or because of differences between the species studied.

Recent results relating to TGF β1-3 gene location during murine development show wide epithelial expression of TGF β2, suggesting a major role in epithelial differentiation (30, 31). It has also been suggested that TGF β2 may be an inducible homeostatic regulator of epithelial growth and differentiation (26, 27, 30, 42). In our study, TGF β2 mRNA was first (during the 13th gwk) located in the mesenchyme underlying developing oral epithelium and around dental lamina. At later stages (20th gwk), it switched to epithelial cells. Similarly, in developing murine skin, TGF β2 mRNA expression in the dermis preceded that in the epidermis (42). These findings are in accordance with the role proposed for TGF β2 in regulating epithelial differentiation. The shift of TGF β2 mRNA expression to the cells of the dental lamina took place when the dental lamina started to degenerate. This suggests that TGF β2 may first act as a regulator of differentiation, then as an inhibitor of epithelial growth. Accordingly, appearance of TGF β2 mRNA in oral epithelium may reflect a stage of development in which the rate of epithelial cell division is slowing. Interestingly, retinoids, another important regulator of terminal epithelial differentiation, induce TGF β2 gene expression in keratinocytes (53). Consequently, disturbances in vitamin A supply alter TGF β2 function and can lead to dental and craniofacial deformities (54).

Epithelial-mesenchymal interactions are involved in regulation of cell growth and differentiation during human tooth development and genesis of odontogenic tumors (55). Disturbances in intercellular communication during tooth development as a result of inability to respond to TGF β signaling or lack of TGF β2 expression may lead to uncontrolled growth and tumor formation. Ameloblastomas, a heterogenous group of tumors of odontogenic epithelial origin, exhibit complex patterns of epithelial differentiation, as has recently been shown by our studies on keratin gene expression (51). All ameloblastomas studied expressed TGF β2 mRNA in their tumor stroma. The corresponding protein was found to various extents in their neoplastic epithelium. Accordingly, TGF β2 mRNA was found in the mesenchyme surrounding the tooth bud and the polypeptide in the epithelial enamel organ before dental hard tissue formation. Ameloblastomas, which lack capacity to induce formation of dental hard tissue, are thought to remain at the differentiation level of an early tooth germ. In contrast, at least some ameloblastic fibromas may undergo inductive changes to form dental hard tissue and develop towards ameloblastic fibro-odontoma and odontoma (5). We observed TGF β2 mRNA signals focally in the peripheral epithelial tumor cells of one ameloblastic fibroma, resembling expression of differentiating ameloblasts in a developing tooth before the onset of dentin matrix production. Two odontogenic myxomas studied lacked both TGF β2 mRNA signals and protein.

TGF βs induce ECM formation (8). We have previously demonstrated an extradomain sequence-A-containing form of cellular fibronectin in mesenchymal and ectomesenchymal components of the developing teeth and odontogenic tumors (55). Cellular fibronectin has been shown to be transcribed by epithelial tumor cells and expressed in stroma (56). The contrary expression of TGF β2 mRNA in mesenchyme and polypeptide in epithelial cells found in the study reported here suggests that TGF β2 may induce production of cellular fibronectin in the tooth germ, and in the stroma of odontogenic tumors through a paracrine mode of action. This is in line with the observation that TGF β2 plays an important role in the genesis of diseases characterized by excessive deposition of ECM proteins; e.g., systemic sclerosis and intraocular fibrosis (10, 11).

We report here, for the first time, expression of a single growth factor, TGF β2, during development of the human teeth and various odontogenic tumors. Increasing evidence suggests that TGF β1-3 are involved in the regulation of tooth development and exhibit a characteristic, partly overlapping expression patterns, suggesting several coordinated roles for TGF βs. Our results indicate that TGF β2 participates in epithelial growth and differentiation, epithelial-mesenchymal interactions, and matrix formation in normal and neoplastic odontogenic tissues.

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