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Original Article

EGF receptor and its ligands, EGF and TGF-α, in developing and neoplastic human odontogenic tissues

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ABSTRACT Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) regulate cell proliferation and functional maturation through the EGF receptor (EGF-R). Their roles in human tooth development and odontogenic tumorigenesis have not been explored. We studied the expression of EGF, TGF-α and EGF-R in human fetal teeth (cap stage to early hard tissue formation) and various odontogenic tumors. EGF-R mRNA and immunoreactive cells were mostly located in odontogenic epithelium. EGF-R expression was subject to temporospatial variation at different stages of tooth development. EGF and TGF-α mRNAs were detected in fetal teeth only by the reverse transcription polymerase chain reaction (RT-PCR). However, EGF and TGF-α immunoreactive cells were demonstrated in epithelial elements of tooth germ, suggesting that the peptides partially originate from non-odontogenic sources. In odontogenic tumors, EGF-R mRNA and immunoreactivity were confined to neoplastic epithelium. Transcripts for TGF-α but not for EGF were detected in tumors of odontogenic epithelial, epithelial-ectomesenchymal and ectomesenchymal origins. It is concluded that regulation of EGF-R expression is developmentally regulated in human odontogenesis. Furthermore, the odontogenic epithelium is the main target tissue for both EGF and TGF-α during tooth development. TGF-α and its receptor may also be involved in odontogenic tumorigenesis.

KEY WORDS: *human tooth germ, growth factors, immunocytochemistry, molecular probe techniques, odontogenic tumors*

Introduction

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) are structurally related mitogens that regulate cell growth and differentiation (Cohen, 1962; De Larco and Todaro, 1978; Derynck *et al.* 1984). They exert their effects via the epidermal growth factor receptor (EGF-R; Ullrich *et al.*, 1984). Other EGF-R activating peptides are vaccinia-virus growth factor (VVGF; Stroobant *et al.*, 1985), amphiregulin (AR; Shoyab *et al.*, 1989), and heparin-binding epidermal growth factor (HB-EGF; Higashiyama *et al.*, 1991). Overexpression of TGF-α and EGF-R has been associated with neoplasia (Ozanne *et al.*, 1985; Derynck *et al.*, 1987; Partridge *et al.*, 1989; Karnes *et al.*, 1992) but EGF is rarely found in tumors (Carpenter and Wahl, 1991). Since antibodies to EGF-R or TGF-α antisense oligonucleotides have been shown to inhibit the proliferation of several carcinoma cell lines, it is suggested that co-expression of TGF-α and EGF-R in neoplastic cells gives them a growth advantage over normal cells (Rodeck *et al.*, 1990; Sizeland and Burgess, 1991; Derynck, 1992; Karnes *et al.*, 1992).

The developing tooth organ as compared with odontogenic tumors provides a biological model for 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 sequential and reciprocal epithelial-mesenchymal interactions (Avery, 1992). Mediators of these interactions include extracellular matrix molecules, and diffusible growth factors. Impaired signaling during tooth development might lead to odontogenic tumor formation. Odontogenic neoplasms arise from tissue remnants of the developing tooth or from the tooth germ proper, thus histologically reflecting different stages of tooth development (Regezi and Sciubba, 1989; Kramer *et al.*, 1992).

Several strands of evidence suggest important roles for EGF, TGF-α and EGF-R during rodent tooth development. Administration

Abbreviations used in this paper: bp, base pairs(s); BMP, bone morphogenetic protein; cpm, counts per minute; ECM, extracellular matrix; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; gwk, gestational week(s); mRNA, messenger ribonucleic acid; PFA, paraformaldehyde; RT-PCR, reverse transcription-polymerase chain reaction; TGF-α, transforming growth factor alpha; TGF-ß, transforming growth factor beta.

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TABLE 1

SEQUENCES OF EGF, TGF-α AND ß-ACTIN AMPLIFICATION PRIMERS

Primer		Sequences	Nucleotides	Product	Reference
EGF	sense	5'-GACGCCTGTCTGAACCAGGA-3'	3261-3628	368 bp	Bell *et al.*, 1986
	antis.	5-CGATAGCAGCTTCTGAGTCC-3'			Haining *et al.*, 1991
TGF-α	sense	5'-TGTTCGCTCTGGGTATTGTG-3'	63-383	321 bp	Derynck *et al.*, 1987
	antis.	5'-TGATGATAAGGACAGCCAGG-3'			Haining *et al.*, 1991
ß-actin	sense	5'-CCCAGGCACCAGGGCGTGAT-3'	153-415	263 bp	Ponte *et al.*, 1984
	antis.	5'-TCAAACATGATCTGGGTCAT-3'			

The expected size of PCR products is shown.

of EGF or TGF-α to newborn mice accelerates incisor eruption (Cohen, 1962; Tam, 1985; Rhodes *et al.*, 1987). Exogenous EGF inhibits early tooth morphogenesis and cytodifferentiation and decreases tooth size when given to neonates (Partanen *et al.*, 1985; Rhodes *et al.*, 1987; Abbott and Pratt, 1988; Hu *et al.*, 1992). EGF and EGF-R are developmentally regulated in cultured mouse tooth organs (Partanen and Thesleff, 1987; Abbott and Pratt, 1988; Hu *et al.*, 1992). Furthermore, dysregulation of EGF or EGF-R during tooth development may result in dental deformities (Abbott and Pratt, 1988). More recently, EGF has been implicated in the induction of murine odontogenesis (Kronmiller *et al.*, 1991b).

The role of the EGF system has not been studied in human dental tissues. To better understand the functions of EGF-R and its ligands EGF and TGF-α in dental growth and differentiation, we studied their expression during normal human tooth development and in various benign odontogenic tumors.

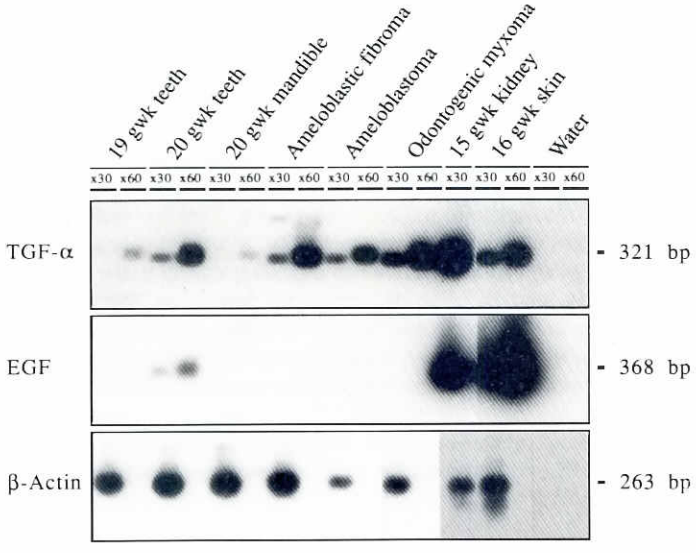

Fig. 1. PCR amplification of TGF-α and EGF in fetal tissues and odontogenic tumors. *RNA samples (1 µg) were subjected to RT and PCR for 30 (x30) and 60 (x60) cycles of amplification followed by Southern transfer and hybridization. When a human TGF-α cDNA probe was used, bands of expected size (321 bp) were evident in tooth germs (19 and 20 gestational weeks, gwk), fetal mandibles without tooth germs, odontogenic tumors, fetal kidney, and fetal skin. An internal EGF-mRNA-specific oligoprobe gave an expected band of 368 bp in 20 gwk tooth germs, fetal kidney and fetal skin. All tissue samples were positive with human internal ß-actin oligoprobe. Water was used as a negative control.*

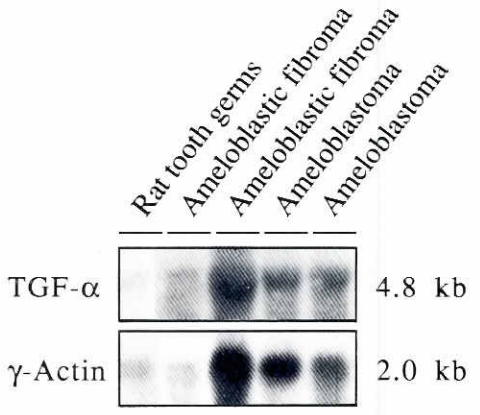

Fig. 2. Northern blot analysis with a TGF-α cRNA probe shows a 4.8 kb band in rat tooth germs (21 gestational days) and odontogenic tumors corresponding to TGF-α mRNA. *A cDNA probe for γ-actin giving a band of 2.0 kb was used as a control for RNA integrity.*

Results

The sensitive RT-PCR was used to amplify EGF and TGF-α mRNA sequences in developing teeth (16-20 gwk) and odontogenic tumors, using specific human EGF and TGF-α primers (Table 1). Amplification of the samples yielded a single band of expected size for TGF-α (321 bp) in 5/5 tooth-germ samples (fetal ages 16, 17, 18, 19, and 20 gwk (Fig. 1). All but the 19 gwk tooth germs were positive for EGF (368 bp) transcripts. Five out of five fetal mandibular samples without tooth germs were negative for TGF-α and EGF mRNA after 30 cycles of amplification. All odontogenic tumors contained TGF-α transcripts after the PCR (Fig. 1). EGF mRNA was not detected. Control tissues, including fetal kidney (15 gwk) and skin (16 gwk) (Fig. 1), fetal (21 gwk) and adult submandibular glands, fetal pancreata (17 gwk) and duodena (17 gwk) were positive for EGF and TGF-α mRNAs (data not shown). RNA integrity was verified by ß-actin PCR (Fig. 1).

The presence of EGF and TGF-α mRNAs in developing teeth and odontogenic tumors was also studied by Northern blot analysis. Total RNA from fetal rat first molars at the bell stage (21 gestational days) was included in the Northern experiments, because sufficient amounts of human tooth germ RNA were difficult to obtain. No EGF transcripts (4.7-4.9 kb) were detected in 15-30 µg of total RNA from rat tooth germs and from four odontogenic tumors (data not shown). However, Northern blot analysis revealed a 4.8 kb band corresponding to TGF-α mRNA in these tissue samples (Fig. 2). A431 vulvar epidermal carcinoma cells were positive for TGF-α and human term placenta was positive for EGF (data not shown). Integrity of the RNA samples of the tissues studied was verified by hybridization with a γ-actin probe (Fig. 2).

EGF-R in developing teeth

Tooth development is initiated by formation of an epithelial placode in the oral epithelium, under which condensed neural-crest-derived ectomesenchymal cells are gathered (bud stage, not studied here). This is followed by invagination of the dental lamina into the mesenchyme leading to formation of the cap stage (Fig. 3A-C). At this stage, EGF-R mRNA and polypeptide were detected in the epithelial cells of the developing tooth germ, i.e. in the dental lamina and the enamel organ (Fig. 3B-C). In addition, low expression of EGF-

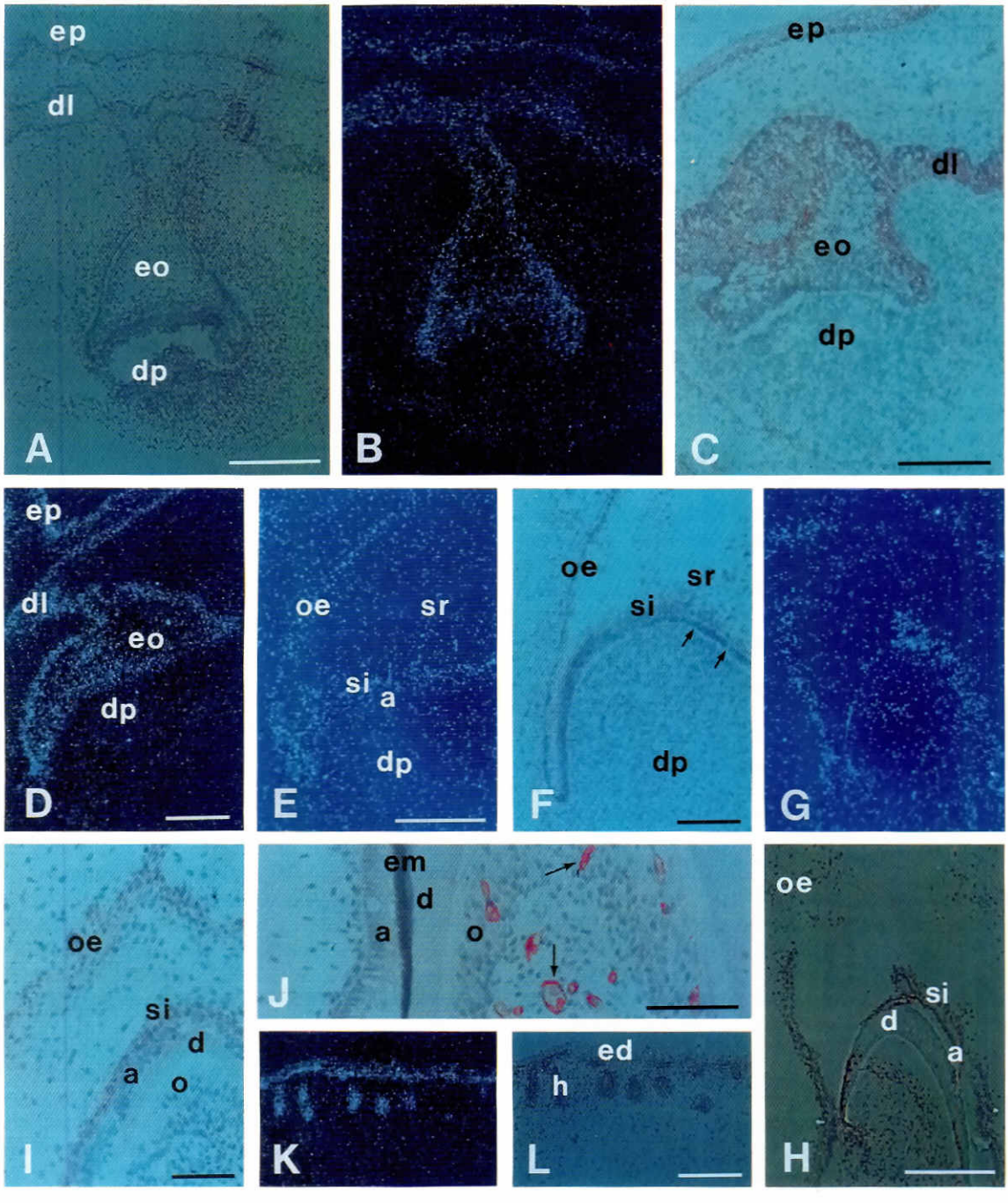

Fig. 3. EGF-R mRNA (B,D,E,G,K) and EGF-R immunoreactivity (C,F,I,J) in developing tooth detected by *in situ* hybridization and immunocytochemistry. **(A)** *Bright-field image of developing tooth at cap stage. (ep= oral epithelium; dl= dental lamina, eo= enamel organ, dp= dental papilla).* **(B)** *Dark-field illumination of the same section shows hybridization signals for EGF-R mRNA in the epithelial cells of the dental lamina, the enamel organ and in the oral epithelium.* **(C)** *Weak immunostaining in the same epithelial structures was obtained with EGF-R antibodies.* **(D)** *In the early bell stage, EGF-R mRNA was seen in the tooth-germ epithelial components.* **(E)** *During the late bell stage, EGF-R mRNA was observed in the outer enamel epithelium (oe), and in the stratum intermedium (si) of the enamel organ. The developing ameloblasts (a) and the stellate reticulum (sr) were devoid of EGF-R transcripts.* **(F)** *Very weak immunoreactivity for EGF-R polypeptide was detected in the outer enamel epithelium, stellate reticulum and stratum intermedium but the developing ameloblasts were negative (arrows).* **(G)** *At the early apposition stage, EGF-R mRNA hybridization signals were detected in the outer enamel epithelium and in the stratum intermedium. Ameloblasts were devoid of EGF-R transcripts.* **(H)** *Phase-contrast image of the same section showing early dentin formation (d).* **(I)** *Positive immunoreactivity with anti-EGF-R was seen in the outer enamel epithelium and stratum intermedium at the early apposition stage. Odontoblasts (o) and ameloblasts were negative.* **(J)** *EGF-R immunoreactivity was seen in endothelial cells (arrows) of developing pulp at a later stage of hard-tissue formation with enamel matrix formation (em).* **(K)** *Hybridization signals for EGF-R mRNA in fetal (13 gwk) epidermis and developing hair follicles.* **(L)** *Corresponding phase-contrast image. (ed= epidermis, h= hair follicles). Exposure times: B and K 6 weeks; D, E, and G 3 weeks. Bars: A-H, K and L, 200 μm; I and J, 100 μm.*

R mRNA and weakly EGF-R immunoreactive cells were observed in the dental follicle, i.e. in the dental mesenchyme surrounding the tooth germ.

As the tooth germ grows it enters the bell stage, during which the tooth crown is formed (Fig. 3D-F). In the early bell stage, EGF-R mRNA was visualized in tooth-germ epithelial components by *in situ* hybridization (Fig. 3D). A low level of EGF-R transcripts was also detected in the dental follicle. EGF-R immunoreactivity was observed in epithelial and follicle cells of developing tooth. During the intermediate and late bell stages, a low expression of EGF-R mRNA (Fig. 3E) and weakly EGF-R immunoreactive cells (Fig. 3F) were restricted to the outer enamel epithelium and in the stratum intermedium of the enamel organ. The developing ameloblasts and the stellate reticulum were devoid of EGF-R transcripts, whereas a faint EGF-R immunoreactivity was seen in the stellate reticulum (Fig. 3E-F). As the tooth development progressed the EGF-R mRNA and immunoreactivities persisted in the dental lamina, whereas dental papilla and follicle were negative.

When the odontoblasts start to secrete dentin matrix at the tip of the cusp, low levels of EGF-R mRNA hybridization signals were detected in the dental lamina, outer enamel epithelium and stratum intermedium (Fig. 3G). In addition, these epithelial cell layers were weakly EGF-R immunoreactive (Fig. 3I). As matrix deposition progressed, dental epithelial cells lost EGF-R mRNA and immunoreactivity. In the dental papilla, the capillary endothelium, previously negative for EGF-R (Fig. 3C,F, and I), was now EGF-R immunoreactive (Fig. 3J).

In all of the fetal mandibular specimens (between 13th and 20th gwk), the oral surface epithelium (Fig. 3A-D) and the epidermis, together with developing hair follicles (Fig. 3K, and L), expressed EGF-R mRNA and EGF-R immunoreactivity. Non-dental connective tissues, including developing alveolar bone, were negative. All tissues were negative with the EGF-R sense probe and the non-immune EGF-R control sera (data not shown).

EGF and TGF-α in developing teeth

All tooth germs gave only background hybridization with both antisense and sense probes for EGF and TGF-α (Fig. 4A-B). Positive immunoreactivity with anti-TGF-α (Fig. 4C and G) and anti-EGF (Fig. 4D and F) was seen in the dental lamina and enamel organ at the cap and bell stages. The dental papilla and the follicle, the presecretory odontoblasts and stellate reticulum did not exhibit immunoreactivity for EGF or TGF-α. However, capillaries of dental papilla and some areas of dental follicle were positive for TGF-α. Presecretory ameloblasts and outer enamel epithelium were variably positive for EGF and TGF-α.

When dentin and enamel secretion had started, the secretory odontoblasts and ameloblasts were immunopositive for both EGF (Fig. 4H) and TGF-α (Fig 4J). The cells of degenerating dental lamina contained EGF and TGF-α immunoreactive cells. The dental papilla, the dental follicle, enamel and dentin were negative with both EGF and TGF-α antibodies. However, capillaries in the dental papilla were continuously positive for TGF-α.

In all of the fetal mandibular specimens studied (between 13th and 20th gwk) the developing oral surface epithelium (Fig. 4F and G) and the developing epidermis (Fig. 4K) expressed EGF and TGF-α. Occasional EGF and TGF-α immunoreactive cells were also detected in developing hair follicles (Fig. 4K). Developing muscle cells and the osteoblasts around developing alveolar bone were positive with EGF- (Fig. 4L) and TGF-α-antibodies.

Positive control tissues, including adult and fetal (16 gwk) submandibular glands (Fig. 4M), pancreas and duodenum contained cells giving positive immunoreactions with EGF and TGF-α antibodies. There was no immunostaining when primary antibodies were replaced by non-immune control sera (Fig. 4E and I). Immunoreactivity was abolished by pre-absorption of EGF and TGF-α antibodies with excess of recombinant EGF and TGF-α (Fig. 4N and O).

EGF-R, EGF and TGF-α in odontogenic tumors

The two ameloblastic fibromas studied consisted of fairly immature cellular connective tissue with plump nuclei and islands, and strands of odontogenic epithelium with cuboidal peripheral cells (Fig. 5A). Ameloblastoma is characterized histologically by neoplastic odontogenic epithelium within mature connective tissue stroma. Of the four ameloblastomas studied, two were plexiform, one was follicular and one was of the granular cell type. In the follicular variant, follicles of odontogenic epithelium with central cells resembling stellate reticulum were seen (Fig. 5E). Plexiform ameloblastomas exhibited strands and sheets of epithelial tissue with prominent cystic degeneration. Collections of large granular cells (Fig. 5L) within neoplastic epithelial follicles were detected in the granular cell type. A feature common to all ameloblastomas was that peripheral cells of neoplastic epithelium were columnar or cuboidal, morphologically resembling ameloblasts or pre-ameloblasts, respectively. Both myxomas studied contained stellate or spindle-shaped cells in myxomatous, poorly collagenous background. No epithelial tissue was seen in the myxomas.

In situ hybridization detected high expression of EGF-R transcripts in the epithelial tumor cells of the two ameloblastic fibromas (Fig. 5B) and four ameloblastomas (Fig. 5F and J) studied. The ectomesenchymal tumor component of the ameloblastic fibromas and the stroma of the ameloblastomas, including capillaries, were negative (Fig. 5B,F, and J). The epithelial tumor cells exhibited positive immunostaining with anti-EGF-R antibodies (Fig. 5C and K). The ectomesenchymal tumor component of the ameloblastic fibromas (Fig. 5B and C) and the stroma of the ameloblastomas (Fig. 5F and J), including capillaries, were negative for EGF-R mRNA and immunoreactivity. No EGF-R transcripts were detected nor was immunoreactivity observed in the two odontogenic myxomas studied (not shown).

In none of the odontogenic tumors studied were EGF transcripts detected by *in situ* hybridization. The two ameloblastic fibromas and four ameloblastomas (Fig. 5L) studied were mostly negative with EGF antibody. Central cells of epithelial tumor follicles occasionally exhibited weak immunoreactivity for EGF but peripheral cells resembling pre-ameloblasts were constantly negative. The ectomesenchymal tumor component of the ameloblastic fibromas, the stroma of the ameloblastomas, including capillaries, and the two myxomas were negative.

A low copy number of TGF-α mRNA was observed in both epithelial and mesenchymal components of all odontogenic tumors studied. Immunohistochemistry revealed immunoreactivity for TGF-α in the neoplastic epithelium of the ameloblastic fibromas and ameloblastomas studied (Fig. 5D,H and M). Weakly TGF-α immunoreactive cells were occasionally detected in the neoplastic ectomesenchyme of the ameloblastic fibromas (Fig. 5D) and in the stroma of the ameloblastomas. Spindle-shaped tumor cells in odontogenic myxomas were positive for TGF-α (Fig. 5N). Capillaries in the tumor specimens were positive for TGF-α.

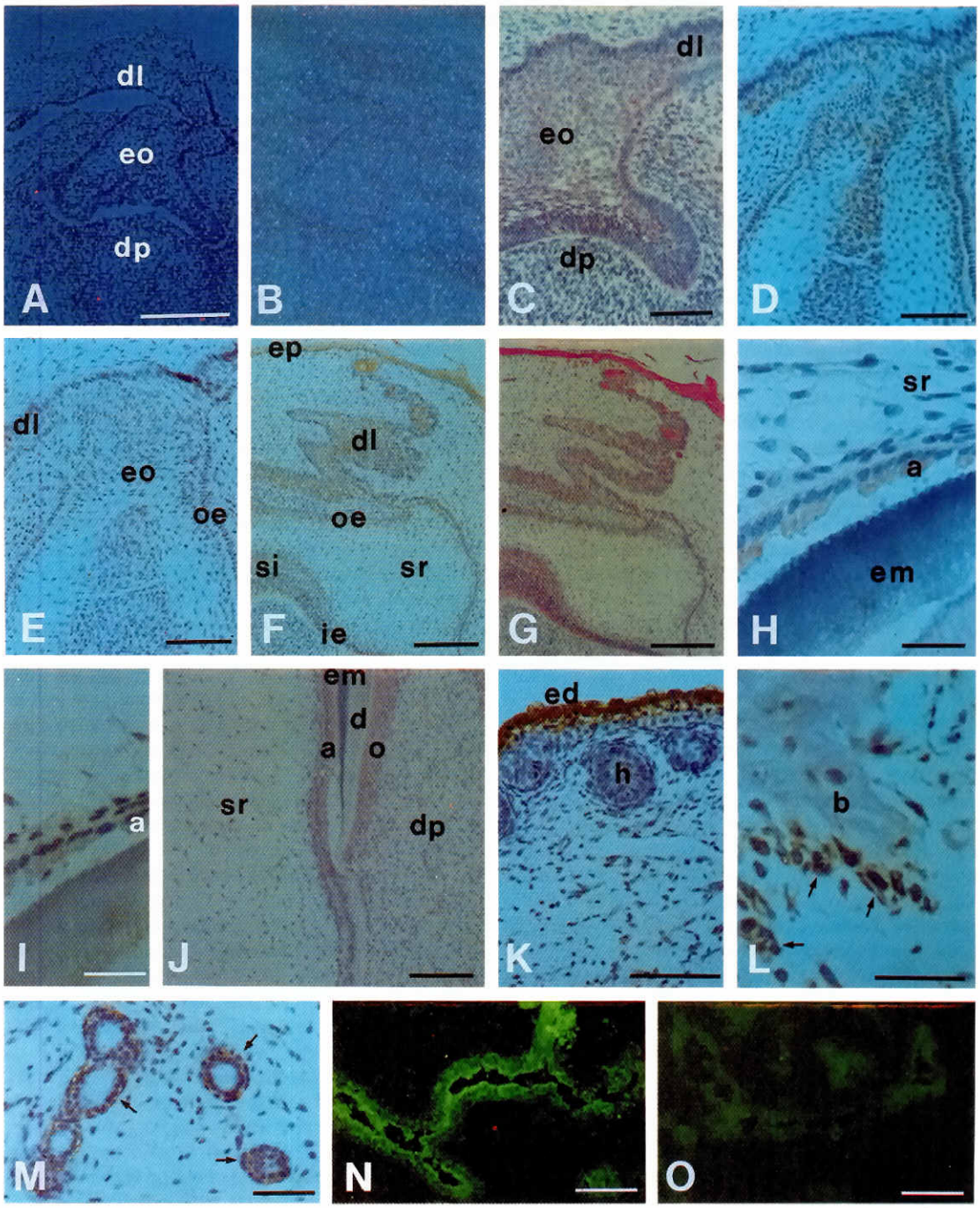

Fig. 4. TGF-α and EGF mRNAs and TGF-α and EGF immunoreactive cells in developing tooth detected by *in situ* hybridization and immunocytochemistry. **(A)** *Bright-field illumination of developing tooth at cap stage.* **(B)** *Only background signal was seen in the dark field image of the same section hybridized with antisense TGF-α cRNA probe.* **(C)** *Weak immunoreactivity was detected in dental lamina and the enamel organ with anti-TGF-α at the cap stage.* **(D)** *Anti-EGF gave a weak positive reaction in the dental lamina and enamel organ at the late cap stage.* **(E)** *A control section of the same tissue reacted with normal rabbit serum remained negative.* **(F)** *At the early bell stage, a positive immunoreaction with anti-EGF was seen in the oral epithelium, dental lamina and outer and inner enamel epithelium.* **(G)** *Positive immunoreactivity with anti-TGF-α was seen at the bell stage in oral epithelium, dental lamina, and outer and inner enamel epithelium.* **(H)** *Secretory ameloblasts were positively stained with anti-EGF at the apposition stage.* **(I)** *No staining was seen in secretory ameloblasts in a control section reacted with normal rabbit serum.* **(J)** *At the apposition stage, anti-TGF-α gave a positive immunoreaction in secretory ameloblasts and odontoblasts.* **(K)** *Positive staining was seen with anti-EGF in the fetal epidermis (13 gwk). Developing hair follicles remained negative.* **(L)** *Osteoblasts (arrows) of developing alveolar bone (b) exhibited a positive immunoreaction with EGF antiserum.* **(M)** *Immunoreactivity for anti-EGF was seen in the ductal cells (arrows) of a fetal submandibular salivary gland (13 gwk).* **(N)** *Anti-TGF-α gave positive immunofluorescence in ductal cells of a developing salivary gland (16 gwk).* **(O)** *Abolition of immunoreactivity was seen with TGF-α antiserum pre-absorbed with an excess of TGF-α polypeptide. Exposure time: B 6 weeks. Bars: A,B,F,G, and J, 200 μm; C-E, and K, 100 μm; H,I,L-O, 50 μm.*

In all of the tumors studied, sense probes for EGF-R (Fig. 5G), EGF and TGF-α gave a background signal. Negative staining was constantly obtained with pre-absorbed primary antibodies and non-immune sera (Fig. 5O).

Discussion

We report here the expression of EGF, TGF-α and their receptor EGF-R in human fetal teeth and various odontogenic tumors. Despite numerous studies on EGF-R in developing and erupting rodent teeth, there is still disagreement about which dental cells express EGF-R. EGF-binding studies have suggested that both dental mesenchyme and epithelium may be target tissues for EGF (Partanen and Thesleff, 1987; Topham *et al.*, 1987; Abbott and Pratt, 1988; Martineau-Doizé *et al.*, 1987, 1991). Previous studies have demonstrated EGF-R immunoreactive cells in the enamel organ of the developing tooth (Abbott and Pratt, 1988; Cam *et al.*, 1990) as well as in ameloblasts and odontoblasts of erupting rat incisors (Shore *et al.*, 1992). Recently, EGF-R mRNA was detected in both dental mesenchyme and epithelium by PCR (Hu *et al.*, 1992). The study we report here shows that the enamel organ expresses both EGF-R transcripts and EGF-R immunoreactive cells, indicating that dental epithelium is the main target tissue for both EGF and TGF-α during human tooth development.

In our study EGF-R was strongly expressed at cap and early bell stages but decreased as odontogenesis progressed denoting that EGF-R is temporospatially expressed. This is in line with previous studies suggesting that only early tooth-germs respond to EGF and that high amounts of EGF-R might be associated with proliferative activity of odontogenic cells (Partanen and Thesleff, 1987; Abbott and Pratt, 1988; Shore *et al.*, 1992).

The tissue sources of EGF and TGF-α during fetal development are as yet not well characterized (Carpenter and Wahl, 1991). We could not detect EGF or TGF-α transcripts in fetal tooth germs (13th to 20th gwk) by *in situ* hybridization. However, using the sensitive RT-PCR assay we were able to demonstrate EGF and TGF-α mRNA in most tooth-germ RNA samples studied (16th to 20th gwk). The level of transcription may be so low that the detection threshold with *in situ* hybridization fails to match that with RT-PCR assay. The possibility that the PCR amplified EGF and TGF-α transcripts originating from blood-carried migratory cells, i.e. eosinophils and macrophages (Madtes *et al.*, 1988; Wong *et al.*, 1990), cannot be excluded. Though mRNA for EGF and TGF-α was detected only by the PCR assay, immunoreactivity was demonstrated in various dental cell populations. These findings suggest that locally acting EGF and TGF-α in the fetal tooth germ may be derived partially from non-odontogenic sources, e.g. amniotic fluid (Goodyer *et al.*, 1991), fetal oral epithelium or blood-borne cells.

Several strands of evidence suggest that levels of EGF transcription are very low in fetal tissues (Carpenter and Wahl, 1991). Popliker *et al.* (1987) did not detect EGF mRNA in mouse fetuses by dot blot and Northern hybridization techniques. However, Kronmiller *et al.* (1991a) demonstrated EGF transcripts in fetal mouse mandibles (9th to 10th gestational days) by the RT-PCR. Snead *et al.* (1989) also found low expression of EGF-precursor mRNA in developing mouse molar and other fetal tissues by *in situ* hybridization. The conflicting results most likely reflect different sensitivities of the EGF transcript assays used. In our study, EGF mRNA was detected in several human fetal (15th to 20th gwk) tissues including tooth, kidney, skin, submandibular gland, pancreatic and duodenal tissues, by the RT-PCR, but not by less sensitive methods.

Regulation of the proliferation and functional maturation of epithelial cells in particular may be the main function of EGF and TGF-α in the developing fetus (Carpenter and Wahl, 1991; Lee and Hahn, 1991). TGF-α mRNA has previously been detected by *in situ* hybridization in several mouse fetal tissues from the 9th to the 10th gestational days but not in older fetuses (Wilcox and Derynck, 1988). Interestingly, high levels of both TGF-α (Wilcox and Derynck, 1988) and EGF (Kronmiller *et al.*, 1991a) mRNA expression have been reported in mouse oral tissues during the 9th and 10th gestational days, just before onset of tooth development. Exposure of mouse fetal mandibular explants (9 gestational days) to EGF antisense oligomers results in the inhibition of odontogenesis (Kronmiller *et al.* 1991b). Thus, EGF may play a role in the induction of odontogenesis and in the regulation of morphogenesis (Partanen *et al.* 1985; Kronmiller *et al.*, 1991b; Hu *et al.*, 1992). In the study reported here, locations of the EGF and TGF-α, and of their receptors, in the epithelial elements of cap/early bell-stage human tooth germs suggest that both peptides may function as mitogens for the early dental epithelium. However, their roles in the initiation of human odontogenesis (around 6th gwk) remain unknown.

In later stages of tooth development, cells of the dental lamina, outer enamel epithelium and stratum intermedium expressed EGF-R. Immunoreactivity for EGF and TGF-α was found in presecretory and mature ameloblasts. However, no EGF-R were detected in these cells, findings consistent with those in earlier EGF binding studies (Martineau-Doizé *et al.*, 1987). Before the onset of amelogenesis, stratum intermedium cells adjacent to ameloblasts expressed EGF-R. The function of these cells is not known. They are thought to participate in enamel formation (Avery, 1992). Our observations suggest that developing ameloblasts may regulate the growth and differentiation of the stratum intermedium paracrinically, through EGF or TGF-α production.

There is increasing evidence indicating that a coordinated action of several growth factors and extracellular matrix (ECM) molecules regulates tooth development. We have detected TGF-ß2 in odontoblasts of developing human teeth (Heikinheimo *et al.*, 1993). Transcripts for TGF-ß1, TGF-ß2 and bone morphogenetic protein-2 (BMP-2) have also been located in odontoblasts (Lyons *et al.*, 1990; Vaahtokari *et al.*, 1991; Heikinheimo *et al.*, 1993). In the study reported here, we found EGF and TGF-α but not EGF-R in secretory odontoblasts. Recent data indicate that EGF, TGF-α, and TGF-ß1 may stimulate synthesis of ECM proteins in the embryonic palatal mesenchyme (Dixon and Ferguson, 1992). Consequently, the increased ECM protein production seen in dental papilla mesenchyme at the onset of dentin formation (Heikinheimo *et al.*, 1991a) may be activated by odontoblast-derived growth factors such as EGF, TGF-α, and members of the TGF-ß superfamily.

TGF-α and EGF-R are overexpressed in several neoplasms suggesting that they participate in tumor formation (Ozanne *et al.*, 1985; Derynck *et al.*, 1987; Partridge *et al.*, 1989; Karnes *et al.*, 1992). High levels of TGF-α have also been associated with the more undifferentiated carcinomas (Partridge *et al.*, 1989). In the present study, TGF-α mRNA and immunoreactivity were detected in all neoplasms of epithelial (ameloblastoma), epithelial-ectomesenchymal (ameloblastic fibroma) and ectomesenchymal (odontogenic myxoma) origin. In addition, EGF-R mRNA and protein were located in neoplastic odontogenic epithelia. In contrast, none of the tumors studied expressed EGF mRNA. However, EGF

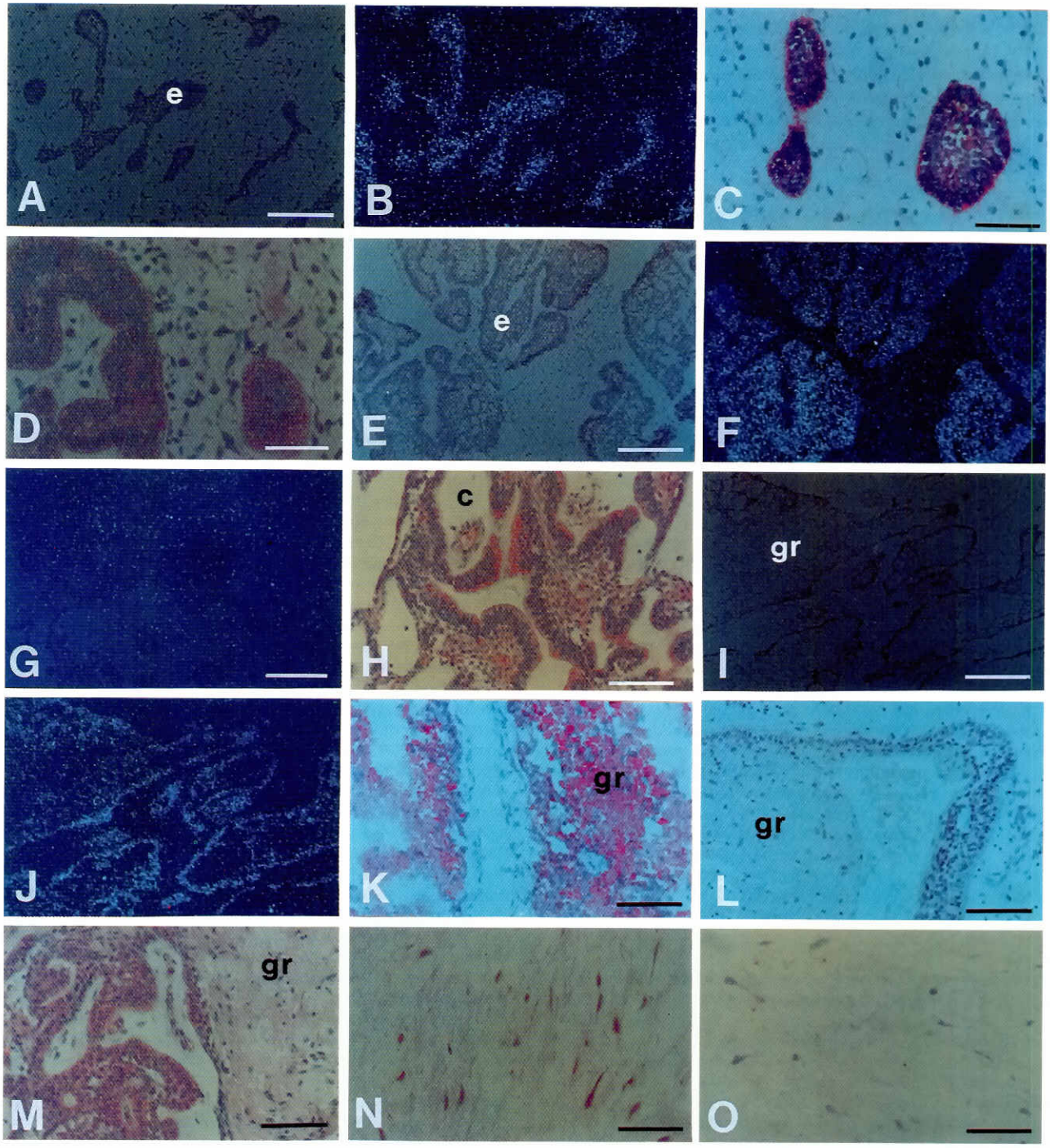

Fig. 5. EGF-R, EGF and TGF-α mRNAs and reactivity with anti-EGF-R, anti-EGF and anti-TGF-α in odontogenic tumors detected by *in situ* hybridization and immunocytochemistry. (A) *Bright-field micrograph of ameloblastic fibroma showed strands of odontogenic epithelium (e) in immature ectomesenchymal tissue.* **(B)** *Dark-field image of the same section demonstrated hybridization signals for EGF-R mRNA in the epithelial tumor component.* **(C)** *Anti-EGF-R showed positive immunoreactivity in the epithelial islands of ameloblastic fibroma.* **(D)** *Positive immunoreactivity in epithelial islands of ameloblastic fibroma with TGF-α antiserum was seen.* **(E)** *Bright-field image of follicular ameloblastoma demonstrated epithelial tumor islands in mature connective tissue stroma.* **(F)** *High expression of EGF-R mRNA was seen in epithelial cells in the dark field image of the same section.* **(G)** *No hybridization signals were obtained in the same ameloblastoma using the sense probe for EGF-R mRNA.* **(H)** *Anti-TGF-α gave a positive immunoreaction in epithelial islands of ameloblastoma. Note extensive cystic degeneration (c) in stromal tissue.* **(I)** *Phase-contrast micrograph of granular cell ameloblastoma demonstrated epithelial islands with granular cells (gr).* **(J)** *Dark-field illumination of the same section showed transcripts for EGF-R in the neoplastic epithelial islands.* **(K)** *Granular cells stained positively with anti-EGF-R.* **(L)** *No immunoreactivity was seen in granular cells with EGF antiserum.* **(M)** *Positive staining of epithelial cells of granular cell ameloblastoma was seen with anti-TGF-α. Granular cells exhibited only weak immunoreactivity.* **(N)** *TGF-α antibody gave positive immunoreaction in spindle-shaped cells of odontogenic myxoma.* **(O)** *No staining was seen with normal mouse serum. Exposure times: B,F,G, and J 6 weeks. Bars: A,B,H, and K-M, 100 μm; C,D,N, and O, 50 μm; E-G,I, and J, 200 μm.*

immunoreactivity was occasionally detected. EGF mRNA is rarely detected in human tumors, indicating that EGF is most likely not associated with tumor development (Carpenter and Wahl, 1991). In neoplastic odontogenic epithelium, TGF-α may enhance cell proliferation in an autocrine manner as has been suggested in human cancers and cancer cell lines (Sizeland and Burgess, 1991; Karnes *et al.*, 1992).

It is concluded that EGF-R expression is developmentally regulated during human odontogenesis. Furthermore, the odontogenic epithelium is the primary target for EGF and TGF-α in the developing tooth. The coexpression of TGF-α and EGF-R in the epithelial components of odontogenic tumors supports this hypothesis.

Materials and Methods

Tissues

Human fetal tissues [13th to 20th gestational weeks (gwk)] were obtained via legal abortions, induced with prostaglandins. The study was approved by the Ethics Committee. Gestational ages were estimated from fetal foot lengths (Munsick, 1984). Eight mandibular samples (13th to 20th gwk) were hemisected. One half was snap frozen in liquid nitrogen and stored at -70°C until use. Serial cryostat sections (5 μm) were cut from each jaw sample and mounted on aminoalkylsilane-treated objective slides for *in situ* hybridization (Rentrop *et al.*, 1986). The other half of the jaw was fixed in cold 4% paraformaldehyde (PFA), decalcified in 10% EDTA neutral 10% formalin, and embedded in paraffin. Serial sections (5 μm) were cut on poly-l-lysine-coated (100 μg/ml, Sigma Chemical Co., St. Louis, MO) slides for immunostaining and histological staining with haematoxylin and eosin. Human fetal (16 gwk) and adult submandibular glands, and fetal pancreata (16 gwk), and duodena (16 gwk) were also collected, and processed similarly.

For RNA extraction, five deciduous tooth germs from five hemisected human fetal mandibles (16th to 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 were frozen and stored for comparative purposes. Twenty-one-day old Sprague-Dawley albino rat fetal mandibular first molars (n= 80) at bell-stage of tooth development (Lefkowitz *et al.*, 1953) were dissected and stored as described above. Human fetal skin (16 gwk), kidney (15 gwk), pancreata (17 gwk) and duodena (17 gwk), fetal (21 gwk) and adult submandibular glands, human term placentas and A431 vulvar carcinoma cells were included for control purposes.

Tumors

Samples from eight fresh benign odontogenic 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 four ameloblastomas studied were from the mandible of a 40 year-old woman, 40-year-old man and 73-year-old man, and from the maxillae of a 74-year-old woman. The two ameloblastic fibromas studied were from the maxilla of a 4-year-old boy and a 14-year-old girl. The two myxomas studied were mandibular tumors from a 17-year-old man and a 30-year-old woman.

Probes

A 1.84 kb EcoRI fragment of human EGF-R (phHER-A64-1 cDNA; Ullrich *et al.*, 1984), a 0.925 kb EcoRI fragment of human TGF-α (phTGF1-10-925 cDNA; Murray *et al.*, 1986) and a 0.555 kb EcoRI fragment of human EGF cDNA (Bell *et al.*, 1986) were subcloned into pGEM7 (+z) ribovectors (Promega Biotec, Madison, WI). A 0.27 kb TGF-α cRNA probe was also generated, as described previously (Miettinen and Heikinheimo, 1992). For *in situ* hybridization and Northern analysis the cRNA probes were labeled by *in vitro* transcription (Melton *et al.*, 1984) using SP6 and T7 RNA polymerases (Promega) and [^{35}S]UTP (800 Ci/mmol, Amersham, Buckinghamshire, UK) or with [^{32}P]UTP (800 Ci/mmol), respectively. To verify the integrity of the RNA, a human γ-actin cDNA probe (Gunning *et al.*, 1983) was labeled by random priming using an Oligolabeling Kit (Pharmacia LKB, Uppsala, Sweden) with [^{32}P]dCTP (6000 Ci/mmol, Amersham).

For Southern hybridization, the 0.925 kb TGF-α cDNA was random prime labeled. An internal 27-mer antisense oligoprobe (5'-CTCCCCGATGTAGCCAACAACACAGTT-3') corresponding to nucleotides 3440-3466 of the human EGF precursor cDNA (Bell *et al.*, 1986) was also labeled with [^{32}P]dCTP (6000 Ci/mmol) by 3'-end-labeling, using terminal transferase (Boehringer Mannheim, Mannheim, Germany). Specificity of ß-actin PCR-products was controlled by hybridization with a 3'-end-labeled internal 27-mer antisense oligoprobe (5'-CTCGGGAGCCACACGCAGCTCATTGTA-3') corresponding to nucleotides 312-338 of human ß-actin cDNA (Ponte *et al.*, 1984).

RNA extraction and Northern analysis

Total cellular RNA was isolated by guanidium isothiocyanate extraction and cesium chloride centrifugation (Chirgwin *et al.*, 1979), determined spectrophotometrically at 260 nm and stored at -70°C until use. For Northern analysis, RNA samples (15-30 μg) were denatured in glyoxal and dimethylsulphoxide, run in an 1.5% agarose gel and transferred on to Hybond-N filter (Amersham) by capillary blotting (Thomas, 1980). The filters were hybridized with [^{32}P]UTP-labeled EGF-R, EGF and TGF-α cRNA probes (65°C, 16 hours) and a [^{32}P]dCTP-labeled γ-actin cDNA probe (42°C, 16 hours) in hybridization buffer containing 0.1% SDS, 5xSSPE (1xSSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 50% formamide, 5 x Denhardt's solution (0.1% Ficoll 400, 0.1% BSA, 0.1 % polyvinylpyrrolidone 360), 0.1 mg/ml denatured herring sperm DNA and 0.1 mg/ml torula RNA. The filters were washed in 0.1xSSC (1xSSC is 0.15 M NaCl and 0.015 M trisodium citrate) with 0.1% SDS (three times 20 minutes at 65°C for cRNA and 50°C for cDNA probes) and subjected to autoradiography using Trimax T16 intensifying screens (3M, Ferrania, Italy) and Hyperfilm MP films (Amersham) for 1-4 weeks at -70°C.

Reverse transcription polymerase chain reaction (RT-PCR) and Southern blotting

In the RT-PCR, 1 μg of each RNA sample was reverse transcribed as previously described (Miettinen and Heikinheimo, 1992). The synthetic oligonucleotide primers for human EGF, human TGF-α and human ß-actin designed for the PCR are listed in Table 1. The PCR was performed following instructions provided with the GeneAmp DNA-Amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT). The PCR volume was 50 μl. The reaction mixture contained 25 pmol of 3' and 5' amplification primers, 5 μl of 10xPCR buffer, 4 μl of dNTP mixture (0.2 mM 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 of mineral oil (Perkin Elmer Cetus), denaturation was performed initially at 94°C for 3 minutes. Thirty cycles were used for primary amplification (denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for one and half minutes) using a Hybaid thermal reactor (Teddington, UK). Because of the low EGF and TGF-α mRNA levels, 1 μl of the primary PCR product was subjected to another amplification round of 30 cycles. Ten-μl portions of the PCR products were size-fractionated in 1.6% agarose gel and stained in ethidium bromide. Hae III-digested ØX174RF DNA was used as a size marker. Southern transfer was performed by capillary blotting the DNA on to Hybond-N nylon membranes. The membranes were hybridized with [^{32}P]dCTP-labeled TGF-α cDNA, internal EGF and ß-actin oligoprobes as described above.

In situ hybridization

In situ hybridization with [^{35}S]UTP-labeled EGF-R, EGF and TGF-α riboprobes was performed on cryostat sections as previously described (Heikinheimo *et al.*, 1991b). Briefly, the sections were hybridized with 1-2x10^6 cpm of ^{35}S-labeled antisense or sense cRNA probes in hybridization buffer containing 60% formamide at 50°C. After posthybridization washes and RNase A (Sigma) and T_1 (Boehringer Mannheim) treatments, the sections were subjected to autoradiography by dipping the slides into NTB-2 film emulsion (Eastman Kodak Co., Rochester, NY) and exposing them for up to 6 weeks

in light-tight boxes at +4°C. After developing the slides in D-19 developer (Kodak), the sections were lightly counterstained in Harris' haematoxylin and assessed using dark- and light-field microscopy.

Immunocytochemistry

A biotin-streptavidin staining kit (StrAviGen Super Sensitive, BioGenex Laboratories, San Ramon, CA) with alkaline phosphatase and monoclonal antibodies to EGF-R (0.5 μg/ml) (BioGenex) and to TGF-α (5 μg/ml) (Oncogene Science Inc., Uniondale, NY) was used on paraformaldehyde (PFA)-fixed, paraffin-embedded tissue specimens. Immunolocation of TGF-α was also undertaken on frozen tissue sections, employing an indirect immunofluorescence detection system using affinity-purified sheep antibodies (200 μg/ml) to N-terminal amino acid residues of human TGF-α (Chemicon International Inc., Temecula, CA). FITC-conjugated donkey anti-sheep IgG (3.75 μg/ml) (Jackson Immunoresearch Laboratories Inc., West Grove, PA) was used as secondary antibody. EGF was detected on PFA-fixed tissue sections with affinity-purified rabbit polyclonal antibody (5 μg/ml) (Oncogene Science) with an ABC-detection kit (OSI Immunohistochemistry System, Oncogene Science) and diaminobenzidine tetrahydrochloride as chromogen. Specificity controls for EGF-R, TGF-α, and EGF included replacement of the primary antibodies with normal mouse, sheep, and rabbit sera, respectively. EGF and TGF-α antibodies were pre-absorbed using 20-fold excess by weight of recombinant EGF (Promega) and TGF-α (Bachem, Bubendorf, Switzerland).

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