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Original Article

Differential expression of the full-length and secreted truncated forms of EGF receptor during formation of dental tissues

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ABSTRACT The developmental regulation of various receptor forms may be a key-element in the local fine tuning of growth factor effects. The present study focuses on the tissue- and stage-specificity of the alternative splicing of EGF receptor transcripts in the rat incisor. *In situ* hybridization, as well as light- and electron-microscopic immunolocalization were performed with a set of tools which enabled us to discriminate the full-length and secreted truncated forms of EGF receptor. Our data show that, apart from a transient expression in differentiating odontoblasts, EGF receptor expression was predominantly observed in the dental epithelium. In the crown, the expression of the full-length EGF receptor was maximal during preameloblast proliferation and differentiation, decreased in differentiated ameloblasts, and remained low throughout enamel secretion. On the other hand, maturation stage ameloblasts, which regulate the final mineralization of enamel, express high levels of the full-length EGF receptor. In contrast with ameloblasts, epithelial supra-ameloblastic cells, which are not directly involved in the deposition of enamel matrix, showed an alternating predominance of the secreted truncated form during the secretion stage, and the full-length form during the maturation stage. The presence of the secreted truncated EGF receptor form was supported by the electron microscopic detection of extracellular aggregates of immunoreactive EGF receptor. Finally, Northern-blotting of enamel organ samples confirmed the presence of transcripts corresponding to mRNAs of both EGF receptor forms. During root formation, a decreasing gradient of full-length EGF receptor form expression was observed from the apical loop to the disrupting zone in root epithelium. The secreted truncated EGF receptor form was essentially detected in epithelial cells of the disrupting zone of root epithelium. During crown formation, the secreted truncated EGF receptor form, which appears to be synthesized by epithelial supra-ameloblastic cells and secreted toward ameloblasts, may competitively bind EGF receptor ligands and modify activation of the full-length EGF receptor.

KEY WORDS: *EGF receptor, EGF truncated receptor, differentiation, tooth, ameloblasts*

Introduction

EGF is the main member of a large family of growth factors and exerts its action by binding to a receptor. EGF receptor (EGFr) is a 170 kDa transmembrane glycoprotein. Its intracellular domain contains tyrosine kinase activity regulated by EGF binding (Carpenter and Wahl, 1991). The major physiological consequences of EGFr activation include increased cell proliferation (Barandon and Green, 1987), effects on the differentiation process of many cells, such as bone (Aubin *et al.*, 1992), and transformed cells (Kim *et al.*, 1987), and electrolyte transport (Opleta-Madsen *et al.*, 1991). These different responses may occur in the same cells (Aubin *et al.*, 1992).

Specific developmental patterns of EGFr expression appear to play important roles in EGF responses (Adamson, 1990; Lee and Hahn, 1991), as observed during lung (Schuger *et al.*, 1993), kidney (Partanen and Thesleff, 1987) and bone (Davideau *et al.*, 1995) formation. Numerous EGFr transcripts have been shown in human (Ullrich *et al.*, 1984; Ilekis *et al.*, 1995) and rat cells (Petch *et al.*, 1990; Das *et al.*, 1994). Full-length receptor is synthesized from three major transcripts: 5.0, 6.5 and 9.6kb (Ullrich *et al.*, 1984; Petch *et al.*, 1990). Among the

Abbreviations used in this paper: EGF, epidermal growth factor; EGFr, EGF receptor.

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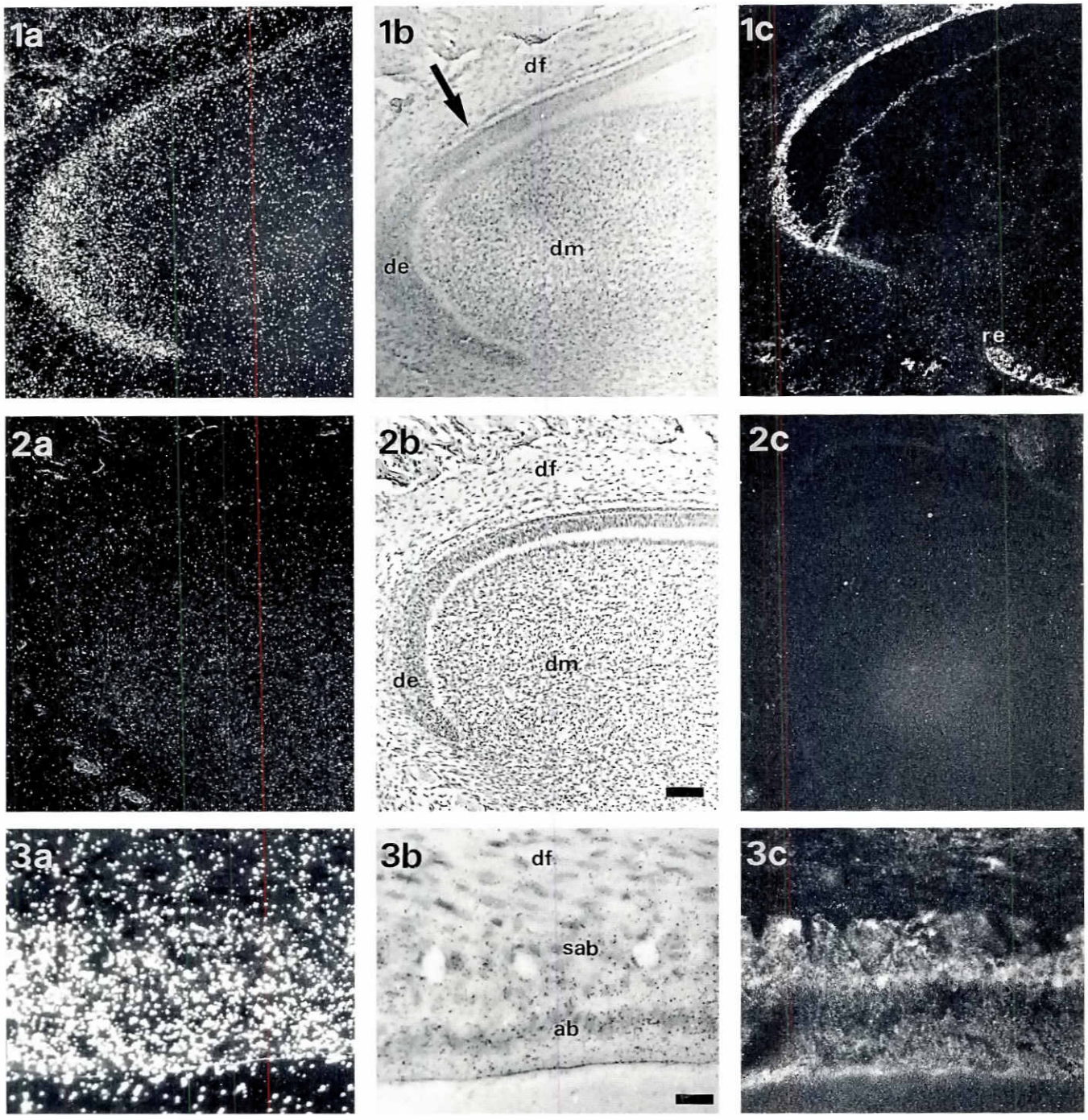


Fig. 1. Expression of full-length EGFr form mRNAs and proteins: the enamel presecretion, and enamel secretion zones of 7 day-old rat incisor. *Dark field* **(a)** *and bright field* **(b)** *views of the same section hybridized with ^{33}P-labeled FU/TR probes (detecting both the full-length and truncated EGFr forms). Dental epithelium (de) is strongly labeled. Labeling decreases in polarizing ameloblasts of the inner dental epithelium (arrow). The hybridization signal is also detected (*) in the apical part of the dental ectomesenchyme (dm). Dental follicle (df) appears negative.* **(c)** *Immunofluorescence detection with FU/TR antibodies. Strong immunostaining of dental epithelium is observed in the presecretion zone. Dental mesenchyme and dental follicle are not stained. Root epithelium (re) is also strongly labeled.*

Fig. 2. Controls on serial sections. *Dark field* **(a)** *and bright field* **(b)** *views of an in situ hybridization control using ^{33}P-labeled FU/TR sense riboprobe.* **(c)** *Immunocytochemistry control by omitting primary antibodies. Bar, 80 μm.*

Fig. 3. Expression of full-length EGFr form mRNAs and proteins: the enamel maturation zone of a 7 day-old rat incisor. *Dark field* **(a)** *and bright field* **(b)** *views of the same section hybridized with ^{35}S-labeled FU riboprobe (detecting full-length EGFr form). Dental epithelium including ameloblasts (ab) and epithelial supra-ameloblastic cells (sab) is strongly labeled.* **(c)** *Immunofluorescence detection by using FU antibodies. Immunostaining is observed in epithelial supra-ameloblastic cells (sab) and ameloblasts (ab). Bar, 30 μm.*

minor transcripts, the 2.7kb species is produced by alternative splicing of EGFr premessenger RNAs and is translated as a secreted truncated form of EGF receptor with a molecular weight of 95 kDa, corresponding to the extracellular domain of the full-length receptor (Petch *et al.*, 1990). The production of alternatively transcribed soluble receptor has been proposed to represent a biological mechanism for selective and efficient inhibition of cellular responsiveness to specific mitogenic and differentiating factors (Kendall and Thomas, 1993). However, the developmental control of alternative splicing of receptor gene transcripts during cell differentiation, and the respective distribution of the corresponding proteins, have not been investigated.

The tooth is a classical target-organ of EGF (Cohen, 1962; Partanen *et al.*, 1985; Rhodes *et al.*, 1987; Topham *et al.*, 1987; Hata *et al.*, 1990; Kronmiller *et al.*, 1991; Hu *et al.*, 1992; Shum *et al.*, 1993). However, the majority of these studies focused on early events of tooth morphogenesis. The present investigation extended the study of EGF receptor proteins and mRNAs to the whole developmental sequence of tooth formation, from presecretion to secretion and maturation stages of amelogenesis.

Results

Expression of full-length EGFr form depends on the developmental stage of ameloblast differentiation

In the enamel presecretion zone of dental epithelium, a strong EGFr mRNAs signal was observed with FU/TR (detecting full-length and secreted truncated EGFr forms) and FU (detecting full-length EGFr form) riboprobes (Figs. 1a,b, 4a,b, 5a,b). EGFr mRNA labeling of inner dental epithelial cells decreased in polarizing ameloblasts and remained low in the enamel secretion zone (Fig. 1a,b). A similar pattern was observed for protein expression by immunolocalization with FU/TR (Fig. 1c) and FU antibodies. In the enamel maturation zone, EGFr mRNAs increased in ameloblasts, which showed intense labeling with FU (Fig. 3a,b) and FU/TR riboprobes. In enamel maturation stage ameloblasts, immunolabeling was located in proximal and distal poles with FU (Fig. 3c) and FU/TR antibodies. In dental mesenchyme, EGFr mRNAs labeling with FU/TR (Fig. 1a,b) and FU riboprobes was restricted to the area facing the end of the enamel presecretion zone. *In situ* hybridization controls with sense riboprobes showed only background (Fig. 2a,b). Immunocontrols with omission of primary antibodies were negative (Fig. 2c). Strong EGFr expression therefore appeared to be tissue- (essentially dental epithelium) and stage- (presecretion and enamel maturation stages) specific. During ameloblast differentiation, successive peaks of strong full-length expression were observed at the presecretion and enamel maturation stages.

Expression of the secreted truncated EGFr form is observed in epithelial supra-ameloblastic cells during the secretion stage

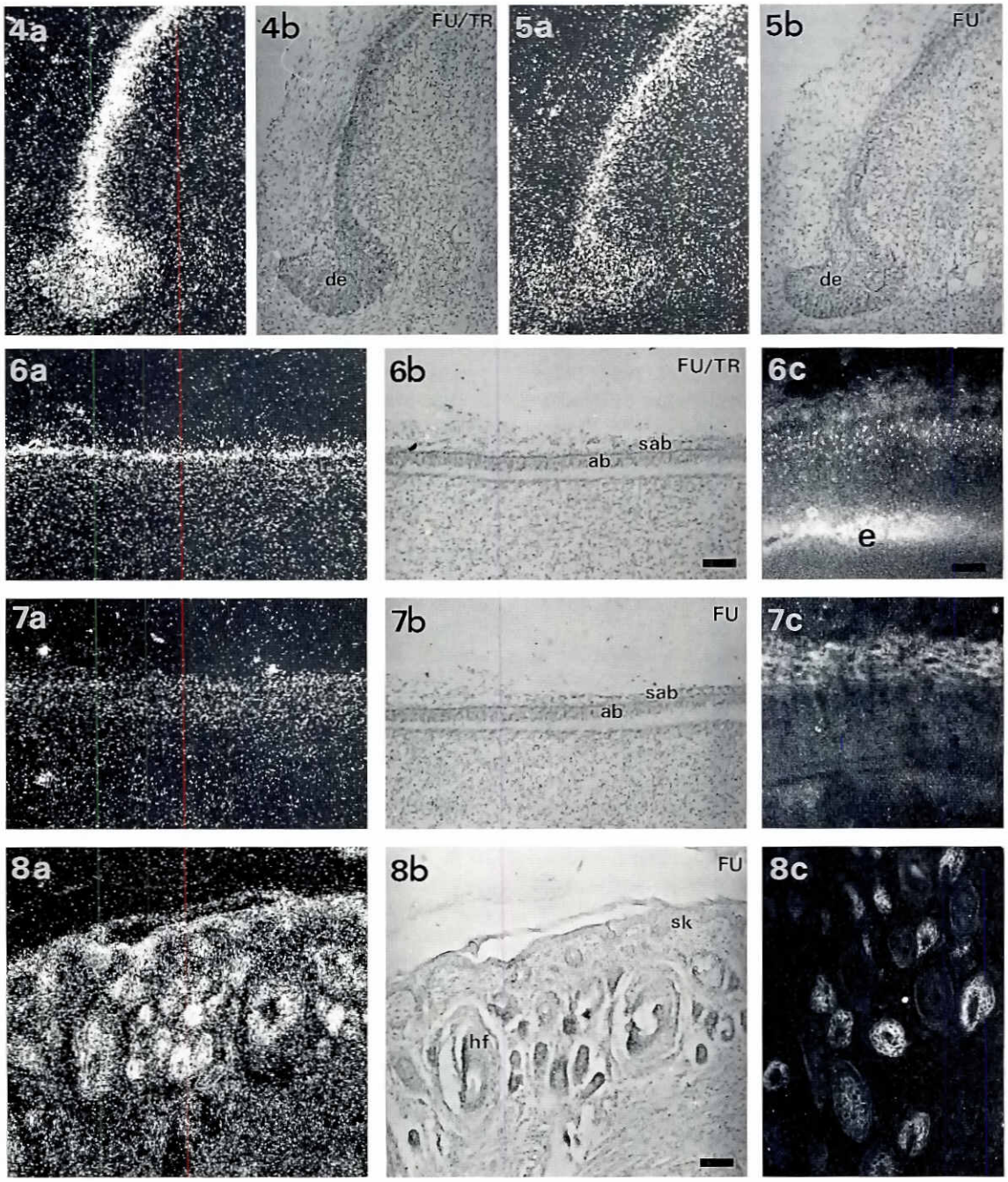
In the secretion zone, FU/TR and FU riboprobes showed different labeling patterns. Epithelial supra-ameloblastic cells showed strong signals with FU/TR riboprobes, whereas the ameloblasts in the secretion zone were weakly labeled (Fig. 6a,b). No difference in FU labeling was observed between the epithelial supra-ameloblastic cells and ameloblasts (Fig. 7a,b). These differences in the labeling pattern with FU/TR and FU probes suggest the presence of additional mRNA species in epithelial supra-ameloblastic cells corresponding to extracellular/transmembrane EGFr domains. FU/TR immunolabeling was located in the cytoplasm and plasma membrane of epithelial supra-ameloblastic cells. Some patches of labeling were observed. Similar patches of immunolabeling confined to the proximal pole of ameloblasts, were also observed (Fig. 6c). No patches were observed with FU immunodetection. Epithelial supra-ameloblastic cells and ameloblasts showed weak labeling (Fig. 7c). However, FU antibodies showed strong staining in hair follicles (Fig. 8c), corresponding to a high *in situ* hybridization signal with the FU probe (Fig. 8a,b). During the secretion stage, the EGFr extracellular domain detection of immunostaining patches with FU/TR antibodies (raised against the extracellular domain of EGFr) and their negative staining with FU antibodies (raised against the intracellular domain of EGFr) therefore suggested the presence of an additional protein in dental epithelium which contained EGFr extracellular domain epitopes without EGFr intracellular domain epitopes.

Immunoelectron microscopy showed epitopes of the EGFr extracellular domain both in cells and in extracellular areas of the enamel organ

Electron microscopic immunodetection was performed to examine the ultrastructural localization of extracellular domain EGFr epitopes. In the secretion zone, immunogold detection with FU/TR antibodies was located on the cell membrane, in the cytoplasm, where it was associated with electron-dense granules, and in the nuclei of ameloblasts and epithelial supra-ameloblastic cells directly in contact with ameloblasts, the stratum intermedium cells (Fig. 9). However, isolated, as well as aggregated gold particles were also detected in the extracellular compartment. They were apparently not associated with the plasmic membrane (Fig. 9). This last result suggests that the additional protein with EGFr extracellular domain epitope observed on light microscopic immunocytochemistry, corresponded to a secreted protein present in the extracellular space around stratum intermedium cells and the proximal pole of ameloblasts.

In situ hybridization analysis indicated that the mRNAs of secreted truncated EGFr form were present in epithelial supra-ameloblastic cells during the secretion stage

To determine whether the extracellular domain epitopes observed correspond to the expression of the secreted truncated form, its transcripts were studied by *in situ* hybridization. The *in situ* hybridization signal with the TR (detecting the secreted truncated EGFr form) oligonucleotide probe was restricted to epithelial supra-ameloblastic cells and more specifically to stratum intermedium cells during the secretion stage of amelogenesis (Fig. 10b,c). No signal was observed along the longitudinal incisor axis in other dental epithelial cells regardless of their stage of differentiation (Fig. 10a,d,e). These data show that the localization of the secreted protein detected with antibodies raised against the EGFr extracellular domain corresponded to the expression of the secreted truncated EGFr form. The expression of this form appeared to be cell- (stratum intermedium cells) and stage- (secretion stage) specific.



Figs. 4 and 5. Expression of full-length EGFr form in a 56 day-old rat incisor in the presecretion zone. *Dark field* **(4a,5a)** *and bright field* **(4b,5b)** *views of the same sections. In situ hybridization with ^{35}S-labeled FU/TR probe* **(4a,b)** *and FU probe* **(5a,b)**. *Both probes show a similar distribution of strong hybridization signal in dental epithelium (de).*

Fig. 6 and 7. Different labeling provided by FU/TR and FU probes and antibodies in the early secretion zone in 7 and 56 day-old rat incisors. *Dark field* **(6a)** *and bright field* **(6b)** *views of the same section. In situ hybridization with ^{35}S-labeled FU/TR probe. Epithelial supra-ameloblastic cells (sab) are strongly labeled, while ameloblasts (ab) show a weak signal. Bar, 80 μm.* **(6c)** *Immunofluorescence detection with FU/TR antibodies. Epithelial supra-ameloblastic cells (sab) are diffusely immunostained. Patches of staining (arrows) are observed at the proximal pole of ameloblasts (ab). Non specific fluorescence of immature enamel matrix is also observed (e). Bar, 20 μm. Dark field* **(7a)** *and bright field* **(7b)** *views of the same section. In situ hybridization with ^{35}S-labeled FU probe. Epithelial supra-ameloblastic cells (sab) and ameloblasts (ab) show similar weak labeling. Bar, 80 μm.* **(7c)** *Immunofluorescence detection with FU antibodies. Epithelial supra-ameloblastic cells (sab) and ameloblasts (ab) are immunostained. Patches of staining are not observed. Bar, 20 μm.*

Fig. 8. EGFr expression in skin and hair follicle of a 7 day-old rat. *Dark field* **(a)** *and bright field* **(b)** *views of the same sections. In situ hybridization with ^{35}S-labeled FU probe. Skin epithelium (sk) and hair follicles (hf) show a high in situ hybridization signal.* **(c)** *Immunofluorescence detection with FU antibodies. Hair follicles are strongly stained. Bar, 80 μm.*

Dental epithelium contains EGFr mRNAs with molecular weights corresponding to the full-length EGFr form and the secreted truncated EGFr form

The RNA extracted separately from isolated epithelial and mesenchymal components of rat incisors were analyzed by Northern blotting. In dental epithelium, using the FU/TR probe, several EGFr mRNA species were detected. In addition to 9.6kb, 6.5kb, and 5.0kb transcripts of full-length EGFr form, the 2.7kb mRNA of the secreted truncated EGFr form was also observed (Fig. 11b). Expression of EGFr transcripts could not be detected in dental mesenchyme (Fig. 11b), when an equal amount of total RNAs was analyzed from dental mesenchyme and dental epithelium (Fig. 11a), confirming the epithelial predominance of EGFr expression. These Northern-blotting data also confirm the presence of both EGFr forms in dental epithelium.

Full-length and secreted truncated EGFr forms are differentially expressed in root epithelium

A decreasing gradient of full-length EGFr mRNAs signal was observed in epithelial cells from the apical loop to the disrupting zone of root epithelium, whereas dental mesenchyme and dental follicle cells were weakly labeled with FU/TR (Fig. 12a,b) and FU riboprobes. The secreted truncated EGFr form mRNA signal was essentially detected in epithelial cells of the disrupting zone of root epithelium, with the TR probe (Fig. 13a,b). Epitopes of both EGFr forms were mainly detected in the epithelium with FU/TR antibodies and FU antibodies. The labeling present in all epithelial layers of the apical loop in the root epithelium was also present in disrupted epithelial areas (Fig. 14a,b). These data show that both EGFr forms are expressed in dental epithelium during root formation. Detection of mRNAs encoding the secreted truncated EGFr form mRNA was restricted to root epithelial cells, especially during the process of epithelium disruption.

Discussion

Previous papers describing EGFr expression in developing teeth by using EGF binding and immunocytochemistry, have reported discordant results (Thesleff *et al.*, 1987; Cho *et al.*, 1991; Martineau-Doizé *et al.*, 1991; Shore *et al.*, 1992; Wise *et al.*, 1992; Davideau *et al.*, 1995). The results of the present study suggest that common epitopes of the full-length EGFr form and secreted truncated EGFr form may have contributed to the ambiguity of previous findings. We used three groups of antibodies and probes which are able to discriminate the full-length and secreted truncated EGFr form mRNAs and the corresponding proteins: FU/TR, which detected the full-length and secreted truncated EGFr forms; FU, which detected only the full-length EGFr form; and TR, which detected only the secreted truncated EGFr form. The labeling patterns obtained with FU and FU/TR antibodies and riboprobes were similar and corresponded to the detection of full-length EGFr form. However, additional labeling was observed with FU/TR antibodies and riboprobes and this was identified as expression of a secreted truncated EGFr form by using the TR oligonucleotide probe. No difference of EGFr expression pattern was observed between pre-eruptive (growing organ) and post-eruptive (continuously growing organ) incisors. The results are therefore presented on 7 day-old (pre-eruptive), as well as 56 day-old (post-eruptive) rat incisors.

Different patterns of EGF receptor expression are observed in dental mesenchyme and dental epithelium

The present study concerning the expression of EGFr mRNAs and proteins confirms the presence of EGFr in dental

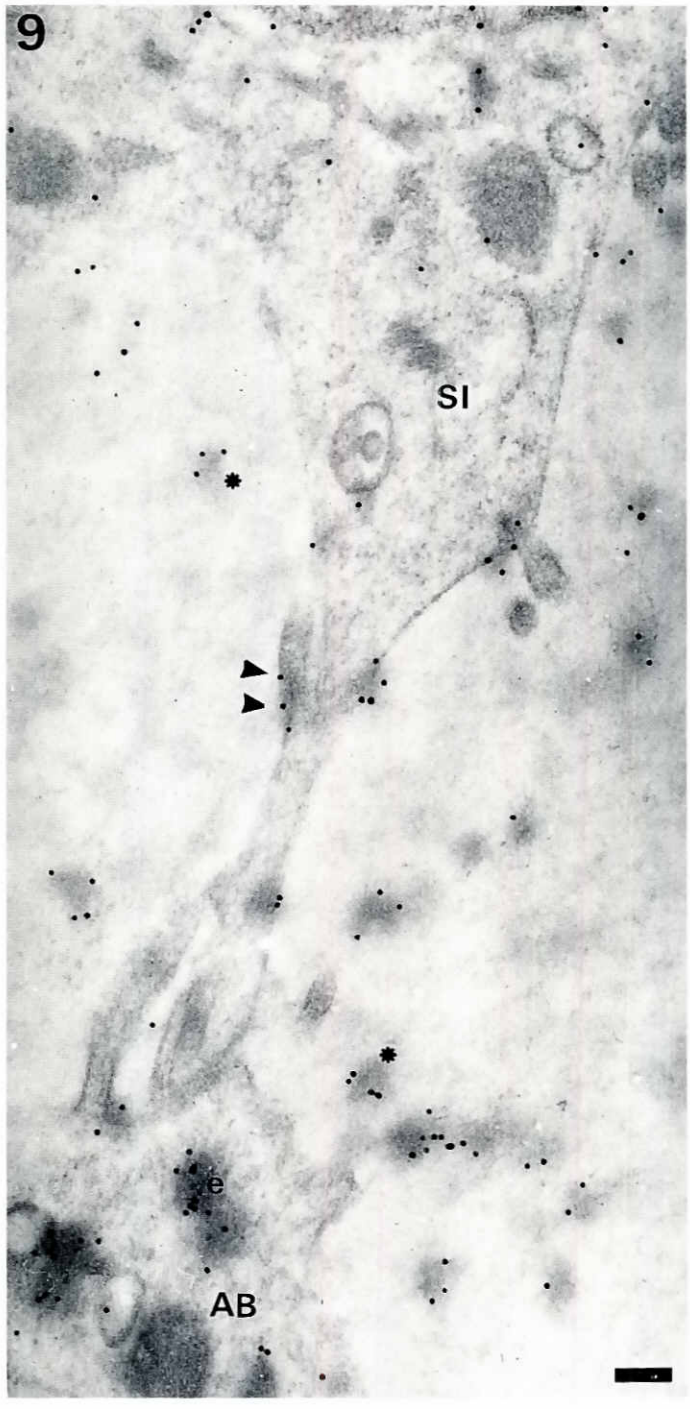


Fig. 9. Immunoelectronmicroscopic detection of full-length and secreted truncated EGFr form with FU/TR antibodies. *15-nm immunogold particles are located on cell membrane (arrows) of stratum intermedium cells (si) and ameloblasts (ab). In the cytoplasm, labeling is associated with electron-dense particles (e). Aggregates (*) and isolated gold particles are also detected extracellularly. They were not associat-*

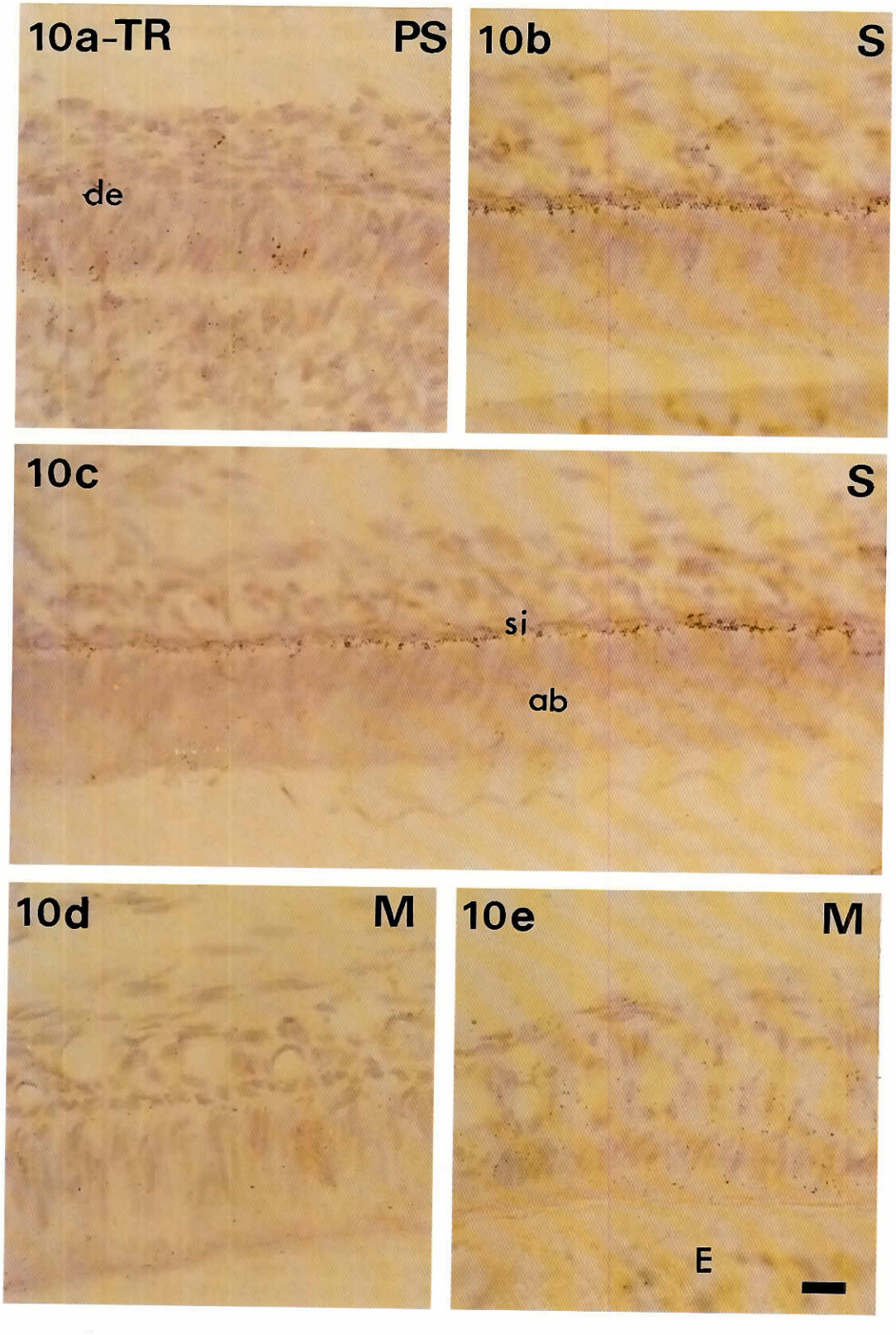


Fig 10. Expression of secreted truncated EGFr form mRNA in a 56 day-old rat incisor throughout the enamel presecretion, secretion and maturation stages. *In situ hybridization with ^{35}S-labeled TR oligonucleotide (detecting secreted truncated EGFr form) at different levels of the long axis of the incisor. TR labeling is absent in dental epithelium (de) of the presecretion (PS) zone* **(a)**. *Strong expression is seen in stratum intermedium cells (si) of the secretion (S) zone while ameloblasts remain negative* **(b,c)**. *Weak or absent labeling is observed in the transition and maturation (M) zones* **(d,e)**. *enamel (E). Bar, 17 μm.*

cells, as previously reported for early stages of tooth development (Partanen and Thesleff, 1987; Cam *et al.*, 1990; Heikinheimo *et al.*, 1993; Shum *et al.*, 1993). In particular, our findings extend this concept to the late stages of enamel mineralization. The effects of EGF have been demonstrated to depend on the origin of dental cells, and epithelial cells appear to be the main cells responsive to EGF (Partanen *et al.*, 1985; Shum *et al.*, 1993). Our results confirmed this epithelial predominance of EGFr expression during tooth formation. In dental mesenchyme, EGFr was detected in preodontoblasts, while post-mitotic odontoblasts appeared to express very low levels of EGFr. A similar pattern of EGFr expression was observed in bone during osteoblast differentiation (Martineau-Doizé *et al.*, 1988; Davideau *et al.*, 1995). In dental mesenchyme, EGFr is therefore principally associated with growing cells, the classical target cells of EGF (Carpenter and Wahl, 1991). The developmental pattern of EGFr expression shows different characteristics in dental epithelium.

We report the existence of two peaks of expression of the full-length EGFr form during the life-cycle of the ameloblast; the first peak was associated with presecretion and the second peak was associated with the enamel maturation stage. These data suggest that EGFr gene expression may include two successive activations during normal development of the same cell (ameloblast). A similar pattern of EGFr gene activation has not been described during the development of other tissues and cells (Lee and Han, 1991).

The first strong expression of mRNAs of full-length EGFr form was located in the presecretion zone of dental epithelium which contains proliferative and differentiating cells (Smith and Warshawsky, 1975; Topham *et al.*, 1987; Nso *et al.*, 1992). EGF increases the proliferation of dental epithelium (Partanen *et al.*, 1985; Topham *et al.*, 1987; Hu *et al.*, 1992). These data support the hypothesis that EGF controls dental epithelial cell proliferation via increased expression of its receptor, as EGF appears to be essential for the initial steps of dental morphogenesis when cell proliferation is determinant (Kronmiller *et al.*, 1991; Shum *et al.*, 1993).

In differentiated ameloblasts, the level of expression of the full-length EGFr form appears to be related to the functional stage of ameloblasts. The expression of the full-length EGFr form decreased at the end of the presecretion stage and remained low throughout the secretion stage. It can be speculated that a decrease in the full-length EGFr form in presecretion stage ameloblasts may allow phenotypic expression of ameloblasts, as the synthesis of amelogenins, which are major proteins of enamel matrix synthesized by differentiated ameloblasts (DenBesten and Li, 1992; Nanci and Smith, 1992), is down-regulated by EGF (Hata *et al.*, 1990). A similar down-regulation by EGF of phenotypic expression, such as type I collagen, has been shown in osteoblasts (Hata *et al.*, 1984). These down-regulations correspond to the inhibitory effects exerted on the differentiation process by EGF observed *in vitro* in dental (Partanen *et al.*, 1985; Hu *et al.*, 1992; Shum *et al.*, 1993) and bone cells (Aubin *et al.*, 1992).

The full-length EGFr form was again strongly expressed in the dental epithelium during the maturation stage, as previously suggested by EGF binding experiments (Martineau-Doizé *et al.*, 1991). The role of the full-length EGFr form during enamel maturation in ameloblasts, which are terminally differentiated cells, can only be speculated. EGF is involved in the control of electrolyte transport, such as transport of sodium ions in the kidney and jejunum (Opleta-Madsen *et al.*, 1991; Warden and Stokes, 1993) and could increase intracellular free ionic calcium via phospholipase C activation (Margolis *et al.*, 1989). Enamel maturation stage ameloblasts regulate the transport of calcium toward maturing enamel (Bawden, 1989). EGF-family growth factors might be involved in the regulation of calcium handling in ameloblasts.

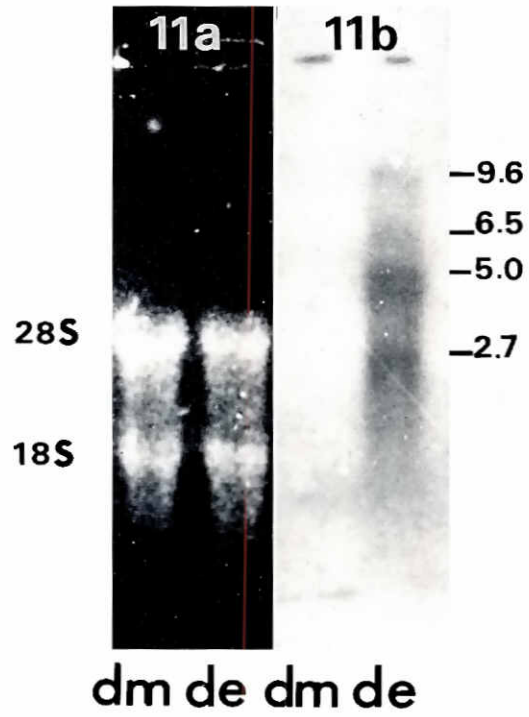


Fig. 11. Northern blotting analysis of a 56 day-old rat enamel organ and dental mesenchyme RNAs. *Total RNAs were separated by agarose-formaldehyde gel electrophoresis, transferred to a nylon membrane, and hybridized with ^{32}P-labeled FU/TR cDNA. Gel stained with ethidium bromide* **(a)** *shows a similar quantity of RNA in dental mesenchyme (dm) and dental epithelium (de). In dental epithelium, the 5.0, 6.5, 9.6 kb transcripts of the full-length EGFr form are observed. The 2.7 kb transcripts of the secreted truncated EGFr form are also detected, with a similar intensity to that of the 5.0 kb full-length EGFr form transcripts. No EGFr transcripts are detected in the dental mesenchyme* **(b)**.

Evidence for cell- and stage-specificity in the alternative splicing of EGF receptor mRNAs and for the presence of secreted truncated EGF receptor protein

The regulation of EGFr gene expression appears to be complex in the epithelial supra-ameloblastic cells. As in the ameloblasts, full-length EGFr mRNAs were strongly expressed only during the presecretion and enamel maturation stages. However, an additional strong expression of the secreted truncated EGFr form mRNA was observed in epithelial supra-ameloblastic stratum intermedium cells and was restricted to the secretion stage. Furthermore, this EGFr form appeared to be specifically expressed in root epithelium during root epithelium

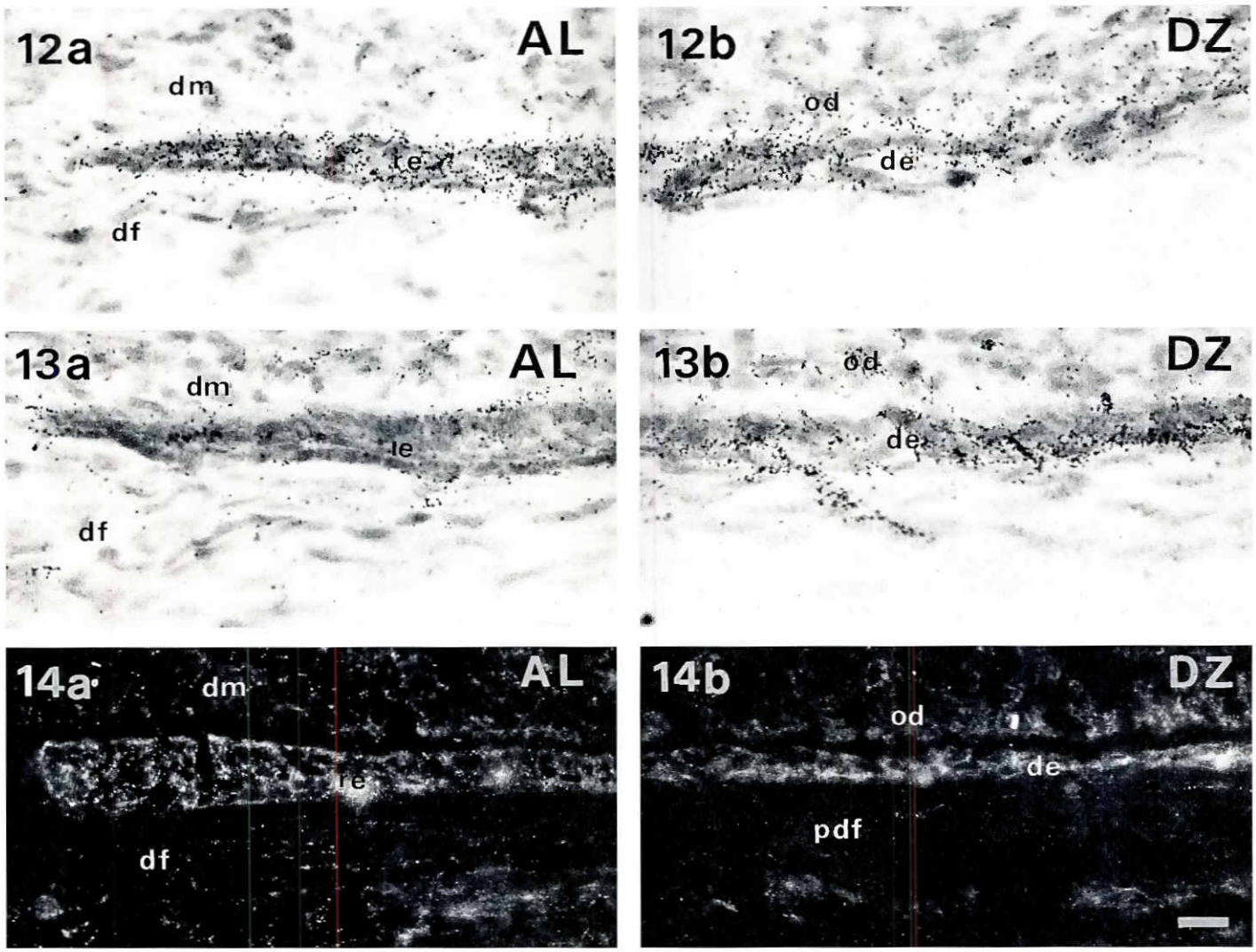


Fig. 12. Expression of full-length EGFr form mRNAs in root epithelium zone of a 56 day-old rat incisor. *In situ* hybridization with ^{35}S-labeled FU/TR antisense probe. (a) *Apical loop (AL) of root epithelium (re) is strongly labeled. No significant signal is observed in dental mesenchyme (dm) and dental follicle (df).* **(b)** *The signal decreases in dislocating epithelial cells (de) of the root epithelium disruption zone (DZ). Some silver grains are also present in differentiating odontoblasts (od).*

Fig. 13. Expression of secreted truncated EGFr form mRNAs in root epithelium zone of a 56 day-old rat incisor. *In situ* hybridization with ^{35}S-labeled TR probe. (a) *Apical loop of root epithelium does not show any significant labeling.* **(b)** *A strong signal is detected in dislocating epithelial cells (de). Dental mesenchyme and follicle are not labeled.*

Fig. 14. Immunofluorescence detection of EGFr with FU/TR antibodies in root epithelium zone of a 7 day-old rat incisor. (a) *Strong immunostaining is observed in the apical loop of root epithelium (re).* **(b)** *Dislocating epithelial cells (de) of the disruption zone of root epithelium are immunostained. Weaker staining is also observed in differentiating odontoblasts (od) and periodontal ligament fibroblasts (pdf). Bar, 15 μm.*

disruption. The secreted truncated form of EGFr is produced by alternative splicing of EGFr gene transcripts (Petch *et al.*, 1990). The corresponding protein has been shown to be expressed by transformed and normal cells (Weber *et al.*, 1984; Petch *et al.*, 1990; Ilekis *et al.*, 1995). Previous Northern-blotting data have demonstrated that the expression of the two EGFr forms is not synchronous in rat embryos between 10 and 14 days of gestation (Petch *et al.*, 1990) and is restricted to epithelium in the uterus (Das *et al.*, 1994). Our data indicate that only one epithelial cell type is able to express the secreted truncated EGFr form. Moreover, we show for the first time that alternative splicing of EGFr (in stratum intermedium and root epithelium cells) appears to be developmentally controlled. Developmental regulation of alternative splicing leading to a balanced expression of active/inactive receptor forms has been observed for other receptors, such as platelet-derived growth factor receptor (Vu *et al.*, 1989), luteinizing hormone receptor (Tena-Sempere *et al.*, 1994), and neurotrophin receptors (Okazawa *et al.*, 1993). The varied expression of truncated receptor proteins could control the responsiveness to growth factor during cell differentiation, as proposed for the expression of various forms of platelet-derived growth factor receptor (Vu *et al.*, 1989).

Our results suggest the existence of a paracrine system between ameloblasts and stratum intermedium cells involving EGFr ligands and the secreted truncated EGFr form. This form is able to bind EGF (Weber *et al.*, 1984), and may control full-length EGFr form activation, as shown during chimeric expression of truncated EGFr form (Wu and Adamson, 1993). The autocrine/paracrine system of EGF family growth factors may therefore involve a complex pattern not only at the level of ligands (EGF, TGFalpha, and presumably matrix molecules such as amphiregulin) as previously proposed (Snead *et al.*, 1989; Hu *et al.*, 1992; Heikinheimo *et al.*, 1993; Thesleff *et al.*, 1995) but also at the level of EGFr via the developmentally regulated expression of full-length and secreted truncated forms.

Materials and Methods

Animals

Fifty-six-day-old (n= 90) male Sprague-Dawley rats (CERJ, le Genest-Saint-Isle, France) were used in this study. Seven-day-old (n=10) Sprague-Dawley rats were also used to study teeth and their surrounding tissues in the mandible.

Antibodies and probes

FU/TR antibodies and probes detecting the full-length and secreted truncated EGFr forms

This group included antibodies raised against a synthetic dodecapeptide of the extracellular domain of human EGF receptor (Cambridge Research Biochemicals, Norwich, UK) and riboprobes (antisense and sense) transcribed on cDNA fragments subcloned into Bluescript plasmids (gift of Dr. H.S. Earp, Chapel Hill, NC, USA). The 2.2 Kbp EcoR-I fragment from clone ER1 for rat EGFr mRNA recognizes a sequence encoding the extracellular and transmembrane part of EGFr and also recognizes the mRNA of EGFr secreted truncated form (Petch *et al.*, 1990).

FU antibodies and probes detecting only the full-length EGFr form

This group included antibodies raised against the intracellular domain of human EGFr (Upstate Biotechnology Inc., Lake Placid, NY, USA) and riboprobes (antisense and sense) transcribed on cDNA fragments subcloned into Bluescript plasmids (gift of Dr H.S. Earp, Chapel Hill, NC, USA). The 700 bp Sac-I fragment from clone ER3 for rat EGFr mRNA only recognizes the sequence of the intracellular part of EGFr (Petch *et al.*, 1990).

TR probe detecting only the secreted truncated EGFr form

This probe is a 50-base oligonucleotide which recognizes the 3'-end untranslated specific sequence of the secreted truncated EGFr form mRNA (Petch *et al.*, 1990).

Northern-blotting

Fifty-six-day-old rats were anesthetized with ether and decapitated. Mandibles were rapidly removed. Tissues were microdissected as previously described in order to separate dental epithelium and mesenchyme (Berdal *et al.*, 1993). Total RNA isolation was performed on 100-200 mg of fresh tissues (Sambrook, 1989). Total RNAs were electrophoretically fractionated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane, Hybond N+ (Amersham, les Ulis, France). Fluorescent staining with ethidium bromide was performed to check the quality of migration and transfer, and to evaluate RNA quantity of each lane by visualizing 28S and 18S RNAs. Membranes were prehybridized, and then hybridized with EGFr cDNA probe FU/TR labeled by random priming (labeling kits were purchased from Boehringer Mannheim, Meylan, France).

Light microscopic immunocytochemistry

Rats were anesthetized with ether and decapitated. Mandibles were removed and the incisors of 56 day-old rats were dissected out, and frozen at -25°C. Mandibles of 7 day-old rats and dissected incisors were then cut into 10 µm sections with a cryostat (MGW Lauda, Leitz) at -25°C, and sections were deposited on 50 mg/ml poly-L-lysine coated slides (Sigma, La Verpillière, France). FU/TR and FU primary polyclonal antibodies were used. All incubations and rinses were performed in 0.1 M phosphate buffered saline pH 7.4 (PBS) at room temperature. Sections were incubated for 30 min in 1/30 non-immune goat serum (Nordic, Tilburg, The Netherlands), for 1 h in 1/100 primary antibodies, and rinsed. After incubation for 1 h in 1/100 biotinylated secondary antibodies (Amersham), sections were rinsed and incubated for 30 min in 1/200 streptavidin FITC (Amersham) containing 2% bovine serum albumin (Sigma). They were finally rinsed for 3 h under constant agitation, mounted in hydrophilic medium for fluorescence (Biosys, Compiègne, France), viewed and photographed with a Leitz Orthoplan photomicroscope. On control sections, primary antibodies were omitted or replaced with non-immune serum (Sigma).

Electron-microscopic immunolocalization

Rats were perfused with 3% PFA plus 0.2% glutaraldehyde in 0.1 M cacodylate buffer containing 0.5% $CaCl_2$, pH 7.4. Mandibles were then dissected out and fixed by immersion in the same fixative for 1 h at 4°C, and rinsed in 0.1 M cacodylate buffer containing 0.5% $CaCl_2$ overnight at 4°C. Incisors and molars were dissected out and processed for embedding in Lowicryl K4M (CWL, Waldkreiburg, Germany) as described by Nanci *et al.* (1989). Ultrathin sections (100 nm) were cut on an LKB Ultrotome III ultramicrotome and mounted on 300-mesh nickel grids. Grids were floated on 0.5 M NH_4Cl, pH 7.4 for 10 min at room temperature. All incubations and rinses were performed in PBS. The grids were first rinsed and incubated for 12 h in 1/10 non-immune goat serum (Nordic) at 4°C, and were then incubated for 90 min in 1/100 FU/TR antibodies at 37°C, and rinsed. The grids were then incubated for 1 h in 1/100 biotinylated secondary antirabbit antibodies (Amersham), rinsed, and incubated for 30 min in 1/20 streptavidin conjugated with 15-nm gold particles (Amersham) at room temperature. The grids were rinsed in PBS and distilled water, counterstained in aqueous uranyl acetate and lead citrate, and examined with a Jeol 100B electron microscope. Controls were prepared by omitting the primary antibodies or by replacing them with the same dilution of rabbit IgGs (Nordic).

In situ hybridization

Whole mandibles of 7 day-old rats were decalcified and embedded in paraffin, while incisors of 56 day-old rats were microdissected and cut undecalcified in a cryostat (Hotton *et al.*, 1995). [^{33}P]-UTP-labeled probes were used to provide a more rapid detection of the *in situ* hybridization signal than [^{35}S]-UTP-labeled probes. Similar results were observed with these different techniques.

Rats were perfused with 4% PFA, 15% sucrose in PBS, for 15 min. Mandibles were then dissected out and fixed by immersion in the same fixative for 1 h at 4°C, and rinsed in 15% sucrose, PBS overnight at 4°C. Incisors of 56 day-old rats were then dissected out. Dissected incisors were cut into 10 µm sections with a cryostat (MGW Lauda, Leitz), and sections were deposited on 50 mg/ml poly-L-lysine (Sigma) coated slides. For paraffin sections, rats were decapitated, mandibles dissected out and directly fixed by immersion in 4% PFA in PBS for 12 h and rinsed in PBS for 24 h at 4°C. Mandibles of 7 day-old rats were decalcified in 4.13% EDTA, 0.1% PFA, in PBS for three weeks, rinsed in PBS, dehydrated by graded series of ethanol and embedded in paraffin. 8 µm sections were deposited on silanized slides. *In situ* hybridization was carried out with FU/TR and FU riboprobes (antisense and sense). FU/TR plasmid was linearized with either BamH-I or Hind-III restriction endonuclease (Boehringer Mannheim), and FU plasmid was linearized with either EcoR-V or BssH II endonuclease (Boehringer Mannheim). [^{35}S]-UTP-

labeled or [^{33}P]-UTP-labeled single-stranded antisense and sense probes were synthesized *in vitro*, using either T3 or T7 polymerase (Promega, Lyon, France), respectively. Oligonucleotides were labeled by tailing (Boehringer Mannheim) with [^{35}S]-dATP (Amersham). *In situ* hybridization was performed as described by Sahlberg *et al.* (1992). Briefly, cryostat sections and deparaffinized sections were pretreated with proteinase K (Sigma), hybridized with 20 µl of labeled probes containing 60000 cpm/µl radioactivity, in a humid chamber overnight at 50°C, and washed under high-stringency conditions. The slides were dipped into NTB2 autoradiographic emulsion (Kodak) and exposed for 10 days at 4°C. After developing the film, sections were stained with hematoxylin, dehydrated and mounted under coverslips. Sections were observed and photographed with a Leitz Orthoplan photomicroscope using bright and dark field illumination.

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