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Expression of epidermal growth factor receptors by odontogenic jaw cysts

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Abstract. The expression of epidermal growth factor receptor (EGFr) by odontogenic epithelium was studied in odontogenic cysts ($n=35$), ameloblastoma ($n=6$), and periapical granulomas containing proliferating epithelial rests of Malassez ($n=7$) using a panel of monoclonal antibodies to EGFr (clone E30, F4 and C11) known to react with formalin-fixed, paraffin-embedded sections. Odontogenic epithelium in all specimens demonstrated immunoreactivity with all three antibodies. Clone E30 consistently gave the most intense, membrane located staining pattern of the three antibodies tested. Generally, staining of epithelial cells progressively diminished with movement away from the basal cell layers toward the most superficial layers of cystic lining or centre of epithelial rests and tumour islands. Developmental odontogenic cysts (odontogenic keratocysts, $n=13$; dentigerous cysts, $n=11$) and ameloblastoma (follicular type, $n=5$; unicystic type, $n=1$) expressed a higher level of EGFr staining than inflammatory cysts (radicular cysts, $n=11$) and the proliferating epithelial rests in periapical granulomas. However, foci of weak EGFr staining of odontogenic keratocyst lining, similar to that seen in radicular cysts, were found in areas associated with inflammation. In addition, epithelial rests not associated with inflammatory cell infiltrates exhibited stronger reactivity for EGFr than proliferating rests within periapical granulomas. These results indicate that the level of EGFr expression by odontogenic cysts and rests is related to the presence of inflammation within adjacent connective tissue and that there is no detectable difference in receptor expression between developmental cysts and ameloblastoma.

Key words: Epidermal growth factor receptor – Odontogenic cysts – Ameloblastoma – Immunocytochemistry

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Introduction

Epidermal growth factor (EGF) is a hormone-like, 53-amino acid polypeptide originally isolated from mouse submandibular glands and found to accelerate incisor eruption and eyelid opening (Cohen 1962). It is a potent mitogen which stimulates proliferation of numerous cell types in vitro and of epithelial cells in vivo (Cohen 1962; Carpenter 1987). EGF interacts with the target cells by binding to its receptor (EGFr) which is a 170 kDa transmembrane protein that can be divided into three zones, i.e. extracellular, transmembrane and intracellular zones, on the basis of its structure and function (Cohen et al. 1982). The binding of EGF to the extracellular part of the receptor, leading to activation of a tyrosine kinase at its intracellular part, is considered to be the first step in a chain of reactions that culminate in mitosis (Carpenter 1985, 1987). EGFr is present on epithelial cells at sites of rapid epithelial proliferation including epidermis, gastric and bronchial epithelia, and hepatocytes (Green et al. 1983; Gusterson et al. 1984), as well as on some epithelial cells that do not have proliferative capacity, such as pancreas, prostate and testis (Damjanov et al. 1986). The highest receptor densities are expressed by undifferentiated, proliferating epithelial cells, such as basal cells of epidermis and oral epithelium, and proliferating cells of the adnexal structures (Green et al. 1983; Cho et al. 1988). Interestingly, the autoradiographic localisation of EGFr in mouse embryonic tooth germs has indicated that the distribution of EGFr is related to its developmental stage, suggesting that EGF and its receptors might provide epigenetic control of odontogenesis by modulating cell growth and cell interaction (Partanen and Thesleff 1987, 1989).

There is a general belief that different types of odontogenic cysts arise from odontogenic epithelial remnants formed at different stages of normal tooth development. The potential for further proliferation and differentiation of these epithelial remnants during formation of a cyst is different and thus leads to variations in their epithelial expression and biological behaviour (Browne

and Smith 1991). Proliferation of the epithelial rests of Malassez and formation of radicular cysts is thought to be initiated by mediators (eg. IL-1) released by the periapical inflammatory and immune responses consequent upon pulpal necrosis of the associated tooth (Browne and Smith 1991). By contrast, little is known about the initiation and control of cellular proliferation and differentiation of developmental cysts, namely odontogenic keratocysts and dentigerous cysts. The mechanisms underlying their pathogenesis remain highly speculative.

The present study was undertaken to characterise the pattern of EGFr expression in the epithelial linings of the three major types of odontogenic cysts and the proliferating epithelial rests within periapical granulomas, using a panel of commercially available monoclonal antibodies to EGFr on routinely fixed and processed paraffin sections.

Materials and methods

Cases of odontogenic keratocyst ($n=13$), dentigerous cyst ($n=11$), radicular cyst ($n=11$), ameloblastoma ($n=6$; 5 cases of follicular type and 1 case of unicystic ameloblastoma) and periapical granuloma ($n=7$) containing proliferating epithelial rests were selected from the files of the Oral Pathology Unit, School of Dentistry, University of Birmingham. Except for ameloblastomas, only undecalcified specimens or specimens that had been decalcified in 10% formic acid for less than 6 h were chosen to minimise possible inconsistencies in immunoreactivity due to such processing variables. Ameloblastomas had been decalcified for 24–48 h prior to processing. All had been fixed in 10% neutral buffered formalin (18–24 h) and routinely processed and embedded in paraffin using a Shandon Hypercentre. To act as positive control tissue, 10 specimens of normal oral mucosa overlying the third molar region were collected fresh at operation. Specimens were divided into two; half was snap frozen and stored in liquid nitrogen and the remainder routinely fixed and processed to paraffin.

A sensitive biotin-streptavidin immunoperoxidase technique was used on serial 5 μm paraffin sections. Sections were deparaffinized and rehydrated through xylene and graded alcohols and treated with 0.1% trypsin prior to immunostaining. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in buffer (10 min), diluted monoclonal antibodies were applied to the sections for overnight at 4 °C (Table 1). After washing, sections were then overlaid with biotinylated anti-mouse immunoglobulin (1 h; BioGenex, 1/100 dilution in buffer containing 10% normal human serum). Unbound conjugate was removed by washing and the sections were overlaid with peroxidase-labelled streptavidin (1 h; BioGenex, 1/100 dilution in buffer). Excess peroxidase-labelled streptavidin was removed by washing and bound peroxidase visualised using diaminobenzidine reagent. After washing in water, the reaction product was darkened by treatment with 0.5% copper sulphate (W/V in saline) for 5 min. Stained sections were lightly counterstained in Mayer's haematoxylin and mounted in Xam. All stages of the method were performed at room temperature and reagent dilutions and washings were performed with 0.01 M phosphate-buffered saline, pH 7.6. Negative staining controls consisted of omission of the primary antibody and replacement of the primary layer with normal mouse IgG (5 and 10 μg/ml) or normal mouse serum (1/100). Acetone-fixed frozen sections of control oral mucosa (5 μm) were stained as described previously (Matthews et al. 1988) using the monoclonal anti-EGFr antibodies at the same dilutions as for paraffin sections. Essential methodological differences were that monoclonal antibodies were applied to dry sections, and neither trypsin nor blocking of endogenous peroxidase activity were performed.

Overall EGFr staining intensity within the epithelial linings of odontogenic cysts was assessed subjectively using a semi-quantitative scale: positive (+++), medium (++), weak (+) and negative (−). In addition, the distribution of the staining within the cyst linings was scored to indicate which epithelial layer(s) exhibited the strongest reaction: basal, suprabasal, basal and suprabasal, and uniform staining of all layers. All scoring of staining was performed in a blind fashion without prior knowledge of section treatment.

Results

Prior to investigating EGFr staining of the paraffin specimens of odontogenic cysts the four monoclonal antibodies were tested on fresh frozen and paraffin embedded specimens of normal oral mucosa (Table 1). All antibodies showed staining of oral epithelium in frozen sec-

Table 1. Details of antibodies to EGFr, use of enzyme pre-treatment and reactivity on frozen and paraffin embedded sections

Antibody clone	Source	Specificity[a]	Dilution[b] (μg/ml)	Trypsin[c] pretreatment (min)	Epithelial staining pattern[d]	
					Frozen	Paraffin
E30	Bio Diagnostics	Extracellular domain	1/60 (0.4)	30	M+++ C−	M+++ C−
C11	Serotec	Extracellular domain	1/600 (2)	30	M++ C++	M+ C++
EGFR1	Amersham	Extracellular domain	1/10 (10)	0, 5, 10, 15, 20, 30, 45	M+++ C−	M− C−
F4	Sigma	Intracellular domain	1/200 (4)	10	M+ C++	M+ C++

[a] Antibody directed toward the peptide portion of EGFr and raised to denatured EGFr (E30), a synthetic peptide (C11, F4) or intact A-431 cells (EGFR1)
[b] Optimum dilution determined by titration studies on normal oral mucosa
[c] Paraffin sections only; optimum times determined by experiment
[d] Staining pattern on normal oral epithelium − M, membranous & C, cytoplasmic patterns of staining; +++, strong, ++, medium & +, weak staining; −, no staining

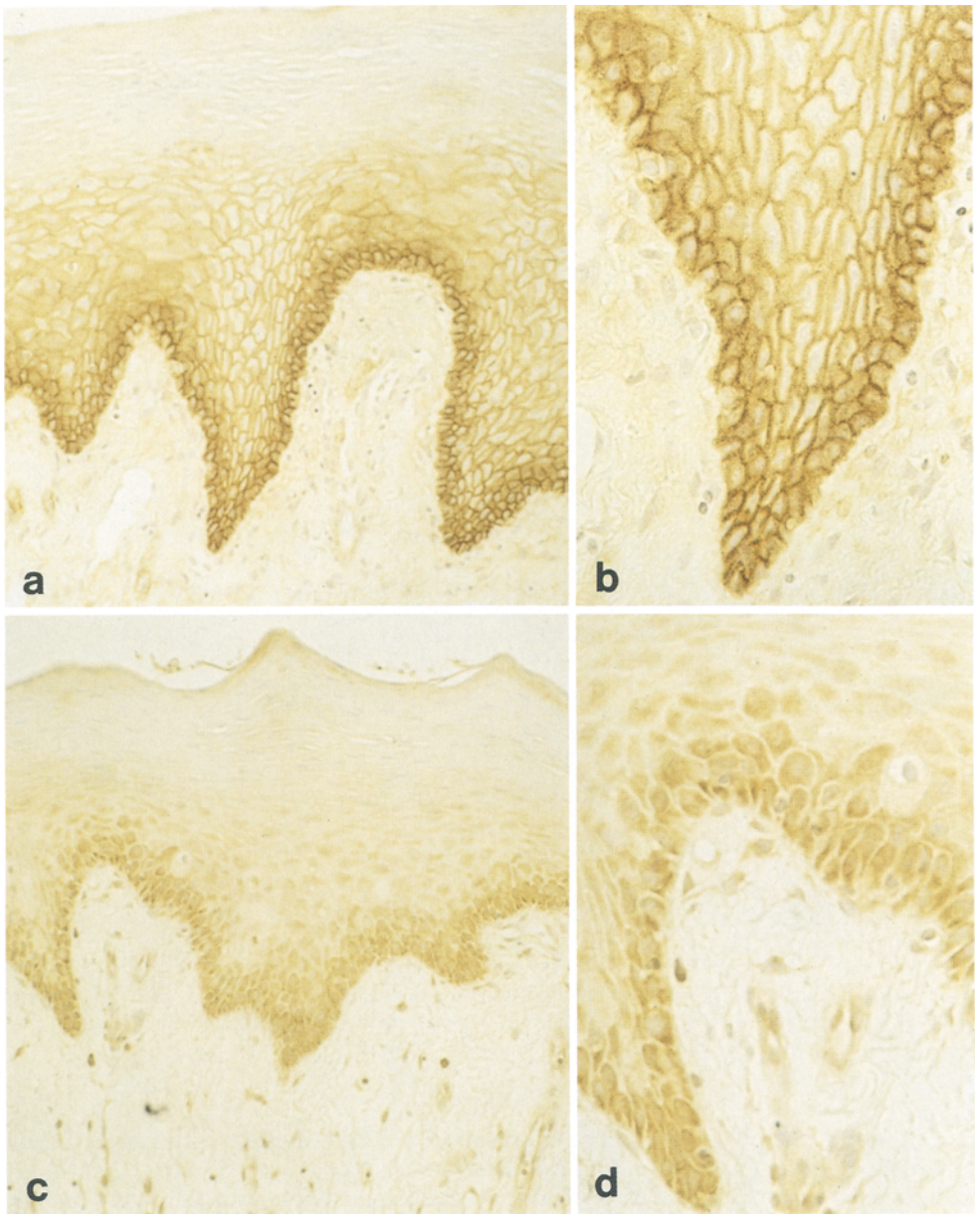

Fig. 1. Formalin-fixed, paraffin-embedded sections of normal oral mucosa stained for EGFr using monoclonal antibody E30 (**a**, ×480; **b**, ×1130) and F4 (**c**, ×480; **d**, ×1130) showing the different localisation patterns obtained with these antibodies

tions. E30 and EGFR1, directed towards the extracellular domain of EGFr, gave intense, exclusively membranous staining of epithelial cells which progressively diminished from the basal toward the most superficial layers. Although the same pattern of reactivity was obtained in paraffin sections with E30 (Fig. 1), EGFR1 was negative. C11 gave a similar predominant membrane staining pattern on frozen sections to that obtained with E30 and EGFR1 consistent with the recognition of the extracellular domain of EGFr. However, reactivity in paraffin sections was patchy and characterised by weak membrane staining together with a stronger cytoplasmic reaction. Clone F4, directed to the intracellular domain of EGFr, gave a mixed, patchy staining pattern with weak membrane plus stronger cytoplasmic reactivity on both frozen and paraffin sections (Fig. 1). As with E30 and EGFR1 the staining reaction with both these antibodies was strongest in basal cells and less in the more superficial layers. All antibodies stained vascular endothelium to a variable degree and, in paraffin sections, both C11 and F4 stained fibroblasts, smooth and striated muscle and, in some specimens, erythrocytes. Paraffin and frozen control sections incubated with purified normal mouse IgG or dilutions of normal mouse serum substituted for the primary monoclonal antibody were consistently negative.

The immunocytochemical staining results for odontogenic cyst linings and proliferating epithelial rests within periapical granulomas are summarised in Tables 2 and 3. The epithelium in all specimens was positive with all three monoclonal antibodies known to be reactive in paraffin sections (E30, C11, F4). The most consistent and intense epithelial cell staining was obtained with E30. Although all cyst linings and proliferating rests

Table 2. Immunoreactivity of odontogenic epithelium with the three monoclonal antibodies to EGFr

Tissue	No.	Staining intensity[a] of epithelium using EGFr antibodies:											
		clone E30				clone C11				clone F4			
		+++	++	+	−	+++	++	+	−	+++	++	+	−
Odontogenic keratocyst	13	85	15	0	0	31	61	8	0	15	77	8	0
Dentigerous cyst	11	73	27	0	0	18	55	27	0	9	55	34	0
Radicular cyst	11	55	45	0	0	9	64	27	0	9	34	55	0
Epithelial rests	7	14	57	29	0	0	57	43	0	0	43	57	0

[a] Percentage of specimens showing the intensity of positive reaction; +++, strong, ++, medium, +, weak staining; −, no staining

Table 3. Staining patterns of odontogenic epithelium with the three monoclonal antibodies to EGFr

Tissue	No.	Staining pattern[a] of epithelium using EGFr antibodies:											
		clone E30				clone C11				clone F4			
		B	B+S	S	U	B	B+S	S	U	B	B+S	S	U
Odontogenic keratocyst	13	69	31	0	0	0	46	54	0	0	69	31	0
Dentigerous cyst	11	100	0	0	0	64	0	0	36	18	0	0	82
Radicular cyst	11	100	0	0	0	100	0	0	0	64	0	0	36
Epithelial rests	7	100	0	0	0	86	0	0	14	86	0	0	14

[a] Percentage of specimens showing predominant staining of: B, basal cells, B+S, basal plus suprabasal cells, S, suprabasal cells. U, uniform staining of all epithelial layers

reacted positively with anti-EGFr antibodies, there was a general trend of decreased staining intensity for a given antibody in the following order: odontogenic keratocyst, dentigerous cyst, radicular cyst and proliferating epithelial cell rests (Table 2). Thus, odontogenic keratocyst and dentigerous cyst linings generally appeared to express higher levels of EGFr staining than those of radicular cyst linings and proliferating rests (Fig. 2). The lower EGFr staining intensity seen in many of the radicular cyst linings and proliferating rests often coincided with the presence of degenerative changes within the epithelium associated with local inflammatory cell infiltrates. This association of reduced EGFr expression and inflammation was also detectable within odontogenic keratocyst walls. Thus, in contrast to the strong staining of the characteristic, regular odontogenic keratocyst lining, focal areas of weak EGFr staining were found in areas of disordered epithelium associated with foci of inflammation (Fig. 3). Furthermore, epithelial rests sometimes present in the capsules of all three types of cyst and not associated with inflammatory cell infiltrates exhibited a far stronger reactivity for EGFr than proliferating rests within periapical granulomas.

The distribution of EGFr staining within the epithelial linings of odontogenic cysts varied with the antibody used (Table 3). E30 reactivity was characterised by strong epithelial cell membrane staining of basal and suprabasal cells, which, except in 4 specimens of odontogenic keratocyst, was most intense on basal cells (Figs. 2a, 4a). Patterns of F4 and C11 staining were more variable between cyst types. Odontogenic keratocyst linings showed either an even staining of the basal and suprabasal cells or, in some cases, suprabasal staining alone (Fig. 4b). By contrast, epithelial linings of the other cyst types and proliferating epithelial rests showed either basal predominant or uniform (Fig. 4c) patterns of reactivity.

All six ameloblastoma specimens gave strong cell membrane reactivity for EGFr with E30, but more variable patchy membrane/cytoplasmic staining with C11 and F4. Follicular ameloblastomas showed intense staining of the basal cells which diminished towards the centres of the tumour islands (Fig. 5a). Reactivity of the lining of the unicystic ameloblastoma was similar to that exhibited by the odontogenic cyst linings (Fig. 5b).

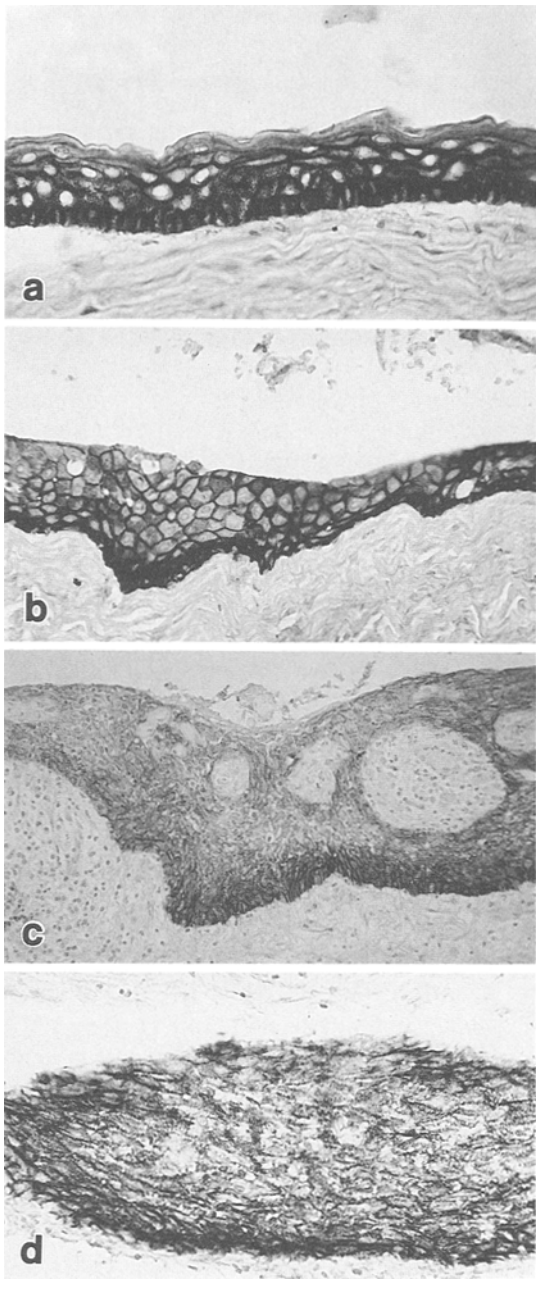

Fig. 2. Odontogenic keratocyst (**a**, ×800), dentigerous cyst (**b**, ×800), radicular cyst (**c**, ×400) and proliferating epithelial rests of Malassez (**d**, ×800) stained for EGFr using monoclonal antibody E30

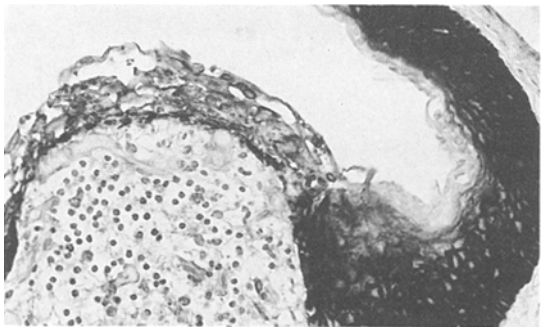

Fig. 3. Reduced expression of EGFr by odontogenic keratocyst epithelium associated with local inflammation in the connective tissue wall (E30, ×800)

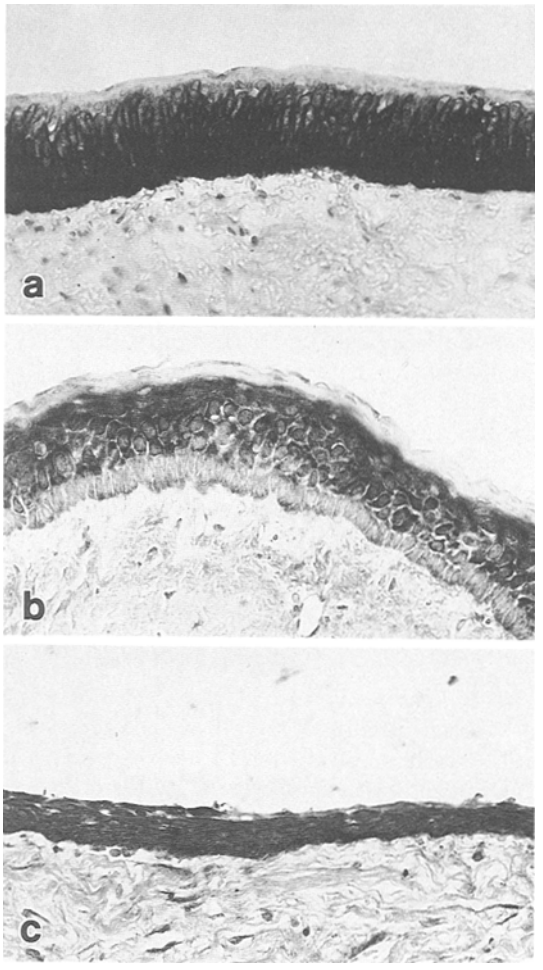

Fig. 4. Odontogenic keratocyst epithelium stained with monoclonal antibodies E30 (**a**, ×800) showing basal and suprabasal predominant reactivity and C11 (**b**, ×800) showing suprabasal predominant reactivity. Dentigerous cyst lining exhibiting a uniform pattern of staining with monoclonal antibody F4 (**c**, ×800) is shown for comparison

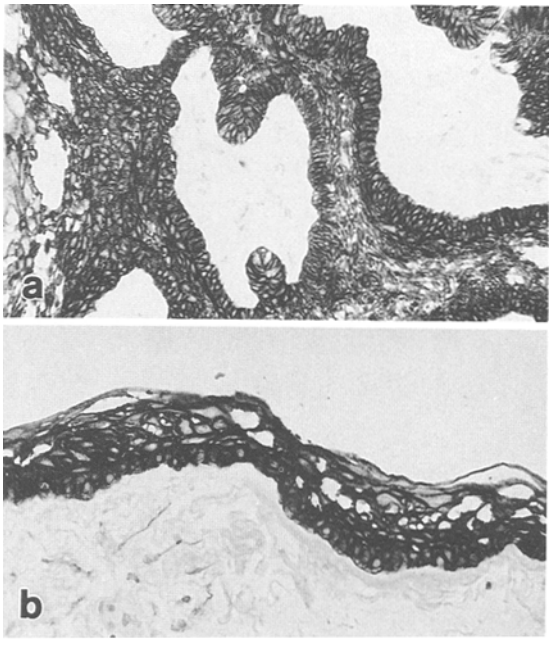

Fig. 5. Reactivity of a follicular ameloblastoma (**a**, ×400) and the epithelial lining of the unicystic ameloblastoma (**b**, ×640) for EGFr using monoclonal antibody E30

Discussion

The potential importance of the effects of EGF and therefore, the distribution and expression of its receptor, in normal developmental and pathological processes, particularly cancer, has stimulated many studies on the immunocytochemical localisation and differential expression of EGFr (Hedler and Ozanne 1984; Libermann et al. 1984; Gusterson et al. 1985; Neal et al. 1985). While the original studies were performed using polyclonal antisera reactive with both frozen and paraffin tissues, most recent work has involved the use of monoclonal antibodies having variable ability to detect EGFr in paraffin sections. Although the extracellular EGFr epitope to which the first monoclonal EGFr antibody was directed is destroyed by routine fixation and paraffin processing (EGFR1), several antibodies (F4, 29.1, C11, E30) have now been developed against stable epitopes able to withstand the rigours of paraffin embedding (Parker et al. 1984; Cerny et al. 1986; Gullick et al. 1986; Berger et al. 1987; Berchuck et al. 1989; Dazzi et al. 1989; C11: Serotec, Oxford, UK; E30: Bio Diagnostics, Upton upon Severn, UK). Unfortunately, some of these antibodies have been found to cross-react with blood group antigens as they are directed to the carbohydrate portion of the extracellular domain of EGFr (eg. clone 29.1; Berchuck et al. 1989). Indeed, even clone C11, raised against a synthetic peptide homologous to part of the extracellular domain of EGFr, appears to cross-react with an epitope on a non-EGFr molecule present on some erythrocytes (Serotec).

Because of these problems we compared the immunoreactivity of four commercially available monoclonal antibodies directed toward the protein portion of EGFr on frozen and paraffin sections of control oral mucosa prior to performing our retrospective study of paraffin-embedded specimens of odontogenic cysts. Our results confirmed the variable reactivity of the antibodies with routinely processed material and only one (clone E30) gave similar staining reactions on both frozen and paraffin sections. The two other antibodies reactive in paraffin sections (C11 and F4) consistently gave high 'background' reactions and weaker specific staining compared to frozen sections. These observations on F4 agree with several previously published reports (Parker et al. 1984; Cerny et al. 1986; Gullick et al. 1986; Berger et al. 1987), although the previous authors still maintained the usefulness of this antibody on paraffin sections. In our opinion the weak, uniform staining of all epithelial layers often obtained was difficult to interpret and did not conform to the expected or observed (with E30) distribution of EGFr.

Our results demonstrate that the epithelial linings of all types of odontogenic cyst express EGF receptors, although the intensity and patterns of EGFr staining varied between cyst types and with the monoclonal antibody used. In particular, the linings of the so-called developmental odontogenic cysts (i.e. odontogenic keratocyst and dentigerous cyst) exhibited more intense staining for EGFr than those of inflammatory cysts (radicular cyst) and the proliferating epithelial rests of Malassez within periapical granulomas (Table 2). This inverse relationship between the presence of inflammation and the staining intensity for EGFr was also observed in the epithelial rests in the walls of radicular cysts (derived from the epithelial rests of Malassez) and odontogenic keratocysts (derived from the remnants of the dental lamina; glands of Serres). Furthermore, disordered areas of odontogenic keratocyst linings which were associated with inflammation were poorly stained in comparison with the adjacent, orderly epithelium not associated with inflammation.

The high levels of expression of EGFr in odontogenic keratocyst epithelium is consistent with the findings in previous studies which have suggested these cysts have an intrinsic growth potential not present in the other types of odontogenic cyst (Main 1970; Browne 1971; Toller 1971; Stenman et al. 1986; Matthews et al. 1988; Li et al. 1992). Similarly, the high level of EGFr expression in non-proliferating rests of Malassez reflects their known EGF-binding ability (Thesleff 1987) and growth characteristics in culture (Brunette 1984). However, the lesser EGFr expression by proliferating rests of Malassez and their derivatives (radicular cysts), contrasts with the maintenance of receptor expression in odontogenic keratocysts which are derived from rests of the dental lamina. The reason(s) for this difference is unknown but may reflect epithelial-mesenchymal interactions and growth factor/receptor modulation. Such processes play an important role during normal odontogenesis (Partanen and Thesleff 1987, 1989) although studies on EGFr expression by the enamel organ are contradictory. Studies based upon ^{125}I-EGF binding found activity restricted to the dental mesenchyme (Partanen and Thesleff 1987) whereas immunohistochemical studies indicate a temporo-spacial pattern of EGFr expression in both the enamel organ and dental mesenchyme (Cam et al. 1990). These latter studies require confirmation as they are reliant on either polyclonal antisera to EGFr (Cam et al. 1990) or paraffin material and monoclonal antibodies (Shore et al. 1992) known to cross-react with blood group antigens (clone 29.1) and/or produce relatively poor results on routinely processed tissue (clone F4).

The association between the loss of the typical epithelial structure and diminution in EGFr expression in areas of odontogenic keratocyst walls containing inflammatory cell foci and similar changes in the rests of Malassez at sites of inflammation indicates the potential importance of the connective tissue. Transplantation studies in nude mice have demonstrated that the connective tissue wall of the odontogenic keratocyst is essential for the growth and differentiation of the characteristic keratocyst epithelium (Vedtofte et al. 1982). It would be interesting to determine if EGFr expression were also dependent on the presence of the correct mesenchyme in this model. In any event the apparently higher expression of EGFr by 'developmental' versus 'inflammatory' cysts supports the view that different mechanisms of initiation and control of epithelial cell proliferation are involved in their formation.

Both in epidermis and oral epithelium EGFr are located primarily on the mitotically active basal cells and

diminish with the degree of differentiation (Green et al. 1983; Cho et al. 1988). The overall intensity of staining of odontogenic epithelia for EGFr did not show any simple relationship with cell proliferation as lesions with known high proliferation rates (odontogenic keratocysts, ameloblastoma) and significantly lower proliferation rates (normal odontogenic epithelial rests, dentigerous cysts; Matthews et al. 1988; Li et al. 1992, 1993) gave similar results. Furthermore, proliferating epithelial rests of Malassez within periapical lesions showed weaker reactivity than non-proliferating islands not associated with local inflammatory changes. However, the apparent lack of basal cell staining (with C11 and F4), or at least basal-predominant staining (with E30), within the linings of some odontogenic keratocysts may suggest some heterogeneity within this group of lesions. Whilst the effect may be processing related, it is equally possible that it reflects the unusual suprabasal position of the majority of proliferating cells within the epithelium of this type of cyst (Browne 1971; Matthews et al. 1988; Li et al. 1992, 1993). The basal cells of odontogenic keratocyst linings are characteristically palisaded and may show some features of pre-ameloblasts simulating the changes in the inner dental epithelium occurring during odontogenesis. Interestingly, there is some evidence that by the bell stage of tooth development in mice the inner dental epithelium has lost all EGFr, but they remain in the stellate reticulum and the outer dental epithelium (Cam et al. 1990).

In our study, all examples of odontogenic epithelium (epithelial rests, cysts and ameloblastoma) gave an apparently specific, positive reaction with all three monoclonal antibodies to EGFr. This result is in agreement with our preliminary studies (Li et al. 1992) but contrasts with the only other study of these lesions (Shrestha et al. 1992) which reported that 36% of odontogenic keratocysts (including primordial cyst), 53% of dentigerous cysts, 65% of radicular cysts and 100% of odontogenic tumours were negative for EGFr using monoclonal antibody clone TL5. This distribution of negative specimens broadly mirrors our finding of the proportion of medium/weak staining found within each cyst group (odontogenic keratocyst < dentigerous < radicular) and may indicate the greater sensitivity of our technique and/or differences in binding affinities for EGFr in paraffin sections of the monoclonal antibodies used. However, the finding that all odontogenic tumours lack EGFr is not supported by our data and is difficult to explain on the basis of sensitivity differences alone. In our study, ameloblastomas are reactive with all three monoclonal antibodies, the reaction with E30 (Fig. 5) being unequivocal. The disparity between results demonstrates the importance of using a panel of antibodies for immunocytochemical studies of a complex and antigenically cross-reactive molecule like EGFr.

In conclusion, our results demonstrate that the epithelium of the three major types of odontogenic cyst, proliferating epithelial rests of Malassez and the follicular and unicystic forms of ameloblastoma express EGF receptors and that the level of expression appears to be related to the presence of inflammation within the adjacent connective tissue rather than epithelial cell proliferation. Furthermore, our data do not support the suggestion, based on differential expression of EGFr (Shrestha et al. 1992), that there is a fundamental difference between odontogenic cysts and tumours in respect of their growth factor requirements.

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