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Expression of Keratinocyte Growth Factor in Periapical Lesions

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Abstract. The epithelial proliferation associated with inflammatory periapical lesions and with periapical cyst formation represents an interesting but poorly understood pathological change. Keratinocyte growth factor (KGF) is a recently identified growth factor that is produced by stromal fibroblasts and acts specifically to stimulate epithelial growth and differentiation. To investigate its possible role in the activation of the normally quiescent rests of Malassez, we examined the expression of KGF by *in situ* hybridization of sections of normal periodontal ligament (PDL) and of 12 periapical granulomas or cysts. Normal PDL and periapical granulomas with scant inflammatory infiltration showed few cells expressing message for KGF. However, KGF-expressing cells were found in the connective tissue stroma close to dense foci of inflammatory cells and to proliferating epithelial elements and cystic epithelial linings. Examination of tissues by the reverse-transcription polymerase chain reaction (RT-PCR) showed KGF expression in 4 specimens of periapical lesions but low or undetectable levels in normal PDL. These observations suggest that the induction of KGF expression in the stromal cells of periapical lesions may play an important role in stimulating the epithelial proliferation associated with cyst formation.

Key words: keratinocyte growth factor, periapical lesions, inflammatory cysts, RT/PCR, in situ hybridization.

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Introduction

The epithelial lining of periapical cysts is generally considered to be derived from proliferation of the epithelial rests of Malassez (Ten Cate, 1972; Shear, 1985), but the factors responsible for the activation of these normally quiescent rests are poorly understood. The changing patterns of expression of epithelial cytokeratins found during the early stages of cyst formation in inflammatory periapical lesions lend support to the general hypothesis of activation of the rests to a new phenotypic pattern (Gao *et al.*, 1988a). Some form of alteration in the connective tissue environment supporting the cell rests is thought to be responsible for this activation (Ten Cate, 1972). Local changes in tissue pH or carbon dioxide tension have been suggested (Grupe *et al.*, 1967), and it is apparent that epithelial proliferation is typically associated with inflammation and the local accumulation of various types of immune cells (Stern *et al.*, 1982; Cymerman *et al.*, 1984; Torabinejad and Kettering, 1985; Gao *et al.*, 1988b). Recent work indicates that tissue concentrations of growth factors are markedly affected by inflammation, due both to the cytokines produced by the inflammatory cells themselves and to the effects of these cytokines on the local stromal cells (Luger and Schwartz, 1994). Growth factors are important mediators of intercellular communication between connective tissues and epithelium; information about the growth factors that are generated in periapical lesions may therefore assist in our understanding of the mechanisms of stimulation of epithelial proliferation and subsequent cyst formation.

Several growth factors have stimulatory effects on epithelial proliferation and differentiation. The recently described keratinocyte growth factor (KGF) appears to be of particular interest. KGF is a stromally derived growth factor with stimulatory paracrine actions specifically targeted to epithelial cells (Finch *et al.*, 1989; Housley *et al.*, 1994; Imagawa *et al.*, 1994; Yi *et al.*, 1994). KGF production is markedly up-regulated during epithelial wound healing (Werner *et al.*, 1992, 1994), and there is increasing evidence that KGF expression by stromal fibroblasts is associated

with the maintenance of normal epithelial structure (Marchese *et al.*, 1990). Little detailed information is yet available about the expression of KGF in oral tissues. It appears to be expressed by fibroblasts beneath the mucosal epithelium but not by those deeper in the submucosa or periodontal ligament (Gao and Mackenzie, 1996a, b). It is possible that quiescence of the rests of Malassez is normally associated with low local levels of KGF or related growth factors in the PDL but that inflammation enhances the expression of growth factors necessary for their activation.

The aim of the present study was to explore the potential role of KGF in the initiation and promotion of epithelial growth during periapical cyst formation by comparing the pattern of KGF expression in various periapical lesions with that of normal periodontal ligament using the techniques of *in situ* hybridization and RT-PCR.

Materials and methods

Specimens for *in situ* hybridization

The material used consisted mainly of archival blocks of wax-embedded biopsies that had been received by a surgical oral pathology laboratory during the previous 12 months. All specimens had been fixed in 10% formalin and processed for routine histopathologic examination. The 12 specimens selected for the study had been previously diagnosed as periapical lesions and included 6 periapical granulomas and 6 periapical cysts. Additional samples of normal PDL were collected by dissection from the roots of extracted vital teeth and were similarly processed. Sections were cut at 6-μm intervals and collected on Probeplus slides (Fisher).

Specimens for RT-PCR

Tissue was obtained from 4 periapical lesions during apicoectomy (2 samples) or from teeth that were extracted with inflamed periapical soft tissue attached to the roots (2 samples). Samples of normal PDL were similarly removed from the apical root surfaces of extracted teeth which showed no evidence of periodontal or periapical pathosis. Half of each sample was fixed in 10% formalin for histological evaluation and the other half placed into cold tissue culture medium (DMEM, Sigma) at 4°C and rapidly transferred to the laboratory for RNA extraction. For RNA analysis, tissues were homogenized, and total RNA was extracted by the RNA Stat 60 method (Lawrence, Newark, NJ). The RNA yield was quantitated by spectrophotometric measurement of absorption at 260 and 280 nm, and RNA samples were kept at -90°C until used for RT-PCR. All samples were collected in accordance with a protocol approved by the Committee for the Protection of Human Subjects at the University of Texas.

In situ hybridization

The general methods used for demonstration of KGF expression by *in situ* hybridization were as previously described in detail elsewhere (Gao and Mackenzie, 1996b). For riboprobe preparation, plasmid containing a 496-bp fragment of KGF cDNA was linearized with appropriate restriction endonucleases as templates. Sense and antisense probes were synthesized with digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, IN) by *in vitro* transcription. The yield of probes was quantitated against supplied standards. Paraffin sections were deparaffinized by treatment in xylene and rehydrated in graded alcohols prior to treatment with proteinase-K for 30 min at 37°C (2 μg/mL). Sections were then hybridized in hybridization buffer (0.3 M NaCl, 10 mM $NaPO_4$, 10 mM EDTA, 10 mM Tris-HCl, 50% formamide, 10% dextran sulfate, 1 mg/mL tRNA, and 1X Denhartd's solution) with digoxigenin-labeled probes at 46°C overnight with 25 ng of labeled probe for each section. After stringency washing in descending concentrations of standard saline citrate (SSC) and RNase A treatment (10 μg/mL at 37°C for 15 min) for removal of unhybridized probe, probe hybridization was detected by binding of an anti-digoxigenin antibody (conjugated to alkaline phosphatase and visualized by an NBT/BICP substrate). In addition to hybridization with anti-sense probes, hybridization procedures for each of the 12 specimens were performed with omission of probes or with the sense probe for KGF as negative controls for confirmation of the specificity of the hybridization of the KGF anti-sense probe. Some specimens were also hybridized with an irrelevant anti-sense probe (for retinoic acid receptor α) as a positive control.

RT-PCR

Reactions for each sample were performed with 1 μg of total RNA and the GeneAmp RNA PCR Kit (Perkin Elmer, Norwalk, CT) used according to manufacturer's instructions. The primers used for amplification of KGF were the same as those previously described by Koos *et al.* (1993) which amplify a fragment of 266 bp and consisted of: upper primer, 5'-TCT GTC GAA CAC AGT GGT ACC T, and lower primer, 5'-GTG TGT CCA TTT AGC TGA TGC AT. Primers for amplification of the KGF receptor were designed to amplify a 174-bp fragment and consisted of: upper primer, GGT TCT CAA CAC TCG GGG ATA, and lower primer, TCT CCT TTT CTC TTC CAG GCG. Negative controls for RT-PCR included reactions performed without the addition of RNA and without the reverse transcription step. RNA extracted from gingival fibroblasts, which have previously been demonstrated to express KGF (Gao and Mackenzie, 1996b), provided a positive control. PCR thermal cycling was carried out in a Strategene Robocycler 40 (La Jolla, CA) according to the following parameters: denaturation for 1 min at 95°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C. A denaturation step of 2 min and an extension step of 7 min were added to the initial and final cycles, respectively. Amplified products from 30 PCR cycles were separated by electrophoresis in 3% agarose gels, with DNA molecular marker VI (Boehringer Mannheim, Indianapolis, IN) used to provide size standards. The gels were stained with ethidium bromide and examined by ultraviolet transillumination. To confirm accuracy of sequence amplification with these primers, we cloned amplified fragments of the expected size for KGF and the KGFr into the PCR II vector (Invitrogene, San Diego, CA) and conducted sequence analysis using the fmol DNA Sequencing System

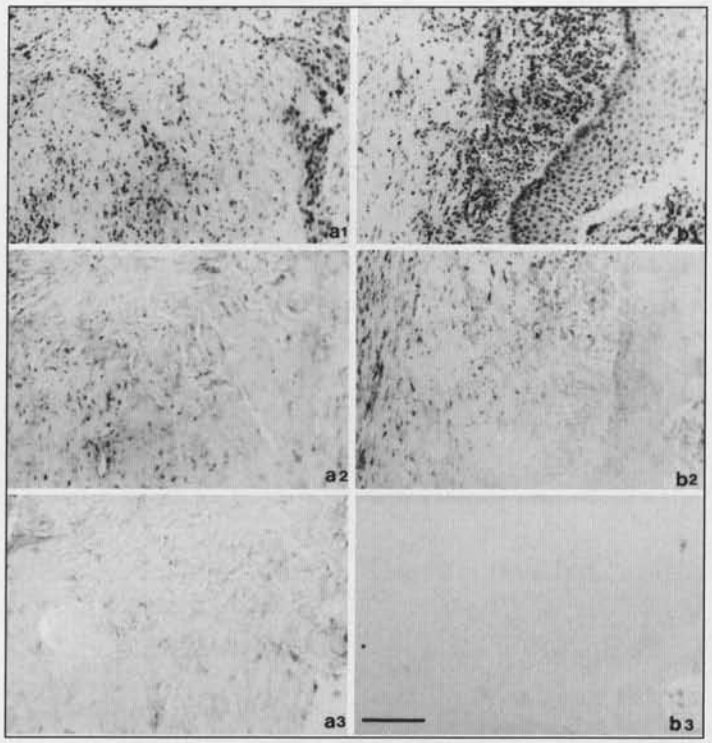

Figure 1. Sections of (a) a periapical granuloma and (b) a periapical cyst which were stained by H&E (a1, b1) or processed for *in situ* hybridization by either the KGF anti-sense probe (a2, b2) or the sense probe (a3, b3). In the sections hybridized with the antisense probe, cells with a spindle-shaped, fibroblast-like morphology are stained positively for probe hybridization to KGF transcripts. Similar cell staining indicative of specific probe hybridization is not seen with the sense probe. (Scale = 200 μm for all panels)

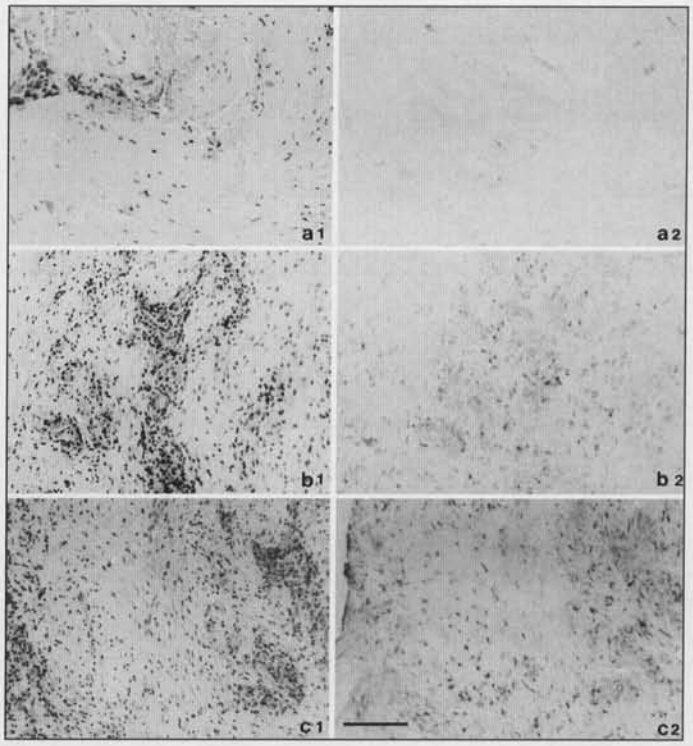

Figure 2. H&E staining of sections of three periapical granulomas shows increasing levels of inflammatory cell infiltration from a1 to b1 to c1, respectively. Adjacent sections of these specimens processed for *in situ* hybridization with KGF antisense probe (a2, b2, c2) show increased numbers of cells reacting positively for KGF transcripts as the inflammatory infiltrate increases. The proliferating epithelial elements identifiable by H&E staining in b1 and c1 are associated with cells expressing KGF mRNA, as seen in b2 and c2. (Scale = 200 μm for all panels)

(Promega, Madison, WI) for comparison with published sequence data for KGF (Finch *et al.*, 1989).

To provide a measure of the amount of KGF mRNA expressed, we also performed some reactions using a competitive PCR method. We constructed a cDNA internal standard, basically according to van den Heuvel *et al.* (1993), by PCR amplification of cDNA for the retinoic acid receptor α (RARα), using a pair of oligonucleotide primers synthesized to include KGF primer tails. This produced a cDNA with short stretches of sequence corresponding to the upper and lower KGF primers positioned at each end of a 185-bp stretch of the RARα cDNA, which acted as an irrelevant spacer sequence. Various amounts of the internal standard cDNA, ranging from 0.1 to 50 pg, were added to reaction tubes after the reverse transcription step was performed with 1 μg of total RNA for each tissue sample. In the subsequent PCR reaction, the internal standard cDNA and the KGF cDNA derived from the RT step compete with similar efficiency for the KGF primers, but the amplified internal standard, which is 46 bp smaller than the KGF product, is separated from the amplified KGF product in 3% agarose gels. As the concentration of the internal standard decreases, the KGF band appears and increases. When the amount of PCR product for KGF matches that for the internal standard, the known concentration of the added internal standard in the reaction mixture is taken as being equivalent to that for the KGF cDNA.

Results

The staining patterns observed in sections treated by *in situ* hybridization with the anti-sense probe for KGF indicated hybridization of probe to stromal cells in most periapical lesions. *In situ* hybridization procedures performed either without probes or with the KGF sense probe showed low levels of diffuse background staining but did not produce the clear patterns of cell staining indicative of specific hybridization. The RARα anti-sense probe, used as a positive control for hybridization, showed clear staining indicative of specific hybridization; this was localized primarily in the epithelium.

Hybridization with the anti-sense KGF probe showed various numbers of positively stained cells which appeared to be stromal fibroblasts rather than inflammatory cells and to represent only a subpopulation of the stromal fibroblasts. Some cells showed weak staining, just above background levels, and others a more intense staining pattern (Fig. 1). Sections of normal PDL (and of 2 periapical granulomas that showed only mild inflammatory infiltration of the connective tissue) exhibited few cells expressing KGF transcripts. Greater numbers of positive cells were found in specimens showing dense inflammatory infiltrates, epithelial proliferation, or periapical cyst formation (Fig. 2). In periapical granulomas, KGF-expressing cells lay adjacent to inflammatory cell foci and close to proliferating epithelial

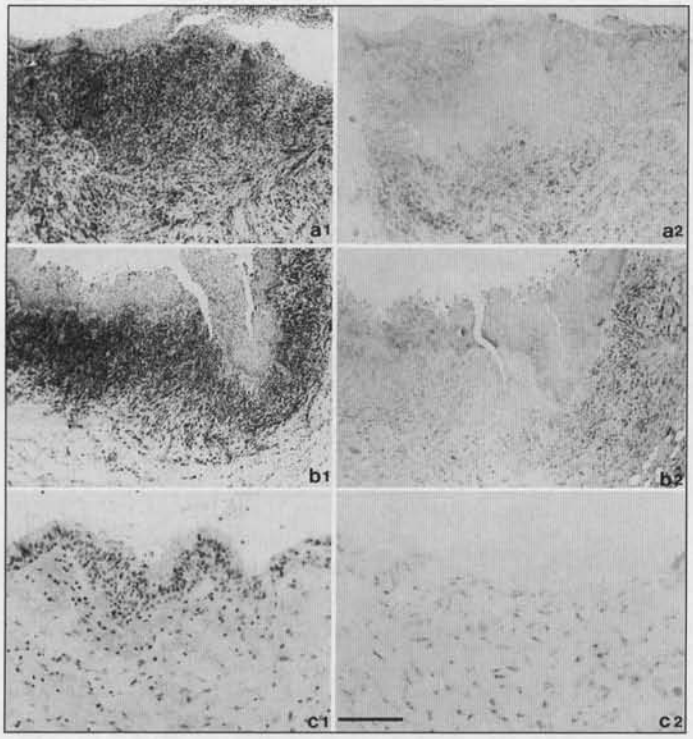

Figure 3. Sections of 3 periapical cysts stained with H&E (a1, b1, c1) or processed for *in situ* hybridization with the KGF anti-sense probe (a2, b2, c2). H&E staining shows dense inflammatory infiltrates subjacent to the epithelial lining in a1 and b1 and a milder inflammatory infiltrate beneath the epithelium in c1. As shown in a2 and to the left of b2, cells expressing KGF mRNA tended to lie around, rather than within, dense infiltrates. However, as seen to the right in b2, strongly reactive cells sometimes lay within the infiltrate itself. Milder infiltrates (c1) tended to be associated with a more generalized pattern of KGF expression of the stromal fibroblasts. (Scale = 0.5 mm for panels a and b and 200 μm for panel c)

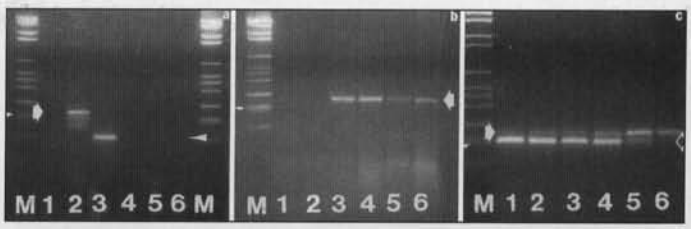

Figure 4. (a) RT-PCR amplification with RNA extracted from a periapical lesion (lanes 1-3) and from non-inflamed apical PDL (lanes 4-6). Lanes 1 and 4 show negative controls run without the reverse transcription step, lanes 2 and 5 show the amplification products with KGF primers, and lanes 3 and 6 the products with primers for the KGF receptor. Amplified transcripts of the appropriate size for KGF (large arrow) and the KGF receptor (small arrow) were produced from the lesional RNA (lanes 2 and 3) but under the same conditions were not detectable for RNA extracted from healthy PDL (lanes 5 and 6). (b) RT/PCR amplification of KGF mRNA from 2 samples of uninflamed PDL and from 4 samples of periapical lesions (lanes 3-6). No product was amplified from normal PDL (lanes 1 and 2), but, from each of the 4 lesional samples, product of the appropriate size for KGF was amplified in various amounts. (c) Competitive PCR (see text) with RNA extracted from a periapical lesion to which an internal standard was added in decreasing quantities from 50 to 0.1 pg (lane 1 to lane 6). The relative densities of the product bands for the internal standard and for KGF reverse between lane 4 (1-pg internal standard) and lane 5 (0.5 pg). The solid arrow indicates bands for the KGF product, and the hollow arrow indicates product derived from the internal standard. For all panels, the small arrow indicates the 234-bp marker band. M = cDNA size marker.

elements. Typically, periapical cysts with a dense inflammatory infiltrate showed KGF-expressing cells at the periphery of the infiltrate, but cysts with milder or diffuse inflammatory infiltrates showed KGF-expressing cells close to the cystic lining (Fig. 3). Examination of H&E-stained sections adjacent to those cut and processed for *in situ* hybridization allowed the tissue regions containing epithelium to be identified. KGF-positive cells could always be identified in those regions of periapical lesions where proliferating epithelium was present.

The periapical lesions evaluated by RT-PCR with specific primers for KGF showed products which appeared as a single band at a position corresponding to the expected length of 266 bp (Figs. 4a, 4b). Samples of normal PDL showed either no detectable PCR product or only a weak product band. Negative controls run without the addition of sample RNA or without the reverse transcription step failed to show product bands. A distinct KGF product band was detected for each of the 4 samples of periapical lesions (Fig. 4b) and also for RNA extracted from cultured gingival fibroblasts, which were used as a positive control. Assessment of the amount of products by competitive PCR (Fig. 4c) indicated that the PCR product from the target RNA (266-bp KGF fragment) increased as the PCR product from the internal standard (241 bp) decreased, and also that the equivalency point between the two products occurred at approximately from 0.5 to 1 pg of added internal standard. RT-PCR with primers for the KGFr resulted in amplified product of the expected size of 175 bp from RNA extracted from the periapical lesions but not from the specimens of normal periodontium (Fig. 4a). The accuracy of PCR amplification for both KGF and the KGFr was confirmed by the exact match with published data for the KGF and KGFr sequences (Finch *et al.*, 1989; Miki *et al.*, 1992) found for samples of amplified product that were cloned into a PCR II vector for sequencing.

Discussion

The results of the *in situ* hybridization studies indicate that KGF mRNA is expressed more frequently and more strongly by the stromal cells of periapical lesions than those of normal periodontal ligament. These observations are also supported by the results of the RT-PCR and competitive PCR which indicated that levels of KGF expression in periapical lesions are quite high and similar to those of gingival fibroblasts *in vitro* (Gao and Mackenzie, 1996b). It thus appears that KGF may thus play a role in the epithelial proliferation associated with the pathogenesis of periapical lesions. Although derived from relatively few samples, and with methods of analysis that did not permit quantification for statistical analysis, the consistency of these observations suggests a general correlation among inflammation, epithelial proliferation, and KGF expression.

KGF was initially isolated as a product of human

fibroblasts that stimulates epithelial growth (Rubin *et al.*, 1989). The subsequent cloning and sequencing of KGF cDNA indicated that it codes for a 194-amino-acid polypeptide with homology to the heparin-binding family of fibroblast growth factors, and that KGF contains a hydrophobic signal sequence in the N-terminal region indicating that it is a secreted protein (Finch *et al.*, 1989). KGF mRNA and the biologically active peptide are expressed *in vitro* by various human fibroblast cell lines, and it is suggested that KGF acts specifically as a paracrine mediator of epithelial proliferation (Finch *et al.*, 1989), a concept that is supported by the expression of the KGF receptor only on epithelial cells (Aaronson *et al.*, 1991) and by the observed mitogenicity of KGF for epithelial but not stromal cells (Le Panse *et al.*, 1994). Further, Guo *et al.* (1993), using a human keratin 14 promoter to target expression of human KGF cDNA to stratified squamous epithelia in transgenic mice, have demonstrated that overexpression of KGF is associated with proliferative epithelial changes *in vivo*.

In view of these roles of KGF in the development, growth, and differentiation of epithelia, the present finding that KGF transcripts are expressed at higher levels and by more stromal cells in periapical granulomas and cysts than in healthy PDL is an interesting one. The rests of Malassez are not normally proliferative, but our present observations, and those of previous studies (Summers, 1974), indicate that proliferating epithelium is commonly found in inflammatory periapical lesions. Inflammation is associated with the generation of multiple interacting cytokine networks, and some of the cytokines produced—such as epidermal growth factor (EGF), transforming growth factor α (TGFα), tumor necrosis factor, and interleukin-6—are known to have stimulatory effects on the proliferation of other epithelia *in vitro* (Luger and Schwartz, 1994). Rests of Malassez express some receptors—for example, the EGF/TGFα receptor (Thesleff, 1987)—through which such cytokines could act, and possibly other factors (such as interleukins and interferon) may play a role in this process (Brown and Smith, 1991). However, the actual magnitude of direct *in vivo* epithelial responses to inflammatory cytokines remains uncertain. The recent findings concerning the actions and patterns of induction of KGF suggest that the major effect of such cytokines may be an indirect one *via* their induction of KGF expression. Examination of the mechanisms of up-regulation of KGF expression in fibroblasts indicates that it is induced by exposure to growth factors such as interleukin 1α and β, TNFα, and PDGF (Brauchle *et al.*, 1994; Chedid *et al.*, 1994), which are produced both by inflammatory cells and by epithelia. The expression of KGF in subepithelial fibroblasts appears to be normally signaled by products of the epithelium itself (Smola *et al.*, 1993).

The observed relationship of KGF-expressing cells to apical inflammatory infiltrates suggests that the inflammatory process induces elevated expression of KGF by periodontal fibroblasts. The association of such KGF expression with proliferating epithelium suggests that KGF may be responsible for activating and maintaining proliferation of the epithelial rests. The detection of message for the KGF receptor in periapical lesions suggests that proliferating periapical epithelia are able to respond to the KGF stimulus. The present study examined KGF expression only at the RNA message level; whether the biologically active peptide is also present in periapical lesions needs to be investigated. Nevertheless, other studies have shown that the expression of KGF message correlates with the production and secretion of KGF (Brauchle *et al.*, 1994; Chedid *et al.*, 1994). Our preliminary observations point to an interesting indirect mechanism by which common inflammatory cytokines could stimulate proliferation and growth of the rests of Malassez by inducing fibroblast production of a potent epithelial mitogen.

Acknowledgments

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