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Expression of bFGF, KGF and FGF Receptors on Normal Oral Mucosa and SCC

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Fibroblast growth factors (FGFs) are involved in the transmission of signals between the epithelia and connective tissue, and influence epidermal growth and differentiation. They are thought to be important in the restoration of normal tissues after injury and aberrant expression may also play a role in tumorigenesis. However, no information is available on the nature of cells within oral mucosa which synthesise and/or respond to FGFs. We have screened normal oral mucosa and oral squamous cell carcinoma (SCC) for expression of bFGF by immunohistology and northern analysis and used RT-PCR to look for transcripts for KGF and the high-affinity FGF receptors FGFR1 and FGFR2. Transcripts for bFGF were detected in normal and malignant oral mucosa and KGF within connective tissue elements. The predominant FGF receptor detected in the epidermis and oral mucosa was FGFR2 which binds KGF with greater affinity than bFGF. Production of KGF by connective tissue components and synthesis of the high-affinity KGF receptor, FGFR2, by oral keratinocytes provides circumstantial evidence for a paracrine growth control loop with KGF synthesised within the lamina propria or tumour stroma influencing the proliferation and maturation of both normal oral epithelium and SCC. Copyright © 1996 Published by Elsevier Science Ltd

Keywords: growth factors, KGF, bFGF, FGFR, tumour, mouth neoplasms

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INTRODUCTION

Fibroblast growth factors (FGFs) are involved in the transmission of signals between epithelia and connective tissue and influence epidermal growth and differentiation. They are important in the restoration of normal tissue after injury. Aberrant FGF expression may also play a role in tumorigenesis [1]. Eleven FGFs are now recognised, the best characterised of which are acidic FGF (aFGF), basic FGF (bFGF) and keratinocyte growth factor (KGF). They are closely related peptides which have pleiotrophic effects and can act as growth or differentiation factors depending on the cell type and local environment. aFGF and bFGF lack a consensus hydrophobic signal peptide for secretion [2] and may remain cell associated in normal tissues although they are released and deposited within the ECM as a consequence of cell damage [3]. KGF is functionally different from all other FGFs as it is mitogenic for epithelial cells but not fibroblasts or endothelial cells.

FGFs exert their effects through two types of receptor, the low-affinity heparan sulphate proteoglycans [4] and the high-affinity FGF receptors (FGFRs) of which there are four major subtypes, FGFR1–4 [5–8]. These transmembrane proteins comprise an extracellular region, with a variable number of immunoglobulin-like (Ig-like) domains and an intracellular tyrosine kinase region. The presence of alternative exons is a mechanism for generating receptor diversity and confers FGF ligand specificity. For example, two isoforms of FGFR1 and 2 can be generated by alternative exons encoding the second half of the third Ig-like loop (see Fig. 1). The bek/111c form of FGFR2 [9] preferentially binds aFGF and bFGF whereas the K-Sam/111b form binds KGF with 15 times greater affinity than it binds bFGF [10]. More than 50 other transcripts have been described. The biological significance of these additional variants is unclear, some may affect signal transduction by influencing receptor oligomerisation or substrate specificity whilst others alter phosphorylation activity [11].

To determine whether FGFs play a role in epithelial–mesenchymal interactions we have screened normal oral mucosa and 25 primary oral squamous cell carcinomas for evidence of production of bFGF, KGF and the high-affinity FGFR1 and 2.

We report the location of immunoreactive bFGF in epidermis and oral epithelia, and the presence of KGF transcripts in dermis and lamina propria. The predominant FGFR synthesised by normal and malignant oral epithelia was

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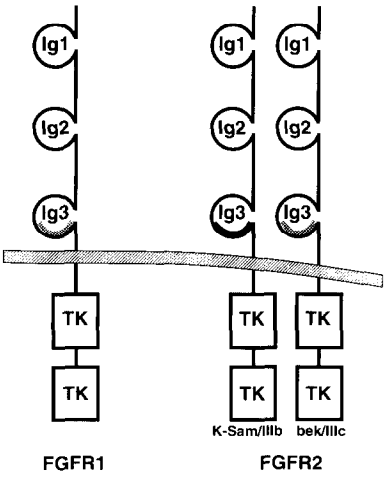

Fig. 1. Simplified schematic diagram showing the structure of polypeptides encoded by FGFR1 and FGFR2. Extracellular immunoglobin-like (Ig-like) domains are shown as loops (Ig 1–3) and the intracellular tyrosine kinase domains as boxes (TK). Both FGFR1 and FGFR1 can be produced without the first immunoglobin-like domains and there are alternate forms of the second half of the third Ig-like domains. FGFR2 variants include the K-Sam/111b and bek/111c receptors.

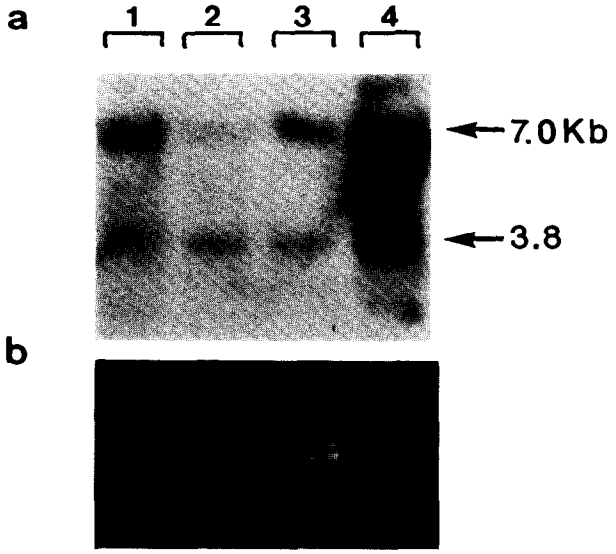

Fig. 2. (a) Detection of bFGF using northern analysis of RNA with a 1.6 kb bFGF fragment. Lane 1, cultured epidermal keratinocytes; lane 2, oral keratinocytes; lane 3, SCC 2; lane 4, SCC 6. (b) EtBr stained gel to show equal loading of RNA.

Table 1. *Primer sequences used for PCR amplification*

FGFR1	5′ CCTCCTCTTCTGGGCTGTGCT 3′ 5′ TCTTTTCTGGGGATGTCC 3′
FGFR2	5′ CCTCCTCTTCTGGGCTGTGCT 3′ 5′ TGTAATCTCCTTTTCTCTTCCA 3′
KGF [18]	5′ ATCAGGACAGTGGCAGTTGGA 3′ 5′ CATAGGAAGAAAGTGGCTGT T 3′

FGFR2 (K-Sam) which preferentially binds KGF [12]. The presence of KGF transcripts within connective tissue elements and expression of FGF receptors FGFR on oral epithelia provides circumstantial evidence for an epithelial–mesenchymal interaction which may represent a paracrine growth regulatory pathway for oral epithelia in health and disease.

MATERIALS AND METHODS

Tissues and cells

Normal epidermis [10], oral mucosa [13] and primary oral squamous cell carcinoma (SCC) [14] were obtained from surgical biopsies and stored in liquid nitrogen. Epidermis was separated from underlying dermis and oral epithelium from the lamina propria by incubation in 10 mg/ml collagenase in Tyrode's buffer at 37°C for 2–4 h [13]. After digestion, the transparent epithelial layer was separated from the underlying connective tissue. Ethical Committee approval for this study was granted at King's College Hospital, London, U.K. Human keratinocytes derived from epidermis and oral mucosa were grown in MCDB 153 (Sigma Ltd) and MRC5 (human fibroblasts) in DMEM supplemented with 10% FCS.

Immunohistology

Polyclonal antibodies recognising bFGF were RB 773 and RB 967 [14]. The antibody recognising all subtypes of the FGFR 06-177 was obtained from Upstate Biotechnology. Cryostat sections (6 μm) were fixed in 4% paraformaldehyde–PBS for 10 min and washed in Tris-buffered saline (TBS). Immunohistology was performed using an APAAP technique [15]. The specificity of the immunoreaction was determined by preincubation of the polyclonal antibodies with either recombinant FGF or the peptide against which the anti-FGFR antibody was raised [16], as appropriate.

Isolation of RNA, northern analysis

Total RNA was extracted from oral SCC and from cultured cells by standard techniques using guanidium isothiocyanate–caesium chloride. Northern analysis was performed using standard protocols and hybridisation with a 1.4 kb ECOR1 bFGF fragment [17] followed by autoradiography at −70°C overnight.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to amplify mRNA-encoding FGFR1 and 2 and KGF. Total cellular RNA (2.5 μg) was reverse transcribed in a final volume of 20 μl containing 500 ng Oligo $(dT)_{12-18}$ primer, 500 mM each dNTP, pH 7.0, in 1 × buffer with 200 U Superscript RNaseH–reverse transcriptase. From this reaction 1 μl of cDNA was amplified in a 100 μl PCR reaction containing 200 μM each dNTP, 45 pmol each primer (see Table 1), 1 × Supertaq buffer and 0.25 U Supertaq DNA polymerase. Forty cycles of PCR were performed at 94°C for 1 min, 45°C for 1 min, 72°C for 1 min (× 40) and 72°C for 10 min for FGFR1 and FGFR2. For KGF the cycling parameters were 94°C for 30 s, 52°C for 30 s and 72°C for 45 s (× 35 cycles).

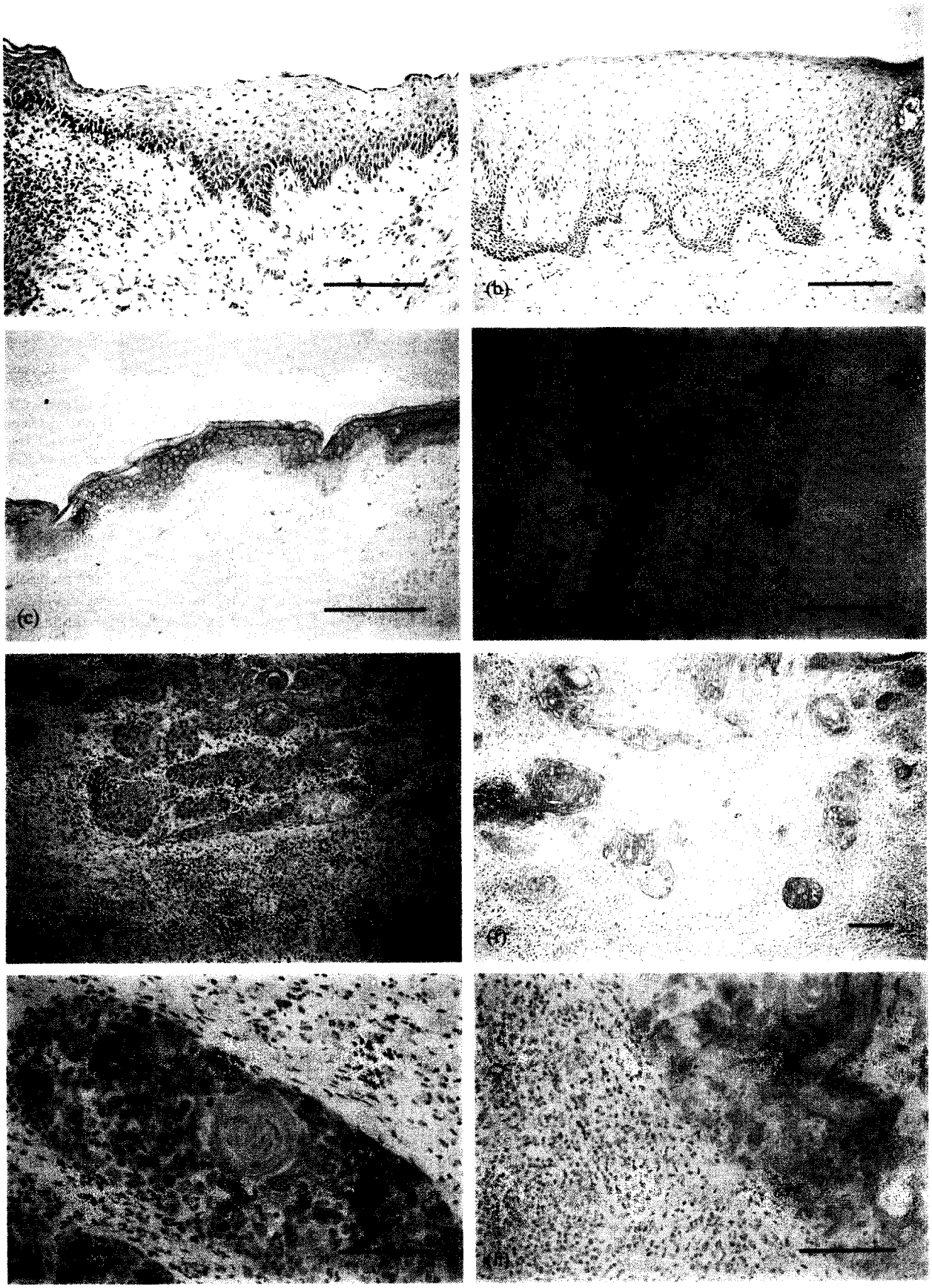

Fig. 3(a)–(h).

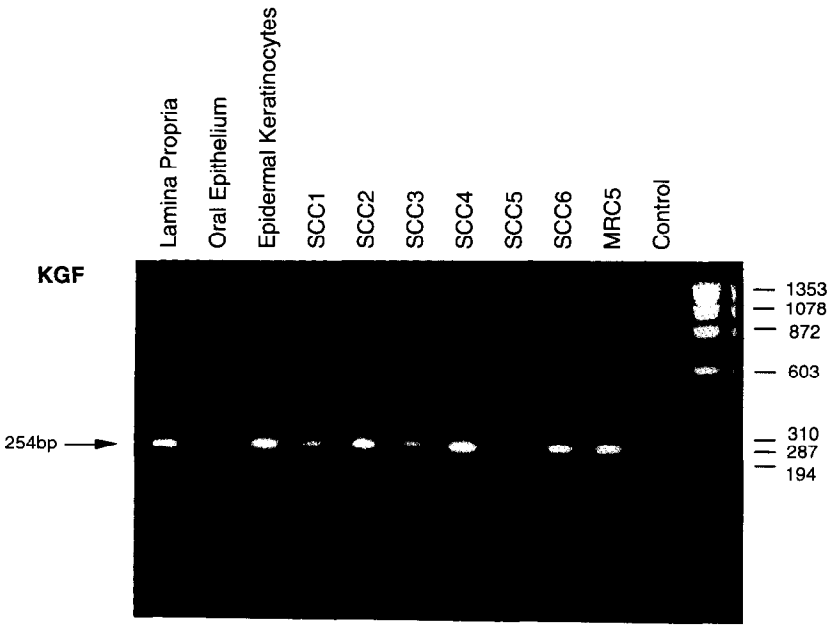

Fig. 4. RNA extracted from lamina propria, oral epithelium, epidermal keratinocytes and five oral SCC was converted into cDNA and amplified with primers to KGF. A 254 bp KGF fragment was detected in lamina propria, oral epithelium, epidermal keratinocytes, SCC 1–6 and human fibroblasts (MRC 5).

Southern blotting and hybridisation

The FGFR2 PCR product (45 μl) was separated by electrophoresis on duplicate 1.5% agarose gels in 0.5 × TBE, denatured with NaOH and transferred to Hybond-N membranes. Twenty pmoles of oligonucleotide, specific for either the bek (CTCTTTGTCCGTGGTGTT) or the K-Sam (TGGAACTATTTATCCCCG) isoform of FGFR2 was end-labelled with 10 U of T4 polynucleotide kinase with 30 μCi of ^{32}P gamma dATP in 1 × kinase buffer. Hybridisation was performed at 42°C overnight using the labelled oligonucleotides at a final concentration of 1×10^6 cpm/ml. Post-hybridisation washes included 1 × 15 min at room temperature in 4 × SSPE, 2 × SSPE and 4 × SSPE. Autoradiography was performed at −70°C overnight with intensifying screens.

RESULTS

Transcripts for bFGF were detected in cultured epidermal keratinocytes, oral keratinocytes and oral SCC by northern blotting, for examples see Fig. 2. Staining with anti bFGF antibodies in epidermis (10 cases) revealed expression of this growth factor in basal and supra basal keratinocytes. Most connective tissue elements were devoid of bFGF although occasional positive blood vessels were seen (Fig. 3a). Incubation of the anti bFGF antibodies with bFGF, but not aFGF, abolished the FGF signal. Lower levels of bFGF were detected for 15 cases of normal oral mucosa examined. At nonkeratinising oral sites (8 cases, buccal mucosa and floor of mouth) bFGF was found throughout the epithelium, whereas keratinised areas (7 cases), for example palate (Fig. 3b) and gingiva, showed a pattern of expression similar to epidermis with only occasional positive cells detected above the level of the rete ridges.

bFGF was principally localised to the malignant epithelial cells within the 25 oral SCC examined although occasional clumps of positive endothelial cells were detected. 18 SCC cases showed homogeneous bFGF immunoreactivity. 10 tumour cases showed strong expression of this growth factor, 5 had moderate levels (Fig. 3e) and 3 cases were only weakly positive. A heterogeneous pattern (different levels of FGF in the same tumour clump) was seen in the other 7 cases (Fig. 3g).

PCR for KGF produced the expected 254 bp KGF fragment in the lamina propria of oral mucosa and cultured fibroblasts (MRC5 cells). Transcripts were also seen in oral epithelium and epidermal keratinocytes and all of the SCC examined (Fig. 4). High molecular weight species observed in lanes 2, 5 and 10 may be owing to contamination of RNA with genomic DNA.

The polyclonal antibody used to localise the FGF receptors does not distinguish between the four major FGFR subtypes. Immunoreactive FGFR was detected throughout the epidermis (7 cases) and oral epithelium at keratinised sites (7 cases) with expression confined to basal cells on non and parakeratinised oral mucosa (8 cases, Fig. 3c and d). FGFR was not detected on the basal cell layers when significant levels of melanin were present (3 cases, for example see Fig. 3c). All 25 oral SCC examined expressed the FGFR (for example see Fig. 3h). However, 11 out of 25 cases contained clumps of tumour which did not express this receptor on the basal-type

Fig. 3. Immunolocalisation of bFGF on (a) normal epidermis and (b) keratinised oral mucosa (palate). Localisation of FGFR on (c) normal epidermis, (d) oral mucosa (buccal mucosa), (e) SCC showing moderate levels of bFGF, (f) an example of an SCC showing reduced expression of FGFR on the peripheral cells of some tumour clumps, (g) heterogeneous pattern of expression of bFGF, SCC 5 and (h) FGFR metastatic SCC within lymph node, strongly positive cells are present at the centre of the tumour mass. Scale bar = 0.1 mm.

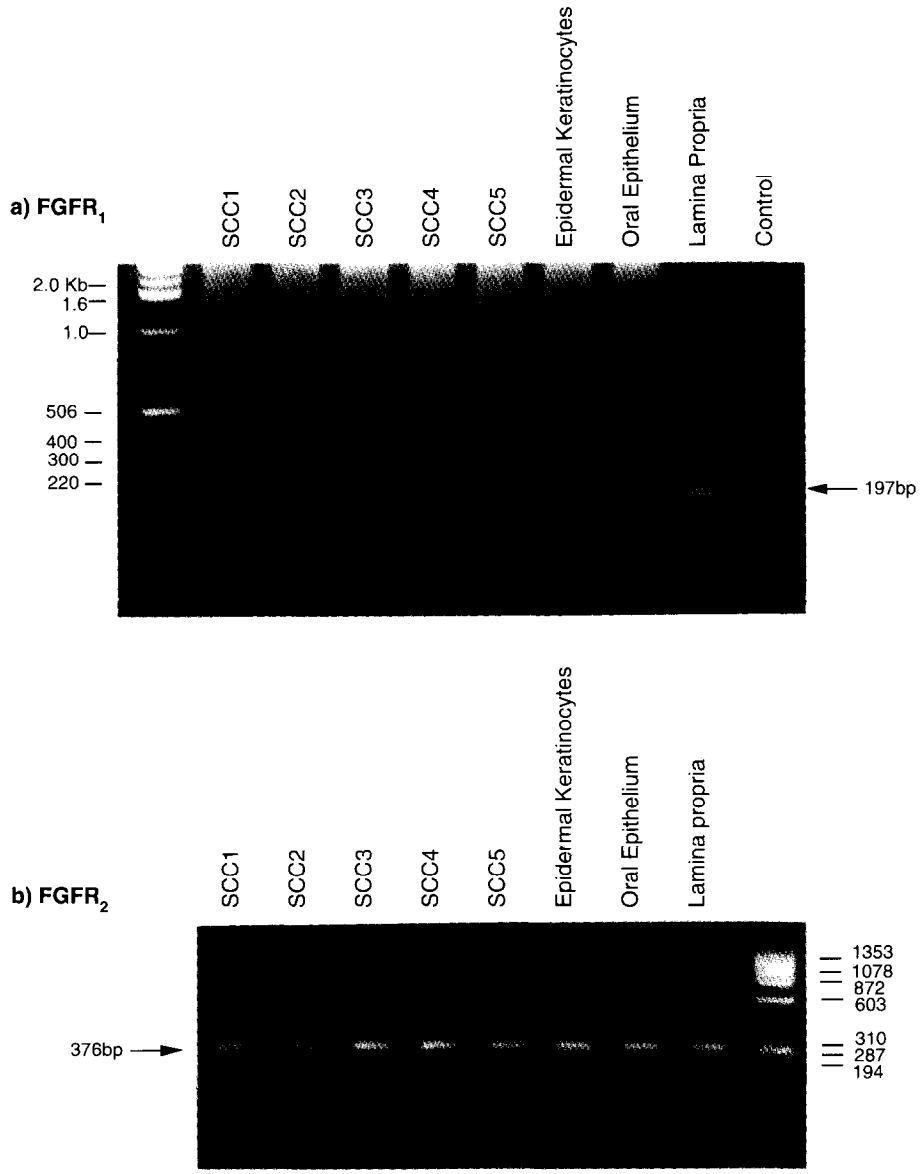

Fig. 5. RNA extracted from five SCCs, epidermal keratinocytes, oral epithelium and lamina propria was converted into cDNA and amplified with primers to either (a) FGFR1 or (b) FGFR2. Lane 9 is a PCR negative control without RNA. A 197 bp FGFR1 fragment was detected in RNA obtained from lamina propria whereas a 376 bp FGFR2 fragment was seen in all SCC examined, epidermal keratinocytes, oral epithelium and lamina propria.

cells, whereas the central, more differentiated cells were always strongly positive (Fig. 3f).

Agarose gel analysis of cDNA showed that a 197 bp FGFR1 fragment was amplified only from RNA obtained from dermis (data not shown) and the lamina propria of oral mucosa (Fig. 5a), whereas a 37 bp FGFR2 fragment was detected in five SCC, epidermal keratinocytes, oral epithelium and lamina propria (Fig. 5b). This suggests that FGFR2 and not FGFR1 is the predominant receptor synthesised by the cells and tissues examined. Further analysis was performed to determine whether the bek or K-Sam variant of FGFR2 was the predominant species. Aliquots of PCR products were electrophoresed on separate agarose gels, transferred to nitrocellulose by Southern blotting and hybridised with oligonucleotide probes homologous to either the bek or K-Sam sequences. These experiments identified K-Sam and not bek as the predominant FGFR2 isoform in 4 out of 6 SCCs as well as in oral epithelium and epidermal keratinocytes (Fig. 6a). One tumour, SCC 5, was strongly positive for the bek isoform of FGFR2 (Fig. 5b).

DISCUSSION

Our data for bFGF in relation to normal epidermis and oral mucosa agree with earlier studies showing bFGF localised to basal cell layers [14, 19]. This growth factor supports keratinocyte growth [21–23]. However, its ability to contribute to the normal physiological process in the epidermis and oral epithelium may be limited by its lack of signal sequence which suggests that it may not be secreted at least by conventionally accepted mechanisms. This has led to suggestions that bFGF is normally stored in keratinocytes and is released into the extracellular environment only during cellular injury. Although only low levels of bFGF are detected

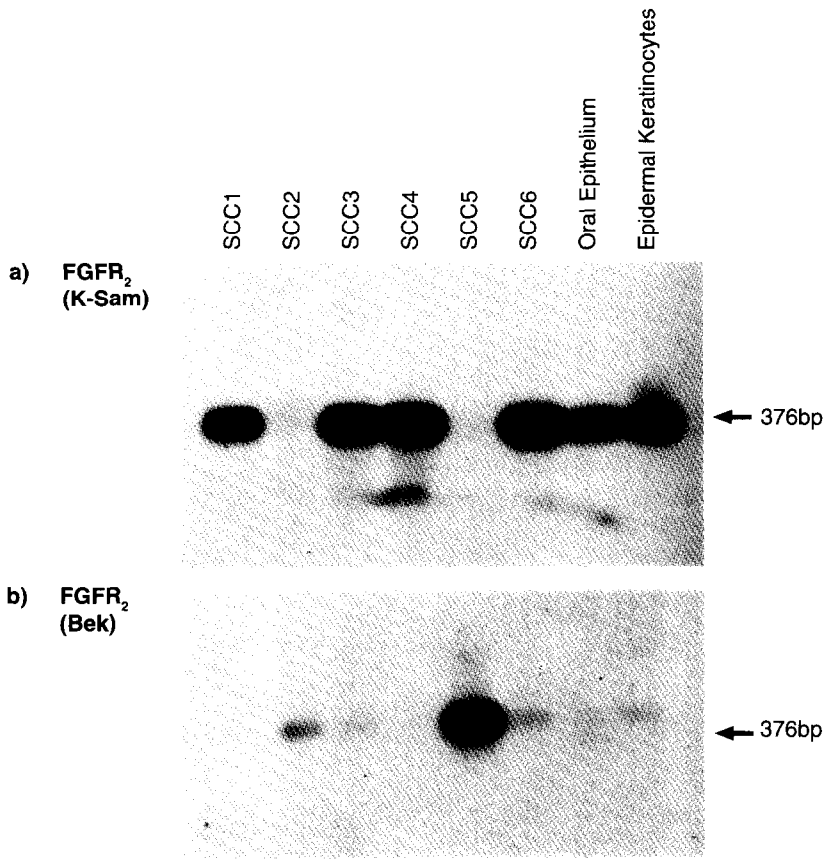

Fig. 6. RNA extracted from the tissues indicated was converted into cDNA and amplified with FGFR2 primers. Aliquots of PCR products were electrophoresed, DNA transferred to Hybond-N membrane and hybridised with ^{32}P-labelled oligonucleotides specific for either (a) the K-Sam or (b) the bek sequences. K-Sam transcripts were detected in SCCs 1, 3, 4 and 6, oral epithelium and epidermal keratinocytes, and transcripts for the bek isoform in SCC 5.

its principal role may thus be to stimulate cell proliferation in tissue repair and inflammation [23]. Levels of bFGF were often high in oral SCC but were not related to clinicopathological parameters of the tumours, including tumour histology or velocity of growth (data not shown).

High-affinity FGFRs were detected by immunostaining in the epidermis and oral mucosa. The RT-PCR data reveals that the predominant receptor transcripts within the normal epidermis, oral mucosa and oral SCC are for FGFR2 (K-Sam, Figs 4 and 5). Different patterns of FGFR expression were seen in the epidermis and normal oral mucosa with the receptor limited to the lower cell layers of nonkeratinised sites (Fig. 3d) suggesting that expression of FGFR may influence the pattern of differentiation in some way. However, the antibody used to localise the FGFR does not distinguish between the different receptor subtypes and further studies with reagents specific for FGFR2 are required to determine the pattern of expression of FGFR2 (K Sam) for the epidermis and oral mucosa. As this isoform binds KGF with 15 times greater affinity than it binds bFGF it seems likely that the K-Sam variant of FGFR2 may influence growth control in the epidermis, oral mucosa and SCC. However, one SCC expressed the bek variant of FGFR2 which may reflect alternative gene usage in this tumour. FGFR1 was detected in oral mucosa but only within the lamina propria (Fig. 4).

KGF is functionally different from bFGF. It is generally considered to be localised exclusively in the dermis [24]. KGF has a signal sequence and is a potent mitogen for epithelial cells both *in vitro* [25] and *in vivo* [26]. Transgenic mice expressing KGF in stratified squamous epithelium show a thickened epithelium and suppression of differentiation. Some animals also developed transformations in the tongue and epidermis [27]. *In situ* hybridisation studies [23] have also shown that KGF mRNA is increased in the dermis at wound edges, whereas levels of bFGF remain low suggesting that KGF may be more important than bFGF in repair processes.

Transcripts for KGF were detected in all SCC examined and within the lamina propria of oral mucosa. Stromal elements are the most likely source of KGF in oral SCC. However, our results also suggest that KGF transcripts may be synthesised by epidermal keratinocytes and within oral epithelia. Although the cultured keratinocytes were grown without a fibroblast feeder layer we cannot exclude the possibility of contaminating fibroblasts in these cultured cells and oral epithelium which has been separated from the lamina propria. However, other studies have detected KGF in epithelial cells [28] and further studies using antibodies which specifically recognise KGF are required to clarify this point.

As KGF influences the proliferation and differentiation of cells which express FGFR2, this growth factor [27] may be able to influence these processes in both normal and malignant epithelia. Thus, the possible interaction of KGF synthesised by cells within the lamina propria of tumour stroma with the FGFR2 (K-Sam) receptor on normal or malignant oral epithelial cells may represent a potential epithelial–mesenchymal interaction, influencing proliferation and differentiation.

During the carcinogenic process prolonged paracrine growth stimulation by KGF may contribute to continued cell proliferation and lead to internalisation and down-regulation of the FGFR2 (K-Sam). This view is supported by the finding of reduced or absent FGFR2 expression on the peripheral, basal-type cells of some tumour clumps. However, this explanation, while attractive, takes no account of other multiple growth factor interactions which may affect receptor expression.

In summary, these results suggest that KGF synthesised within the lamina propria of normal oral mucosa and tumour stroma may influence growth regulatory processes within normal and malignant oral epithelia. The potential interaction between KGF synthesised by connective tissue elements and K-Sam produced by oral keratinocytes may be an example of an epithelial–mesenchymal interaction influencing epithelial cell growth via paracrine mechanisms.

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