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The expression of basic fibroblast growth factor and its receptor in cell lines derived from normal human mammary gland and a benign mammary lesion

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SUMMARY

mRNA for basic Fibroblast Growth Factor (bFGF) was expressed in a series of SV40-transformed human mammary cell lines as molecules of 7.1, 3.6, 2.0 and 1.2 kb. This expression was much weaker in those lines of epithelial morphology than in myoepithelial-like cell lines derived from them. It was confirmed, using northern hybridization to single-stranded RNA probes, that the multiple mRNAs were transcribed from the coding strand for bFGF. bFGF activity was detected in extracts of the cells and the relative amounts of activity corresponded in general to the amounts of mRNA found. Similar results were obtained from spontaneously transformed cell lines derived from a human benign breast lesion. The presence of bFGF protein in the extracts was confirmed by western blotting, which showed a band of 18-19 kDa, migrating in the same position as authentic bFGF; in addition, the myoepithelial-like cells showed prominent bands of bFGF at 24 and 26 kDa. No FGF receptor was detectable by the binding of ^{125}I-bFGF to the SV40-transformed cell lines or to the epithelial cell lines from the benign breast lesion, but both high- and low-affinity receptors were found on myoepithelial-like cells derived from the latter. The results indicate that differentiation to the human myoepithelial-like phenotype in culture is associated with the enhanced expression of bFGF, and it is suggested that bFGF, immunocytochemically detected in the basement membrane of the human breast, may arise, at least in part, from the myoepithelial cells of the mammary parenchyma.

Key words: fibroblast growth factor, receptor, mammary cells

INTRODUCTION

Development of the rat mammary gland has been extensively studied in vivo (Russo et al., 1982) and by the use of clonal cell lines in tissue culture that mimic certain aspects of mammary differentiation seen in vivo (Rudland, 1987). Thus, cultured epithelial cell lines derived from normal rat mammary glands (Ormerod and Rudland, 1985) and benign mammary tumours convert in culture to myoepithelial-like cells (Bennett et al., 1978). The conversion of the epithelial cell lines derived from the benign mammary tumours proceeds through a series of discrete morphological forms (Rudland et al., 1986), which are thought to represent intermediates in the normal differentiation pathway of developing ductal structures in vivo (Ormerod and Rudland, 1984). The conversion of the epithelial cells to the myoepithelial-like cells in these rat mammary cell lines is associated both with discrete changes in their pattern of polypeptides (Barraclough et al., 1982; Paterson and Rudland, 1985) and with changed responses to purified growth factors, particularly to fibroblast growth factor (Fernig et al., 1990a).

With regard to fibroblast growth factor, quiescent rat mammary myoepithelial-like cell lines in culture are stimulated to proliferate by the addition of basic fibroblast growth factor (bFGF), whereas the parental epithelial cell lines fail to respond (Smith et al., 1984b). This is due to the fact that the myoepithelial-like cell lines produce high- and low-affinity receptors for bFGF on their surfaces, whereas the parental epithelial cell lines do not (Fernig et al., 1990b). In addition, rat mammary myoepithelial-like cells produce mRNA for bFGF, and bFGF itself, much of which becomes associated with heparin-like molecules of the extracellular matrix (Barraclough et al., 1990). In contrast, the parental epithelial cell lines, which do not respond to bFGF, do not produce detectable levels of bFGF, nor its mRNA (Barraclough et al., 1990). In cell lines morphologically intermediate between the epithelial and myoepithelial cells (Rudland et al., 1986), the production of bFGF and the levels of its surface receptors increase with increas-

ing myoepithelial characteristics (Barraclough et al., 1990). These results obtained with cultured cell lines suggest that bFGF may have developmental significance in the rat, particularly in view of the recent finding of high levels of bFGF in the growing mammary glands of virgin rats (Barraclough et al., 1990).

Although the previous work with rat mammary cell lines has suggested that the myoepithelial-like cells synthesize bFGF (Barraclough et al., 1990) and produce both high- and low-affinity receptors for the FGF molecules (Fernig et al., 1990b), immunocytochemical detection of bFGF in human breasts has recently located it in the region of the myoepithelial cells/basement membrane/extracellular matrix (Gomm et al., 1991). This result raises the possibility that the bFGF detected in these experiments and its receptors are produced by stromal cells that lie adjacent to the outside of the basement membrane and are embedded in the extracellular matrix surrounding the mammary parenchyma, at least in human breasts. We now use recently isolated human breast cell systems to clarify the cellular site of production of bFGF.

MATERIALS AND METHODS

Cell culture

The derivation of the clonal epithelial cell line, SVE3, from primary cultures of normal human mammary glands transformed with SV40 virus, and the cloned epithelial derivative, Huma 7, have been described previously (Rudland et al., 1989b). Huma 7 cells convert to elongated, myoepithelial-like cells at a frequency of about 0.1%. Cell lines corresponding to myoepithelial-like cells, such as Huma 62 (Rudland et al., 1989b), have been isolated from cultures of Huma 7 cells.

The diploid human breast epithelial cell line HMT-3522 derived spontaneously from human benign fibrocystic breast tissue (Briand et al., 1987) was single-cell cloned and the resultant cell line was again single-cell cloned to yield epithelial cell lines (e.g. Huma 121 and Huma 123) similar to the parent line, and a derivative myoepithelial-like cell line, Huma 109, was isolated. They were all obtained and characterized in vitro and as tumour nodules in nude mice in vivo, using procedures exactly as described previously (Rudland et al., 1989b).

Human mammary fibroblasts (HMF) were obtained as described previously (Rudland et al., 1989a).

To confirm that the more elongated cell lines Huma 62 and Human 109 are related to myoepithelial and not to fibroblastic cells, they were subjected to immunofluorescence and immunocytochemical analyses as reported previously (Rudland et al., 1989a,b). The more elongated cell lines Huma 62 and Huma 109 were stained to a variable degree by monoclonal antibody (mAb) to complex keratins, mAb to cytokeratin 14; mAb to smooth muscle actin and a polyclonal antibody raised in rabbits to laminin, whereas the HMFs were unstained (Table 1; and Rudland, 1993).

Table 1. Immunochemical staining of more-elongated cell lines

Antibody* (determinant)	Immunochemical staining†		
	HMF	Huma 62	Huma 109
mAb PKK2 (complex keratins)	−	+	+
mAb CKB1 (keratin 14)	−	+	+
mAb MA-933 (smooth muscle actin)	−	+	+
Anti-laminin	−	+	+

*All monoclonal antibodies (mAb) except anti-laminin, raised in rabbits.

†Immunochemical staining results are a summation of results obtained by immunofluorescence and immunocytochemistry (Materials and Methods); +, some cells stained; −, no cells stained. HMF, human mammary fibroblasts; Huma 62 was derived from Huma 7; Huma 109 from cloned HMT-3522.

Polynucleotide probes

A synthetic cDNA corresponding to the coding region of human bFGF mRNA was purchased from British Biotechnology Ltd. (Oxford, UK) and propagated in pUC18, in cells of *Escherichia coli* strain DH5α. The cDNA was excised from the plasmid with restriction enzymes *Nco*I and *Eco*RI, and separated from the vector DNA by electrophoresis through a low-melting agarose gel (Sambrook et al., 1989). The band corresponding to the bFGF cDNA was radioactively labelled to a specific activity of between 0.4×10^9 dpm/μg and 1.1×10^9 dpm/μg using the method of random-primed synthesis (Feinberg and Vogelstein, 1984).

For the production of single-stranded RNA probes, the bFGF cDNA was subcloned into the phagemid vector pT7T3 (Pharmacia, Uppsala, Sweden). Single-stranded RNA molecules corresponding to, or complementary to, bFGF mRNA were transcribed by either T3 or T7 RNA polymerase, respectively (Sambrook et al., 1989), incorporating [α-^{32}P]UTP to specific activities of about 5×10^9 dpm/μg. The template DNA was removed by treatment of the transcription mixture with ribonuclease-free DNase and the unincorporated radioactivity was removed by precipitation of the probe with ethanol three times.

A cDNA corresponding to rat non-muscle actin (Barraclough et al., 1987) was radioactively labelled using the method of random-primed synthesis (Feinberg and Vogelstein, 1984) to a specific activity of 1×10^9 to 1.5×10^9 dpm/μg, and was used as a constitutive probe to standardize hybridization results.

Isolation of cellular RNA

Total cellular RNA was isolated from cell lines using the guanidinium isothiocyanate method (Han et al., 1987) and was separated from DNA by centrifugation through 5.7 M CsCl (Chirgwin et al., 1979; Barraclough et al., 1987). Total RNA was subjected to either one (Huma 7 and Huma 62) or two (Rama 29, Huma 109, Huma 121 and Huma 123) rounds of affinity chromatography on oligo(dT)-cellulose to enrich for mRNA (Aviv and Leder, 1972). The integrity of mRNA preparations was assessed by the presence of an undegraded band of actin mRNA following size fractionation by electrophoresis through agarose gels, transfer of the RNA to a nylon filter and incubation of the filter with a probe corresponding to rat non-muscle actin (Barraclough et al., 1987).

Detection of specific mRNAs by filter hybridization

Isolated RNA preparations were subjected to electrophoresis in 1.1% (w/v) agarose gels containing 5 mM methylmercury hydroxide (Bailey and Davidson, 1976). A mixture containing RNA molecules of 9.5, 7.5, 4.4, 2.4, 1.3, 0.24 kb in size was also subjected to electrophoresis to provide molecular size markers. The gel was treated with alkali and neutralized (Alwine et al., 1977; Barraclough et al., 1987), and the RNA was transferred to a nylon filter (Hybond, Amersham International, Amersham, UK) using standard blotting procedures (Sambrook et al., 1989). For hybridiz-

ation to DNA probes, prehybridization of the filter for 4 hours at 42°C and hybridization for 16 hours at the same temperature were carried out as previously described (Barraclough et al., 1990). The filter was washed twice for 10 minutes each in 1× SSPE (SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 0.1% (w/v) SDS at room temperature, and once for 2 hours at 65°C. Radioactivity which had bound to the filter was detected by autoradiography against Kodak XAR-5 or Fuji RX X-ray film. For hybridization to RNA probes, the same buffers were used as for the DNA probes. However, prehybridization and hybridization incubations were carried out at 60°C. Filters were washed in 0.1× SSPE, 0.1% (w/v) SDS at 62°C twice for 1 hour each.

In some experiments, increasing amounts from 0.05 to 1.1 μg of RNA preparations were spotted onto filters (Hybond, Amersham International) using a dot-blot apparatus (Bio-Rad). The RNA was coupled to the filter by a brief exposure to ultraviolet light. Duplicate filters, prepared at the same time, were incubated with radioactive bFGF synthetic cDNA or actin cDNA, washed as described above for the northern hybridizations, and the dried filters were subjected to autoradiography.

Radioactivity bound to filters in the northern and dot-blot hybridizations was quantified by scanning the autoradiographic images using a Shimadzu C9000 scanning densitometer, and measuring the peak area of transmitted light. For the dot-blot experiments, the best-fit straight line of a plot of peak area against the amount of RNA/dot over the linear part of the plot was calculated by linear regression (Dunn, 1977).

The results of the hybridization experiments were normalized for possible loading artifacts using a constitutively expressed mRNA. All the cell lines studied expressed two commonly used constitutive mRNAs, namely non-muscle actin and glyceraldehyde-3-phosphate dehydrogenase. In all cell lines, the ratios of non-muscle actin mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA, detected on northern blots, were virtually identical (1.8 ± s.d. 0.096). Thus the relative levels of bFGF mRNA were the same, whichever constitutive probe was used to correct for loading artifacts.

Binding assays of bFGF

Recombinant human bFGF (rhbFGF) was produced in *E. coli* cells and purified to homogeneity as described (Ke et al., 1992). The rhbFGF was iodinated using IODOGEN (Pierce and Warriner, Chester, UK) to a specific activity of 10-26 μCi/μg and used immediately, as described previously for bovine pituitary bFGF (Fernig et al., 1990b). Analysis of the growth-promoting activity of the iodinated bFGF indicated that essentially 100% of the original bFGF activity was recovered.

The binding of ^{125}I-bFGF to cells in monolayer culture was performed as described previously (Fernig et al., 1990b, 1992). Cells were plated at about 3×10^4 per 1.5 cm-diameter well (24-well plates, Sterilin, UK) in 500 μl of the medium appropriate for the cell line. After 48 hours the near-confluent cell monolayers were incubated with increasing concentrations of ^{125}I-bFGF in 200 μl of Binding Medium (DEM supplemented with 10 mM HEPES, pH 7.4 and 0.1% BSA (w/v)) at 4°C for 2 hours, until binding reached a maximum. Non-specific binding was determined by the inclusion of at least 3.2 μg/ml non-radioactive bFGF. Cell-associated radioactivity was solubilized with 0.2 M NaOH and determined in a Wilj gamma counter.

The binding parameters, K_d, and number and type of receptors, could not be determined by Scatchard analysis, since the specific binding of ^{125}I-bFGF to the human mammary cells, as was observed with rat mammary cells, was not saturable at the concentrations of bFGF used (Fernig et al., 1990b, 1992). Therefore the binding parameters were determined with the modelling programme LIGAND (Munson and Rodbard, 1980) as previously described in detail (Fernig et al., 1992).

Extraction of bFGF from cells

bFGF was extracted from approximately 2×10^7 cells by a modification of the method described previously (Barraclough et al., 1990). The extracts were fractionated on a heparin-Sepharose column (Barraclough et al., 1990). The resulting fractions were assayed for their ability to stimulate the incorporation of [^{3}H]thymidine into the DNA of the fibroblastic Rama 27 cells, exactly as described previously (Smith et al., 1984a). Quantification of the amount of growth-stimulatory activity present in the 2 M NaCl heparin-Sepharose eluates in terms of bFGF was accomplished by constructing dose-response curves which were compared to a standard curve obtained with pure rhbFGF (Barraclough et al., 1990).

Immunoblotting of 2 M NaCl heparin-Sepharose eluates

A scaled-up extract from 2×10^8 cells was prepared as described above. After fractionation of the cell extracts on a 1 ml column of heparin-Sepharose, the 2 M NaCl eluates were desalted on a C2 Bond Elut minicolumn (Analytichem International, California) and the protein was eluted sequentially with acidified (0.1%, v/v, trifluoroacetic acid) 20%, 40% and 60% acetonitrile, and finally with isopropanol; the pooled eluates were freeze-dried. The protein was fractionated by electrophoresis on SDS-containing polyacrylamide gels (Laemmli, 1970; Fling and Gregerson, 1986) prior to electrophoretic transfer to nitrocellulose (Towbin et al., 1979). To identify bFGF, the nitrocellulose filters were incubated with a rabbit antiserum to rhbFGF (Joseph-Silverstein et al., 1988) (kindly provided by Drs D.B. Rifkin and D. Moscatelli, New York Cancer Centre), which had been purified on Protein A-Sepharose (Pharmacia). Bound antibody was located with a horseradish peroxidase-conjugated sheep anti-rabbit IgG, which was visualized using the substrates 4-chloro-1-naphthol or diaminobenzidine (Sigma Chemical Company, Poole, UK). Cross-reaction of the anti-bFGF sera with recombinant acidic FGF (aFGF) (Ke et al., 1990) was observed only at concentrations of aFGF that were 100-fold greater than the concentration of bFGF necessary to generate the observed level of immunoreactivity.

Experiments were carried out under the Control of Substances Hazardous to Health (COSHH) regulations as specified by the United Kingdom Health and Safety Executive. The propagation of recombinant DNA in *E. coli* was carried out under conditions of containment level 1 as specified by the United Kingdom Health and Safety Executive.

RESULTS

Levels of mRNA for bFGF and of bFGF activity in SV40-transformed cell lines

Radioactive cDNA corresponding to bFGF mRNA (bFGF probe) hybridized to a single mRNA of 6.5 kb in rat brain mRNA (not shown), similar to that obtained previously (Barraclough et al., 1990). Under the same conditions of hybridization (Materials and Methods), the bFGF probe hybridized strongly to mRNA molecules of 7.1, 3.6, 2.0 and 1.2 kb, in RNA from the myoepithelial-like cell line Huma 62 (Fig. 1, Table 2). The intensities of these bands of bFGF mRNA relative to the band of 7.1 kb were: 1: 0.5 ± 0.1: 0.2 ± 0.04: 0.8 ± 0.5 (mean ± s.d. of 3 observations). Weak hybridization of the probe to mRNA molecules of

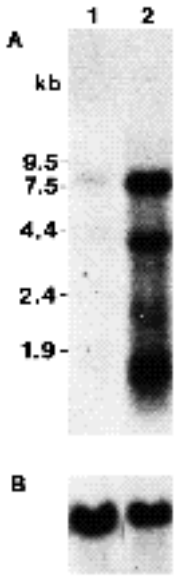

Fig. 1. bFGF transcripts in SV40-transformed human mammary cell lines. Samples of RNA (10 μg) from epithelial Huma 7 (lane 1) and myoepithelial-like Huma 62 (lane 2) cells were subjected to agarose gel electrophoresis, transferred to nylon filters, and incubated with the cDNA corresponding to human bFGF mRNA (A) or with the cDNA corresponding to the mRNA for rat non-muscle actin (B), as described in Materials and Methods. The washed filters were exposed against Kodak XAR-5 film for 12 hours at −70°C with an intensifying screen (A) or against Fuji RX film for 30 minutes at −70°C with an intensifying screen (B). The molecular sizes of RNA markers are shown in kb.

the same molecular size, extracted from the epithelial cell line Huma 7, was also visible (Fig. 1). Quantification relative to actin mRNA of the results of the northern hybridizations and of dot-blot hybridization experiments, incorporating a correction for the variation in the loading of the RNA onto the gel and transfer of RNA to the filter, showed that in three experiments the elongated, myoepithelial-like cell line, Huma 62, contained 23, 21.5 and 16.4 times the amount of bFGF mRNA in the parental epithelial cell line, Huma 7 (Table 2).

Heparin-binding growth factors bind strongly to heparin, so that they are eluted from heparin-Sepharose by concentrations of NaCl between 1.1 M and 2 M (Materials and Methods). When extracts of the same cell lines were fractionated on columns of heparin-Sepharose, growth-promoting activity that stimulated the incorporation of [^{3}H]thymidine into the DNA of rat mammary fibroblasts was observed both in the unbound fraction and in the 2 M NaCl eluate, but not in the 0.6 M NaCl wash (not shown). Quantification of the amounts of heparin-binding growth-stimulatory activity present in the cell extracts, in terms of bFGF, indicated that on a per cell basis the myoepithelial-like cells, Huma 62, contained 80-fold more bFGF-like activity (48 ± 11 ng in 10^6 cells) than the parental Huma 7 cells (0.6 ± 0.2 ng in 10^6 cells) (Table 2).

Levels of mRNA for bFGF and of bFGF activity in cell lines from a human benign breast lesion

To find out whether the pattern of four bands of mRNA for bFGF arose as a consequence of the transformation of the cells by SV40 virus, bFGF mRNA was sought in RNA from spontaneously transformed cloned epithelial cell lines and derivative myoepithelial-like cells isolated from a human benign breast lesion. A single band of hybridization of 6 kb was obtained with RNA from the rat mammary myoepithelial-like cell line, Rama 29 (Fig. 2A). A very strong hybridization signal was obtained with RNA from the representative human myoepithelial-like cell line, Huma 109 (Fig. 2A), whereas it was necessary to expose the film for long periods of time to be able to detect bands of hybrid-

Table 2. Quantification of the relative levels of bFGF mRNA and of bFGF-like activity in SV40-transformed human mammary epithelial cells and their derivative myoepithelial-like cells

	Epithelial cells Huma 7	Myoepithelial-like cells Huma 62
bFGF mRNA		
Northern hybridization		
Total area under bFGF peaks*	21,655	314,809
Total area under actin peak†	106,352	60,810
Relative hybridization†	1.0	23.0
Dot-blots		
Relative hybridization‡		
Experiment 1	1	21.5
Experiment 2	1	16.4
bFGF activity		
bFGF-like activity (ng/10^6 cells ± s.e.m.§)	0.6±0.2	48±11
Ratio¶	1.0	80.0

*RNA was fractionated by agarose gel electrophoresis, transferred to nylon filters and incubated with the human bFGF synthetic gene labelled to high specific activity with [^{32}P]dCTP (Materials and Methods). DNA that hybridized was detected by autoradiography and the autoradiographic images were scanned with a densitometer.

†The filters were subsequently incubated with a ^{32}P-labelled constitutive, actin-specific DNA and the bands of hybridization detected and quantified as for the bFGF. The hybridization to the bFGF mRNA for each sample was corrected for variations in the actin mRNA content, and a ratio of the corrected hybridization of the Huma 62 sample to that of the Huma 7 sample was established.

‡Increasing amounts of RNA were spotted onto Hybond filter membranes using a 'dot-blot' apparatus. The filters were incubated with the human bFGF synthetic gene labelled to high specific activity with [^{32}P]dCTP (Materials and Methods). Radioactivity that hybridized was detected by autoradiography and the autoradiographic images were scanned with a densitometer. Duplicate filters, prepared at the same time, were incubated with ^{32}P-labelled constitutive actin-specific cDNA and the spots of hybridization were detected and quantified as for the bFGF. The best-fit straight line of a plot of scanned peak area/dot against μg RNA/dot was obtained by linear regression (Dunn, 1977). The gradient for the hybridization of the bFGF probe to each RNA sample was corrected for variation in the hybridization to the actin cDNA, and the resultant corrected gradients are expressed relative to the Huma 7 RNA sample for each experiment.

§bFGF-like activity was extracted with 2 M NaCl from the indicated cell lines (Materials and Methods). Dose-response curves were constructed for each 2 M extract, and were compared to a standard dose-response curve for pure human recombinant bFGF, which has an ED_{50} of 100 pg/ml in this assay (ED_{50} is the concentration of bFGF required for half-maximal stimulation of DNA synthesis). The total amount of bFGF-like activity in 1 ml of eluate was calculated. Results are expressed as the mean total ng of bFGF-like activity recovered per 10^6 cells ± s.e.m.

¶Ratio is the ng/10^6 cells bFGF-like activity expressed relative to that of the Huma 7 cell line.

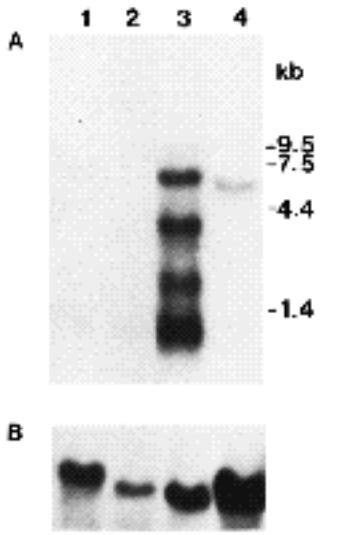

Fig. 2. bFGF transcripts in cell lines from a benign human breast lesion. Samples of RNA (10 μg) from cell lines epithelial Huma 123 (lane 1); epithelial Huma 121 (lane 2); myoepithelial-like Huma 109 (lane 3); and the rat myoepithelial-like cell line Rama 29 (lane 4) were subjected to agarose gel electrophoresis, transferred to nylon filters, and incubated with the cDNA corresponding to human bFGF mRNA (A) or with the cDNA corresponding to the mRNA for rat non-muscle actin (B), as described in Materials and Methods. The washed filters were exposed against Kodak XAR-5 film for 70 hours at room temperature (A) or against Fuji RX film for 30 minutes at −70°C with an intensifying screen (B). The molecular sizes of RNA markers are shown in kb.

ization in the lane loaded with a similar amount of RNA from epithelial cell lines, Huma 121 and 123 (Fig. 2B). The cloned bFGF cDNA hybridized to four bands of mRNA of sizes 6.6 ± 0.4, 3.4 ± 0.3, 1.9 ± 0.2, and 1.3 ± 0.1 kb (mean ± s.d. of 5 determinations) (Fig. 2). The levels of these mRNAs relative to the band of 6.6 kb were: 1: 1.7 ± 0.1: 1.3 ± 0.2: 4.8 ± 2.8, respectively (mean of 3 determinations ± s.d.). Quantification of the northern blots and dot-blot experiments, incorporating a correction for the variation in loading of the RNA and transfer of the RNA onto the filter (Table 3), showed that the myoepithelial-like cell line, Huma 109, contained 100 times the amount of bFGF mRNA in the epithelial cell line, Huma 123.

In common with the level of bFGF mRNA, a higher level of bFGF-like activity was observed in extracts of the myoepithelial-like cell line, Huma 109 (109 ± 79 ng bFGF/10^6 cells), than the epithelial cell line Huma 123 (1.4 ± 0.4 ng bFGF/10^6 cells) (Table 3). The level of bFGF-like activity produced by the myoepithelial-like cell line, Huma 109, relative to the epithelial cell line, Huma 123 (78-fold), reflected the large increase in the relative level of bFGF mRNA in the same cells as determined by either northern hybridization (151-fold) or dot-blot hybridizations (202-fold) (Table 3).

To eliminate the possibility that one or more of the multiple mRNAs for bFGF were transcribed from the opposite strand of the bFGF gene, northern blots were hybridized to single-stranded RNA probes of sequence either identical to, or complementary to, that of bFGF mRNA. The RNA probe

Table 3. Quantification of the relative levels of bFGF mRNA and of bFGF-like activity in epithelial cell lines derived from a benign human breast lesion and a derivative myoepithelial-like cell line

	Epithelial cells		Myoepithelial-like cells
	Huma 121	Huma 123	Huma 109
bFGF mRNA			
Northern hybridization			
Total area under bFGF peaks*	3,983	2,491	392,122
Area under actin peak†	47,006	114,557	119,540
Relative hybridization†	3.9	1.0	151
Dot-blots			
Gradient of slope (±s.d.; cpm/μg RNA)‡			
(a) bFGF	396±96	303±274	97,029±39,063
(b) Actin	12,517±5,524	34,586±4,498	54,863±28,750
Relative hybridization§	3.6	1	202
bFGF-like activity			
bFGF (ng/10^6 cells ± s.e.m.)¶	−	1.4±0.4	109±79
Ratio‖	−	1	78

*RNA was fractionated by agarose gel electrophoresis, transferred to nylon filters and incubated with the human bFGF synthetic gene labelled to high specific activity with [^{32}P]dCTP (Materials and Methods). DNA that hybridized was detected by autoradiography and the autoradiographic images were scanned with a densitometer.

†The filters were subsequently incubated with a ^{32}P-labelled constitutive actin-specific DNA and the bands of hybridization detected and quantified as for the bFGF. The hybridization to the bFGF mRNA for each sample was corrected for variations in the actin mRNA content, and for each experiment a ratio of the corrected hybridization of each sample to that of the Huma 123 sample was established.

‡Gradient of slope is the gradient of the best-fit straight line of a plot of ^{32}P-bound/dot against micrograms RNA applied calculated by linear regression analysis ±s.d. of the slope (Dunn, 1977).

§Relative hybridization is the gradient for each cell line, corrected for variations in the hybridization of the RNA samples to actin cDNA and the resultant corrected gradients are expressed relative to the corrected gradient of the Huma 123 cell line.

¶bFGF-like activity was extracted with 2 M NaCl from the indicated cell lines as described in Materials and Methods. Dose-response curves were constructed for each 2 M NaCl extract, and were compared to a standard dose-response curve for pure human recombinant bFGF, which has an ED_{50} of 100 pg/ml in this assay (ED_{50} is the concentration of bFGF required for half-maximal stimulation of DNA synthesis). The total amount of bFGF activity in lml of eluate was calculated. Results are expressed as the mean total ng of bFGF activity recovered per 10^6 cells ± s.e.m.

‖Ratio is the ng/10^6 cells bFGF for the Huma 109 cell line expressed relative to the Huma 123 cell line.

complementary to the bFGF mRNA hybridized to all four size-classes of mRNA from either the SV40-transformed cell lines or the cell lines of the benign lesion (Fig. 3), whereas there was no detectable hybridization when RNA molecules of identical sequence to the bFGF mRNA were used as a hybridization probe (not shown).

Western blotting of bFGF from the cell lines

To confirm that the activity eluted from heparin-Sepharose by 2 M NaCl was indeed bFGF, extracts prepared from the cells were immunoblotted with an anti-bFGF serum (Materials and Methods). The results clearly demonstrated the presence of an 18-19 kDa band of bFGF migrating at the same position as authentic recombinant bFGF in both the

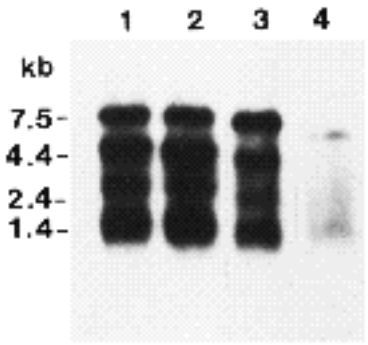

Fig. 3. Northern hybridization of cell-line RNA to strand-specific bFGF probes. Samples of RNA (10 μg) from the human myoepithelial-like cell lines Huma 109 (lanes 1 and 2); Huma 62 (lane 3), and the rat myoepithelial-like cell line, Rama 29 (lane 4), were subjected to agarose gel electrophoresis, transferred to nitrocellulose filters and incubated with a single-stranded RNA probe complementary to bFGF mRNA (Materials and Methods). The washed filters were exposed against Fuji RX X-ray film for 24 hours at −70°C with an intensifying screen. The molecular sizes of RNA markers are shown in kb.

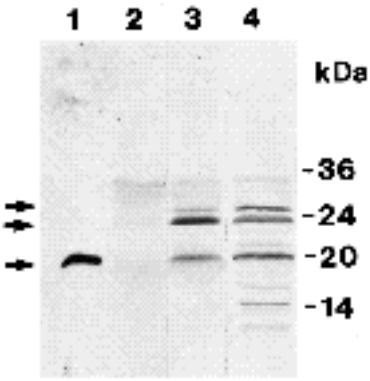

Fig. 4. Detection of bFGF in human cell lines by immunoblotting. Immunoblots of 2 M NaCl eluates of cell extracts fractionated on columns of heparin-Sepharose from an uncloned epithelial cell line HMT 3522 (lane 2); myoepithelial-like Huma 62 (lane 3); myoepithelial-like Huma 109 (lane 4) were incubated with anti-bFGF serum and bound antibodies detected as described in Materials and Methods. 100 ng human recombinant bFGF (lane 1) is present as a marker. All these lanes were from a single immunoblot. Major immunoreactive bands are indicated by arrows. The apparent molecular sizes of protein markers are shown in kDa.

extracts prepared from the myoepithelial-like cells containing relatively high levels of active bFGF (Fig. 4).

Extracts from the myoepithelial-like cell lines (Huma 62 and Huma 109) contained additional prominent bands of bFGF corresponding to molecular weights of 24 and 26 kDa (Fig. 4). In addition, the Huma 109 cells contained low-molecular-weight immunoreactive bands (Fig. 4). Faint bands with a molecular weight between 19 kDa and 34 kDa were also sometimes visible on the immunoblot (Fig. 4).

bFGF-binding sites in cell lines

When the SV40-transformed cell lines were tested for their ability to bind bFGF on their surfaces, none of the cell lines showed detectable binding (not shown), a result consistent with a low level of bFGF receptor mRNA detected in these cells (results not shown).

Saturation binding experiments of recombinant human bFGF failed to detect high- or low-affinity receptors in the epithelial cell line Huma 123, but both high- and low-affinity receptors for bFGF were detected on the myoepithelial-like cell line Huma 109 with K_d values of 24 pM and 11 nM, respectively (Table 4).

Table 4. Dissociation constants and numbers of receptors for bFGF on human mammary cell lines

	Cell line	
	Huma 123 epithelial-like	Huma 109 myoepithelial-like
High-affinity receptors		
K_d (pM) ±s.e.m.*	ne†	24±23‡
Receptors per cell (×10^3) ±s.e.m.	ne†	11±10‡
Low-affinity receptors		
K_d (nM) ±s.e.m.*	ne†	11±10‡
Receptors per cell (×10^6) ±s.e.m.	ne†	0.9±0.8‡

*Mean ± s.e.m. of values calculated from 3 independent experiments.
†No evidence, since no specific ^{125}I-bFGF binding was observed.
‡A two-site model fitted the saturation binding data for the Huma 109 cell line considerably better than a one-site model (P<0.005) with no deterioration in other measures of goodness of fit, when analyzed with the ligand programme (Munson and Rodbard, 1980).

DISCUSSION

We have previously shown that the level of mRNA for bFGF is increased in rat mammary myoepithelial-like cell lines relative to their parental epithelial cells. The increased amount of mRNA is accompanied by a similar increase in the level of bFGF activity within these cells (Barraclough et al., 1990). In the present paper, these observations have been extended to paired cloned epithelial and derivative myoepithelial-like cells derived from two equivalent human systems: one of SV40-transformed cell lines of normal human mammary origin, and the other of spontaneously transformed cell lines derived from a human benign breast lesion. Immunochemical staining has confirmed their myoepithelial rather than fibroblastic origin (Rudland, 1993). In both these human systems, the myoepithelial-like cell lines express abundant levels of bFGF mRNA, whereas the parental epithelial cell lines express almost undetectable levels of bFGF mRNA.

A major difference between the rat and the human cell lines with respect to bFGF mRNA is the presence of a single bFGF mRNA of 5.7-6 kb in the rat cells (Barraclough et al., 1990) compared to four discrete mRNAs in the human cell lines. Within experimental error, the sizes of the four mRNAs in cell lines from the benign lesion are the same as those for mRNA from the SV40-transformed cell lines. These multiple transcripts are present in the myoepithelial cells of the SV40-transformed human cell lines and also in those derived from the human benign lesion. Multiple species of bFGF mRNA of 7.5, 4.4 and 2.2

kb in human mammary epithelial cells (Li and Shipley, 1991), mRNAs of 7, 3.7, 2.9 and 1.8 kb in human adrenal carcinoma cells (Corin et al., 1990) and mRNAs of 7 and 3.7 kb in human astrocytoma cells (Murphy et al., 1989) and human mammary fibroblasts (Valverius et al., 1990) have been reported previously. Heterogeneity at the 3′ end of the mRNA for bFGF in human foreskin fibroblasts has been shown to be due to the utilization of different polyadenylation sites (Kurokawa et al., 1987). In the present experiments, the four detectable mRNAs are shown to arise from transcription of the same strand of DNA; a result that strongly suggests the absence of any stable reverse transcript, such as that of 1.5 kb identified in *Xenopus* oocytes (Kimelman and Kirschner, 1989; Volk et al., 1989).

The immunoblot analysis of the proteins present in the eluates from the columns of heparin-Sepharose clearly shows that immunoreactive 18-19 kDa bFGF is present in all those cell lines tested that express bFGF mRNA and contain heparin-binding growth-stimulatory activity. Moreover, the amount of bFGF immunoreactivity (Fig. 4) is in agreement with the amount of heparin-binding growth-stimulatory activity in the 2 M NaCl eluates (Tables 2 and 3). Since immunoblotting is a qualitative technique, other heparin-binding growth factors, besides bFGF, may also be present in the 2 M NaCl eluates, but they are anticipated to be only minor contributors to the total amount of growth-stimulatory activity recovered from the columns of heparin-Sepharose. Thus the majority of the heparin-binding growth-stimulatory activity in the myoepithelial-like cells is likely to be bFGF. In addition to the immunoreactivity with an M_r of $18\text{-}19\times10^3$, there are also immunoreactive, higher molecular mass forms of bFGF present in the extracts. These additional forms may represent translation products of bFGF mRNA initiated from two of the three alternative CUG codons, upstream of the AUG initiation codon (Florkiewicz and Sommer, 1989; Prats et al., 1989; Sommer et al., 1989).

All these experiments show that in two independent human mammary cell systems, the myoepithelial-like cells produce vastly increased levels of bFGF and its mRNA relative to their parental epithelial cells. Recently, bFGF has been immunocytochemically localized to the extracellular matrix of the normal human breast and benign human mammary tumours (Gomm et al., 1991). The results obtained in the present paper suggest that, as in the rat (Barraclough et al., 1990), the source of some of this extracellular bFGF may be the myoepithelial cells of the mammary parenchyma.

Cell-surface high- and low-affinity receptors for bFGF are detected on the myoepithelial-like Huma 109 cells (Table 3) but undetectable on the cloned epithelial cell line, Huma 123. The binding assay readily detects 10^3 high-affinity and 10^5 low-affinity receptors in other mammary cell lines (Fernig et al., 1990b, 1992, 1993). Although the identity of the low-affinity receptor for bFGF in the myoepithelial-like cell line is not known, in the equivalent rat cells, it has been shown to be heparan sulphate glycosaminoglycans (Fernig et al., 1992), as in other cell systems (Moscatelli, 1987). The heparan sulphate glycosaminoglycan low-affinity receptor for bFGF has recently been shown to be necessary for the activation of bFGF, enabling it to bind at least to the *flg* high-affinity receptor (Rapraeger et al., 1991; Yayon et al., 1991). However, recent evidence (Nugent and Edelman, 1992; Kan et al., 1993) suggests that receptors such as *flg* are not high-affinity in themselves, but only through association with heparan sulphate glycosaminoglycans. Hence the high-affinity receptors identified by binding assays may consist of a complex of FGF tyrosine kinase receptors and heparan sulphate glycosaminoglycans.

The presence of low-affinity receptors on the myoepithelial-like cell lines derived from the spontaneously transformed mammary lesion provides an explanation for the preliminary observation of immunocytochemical staining of the extracellular matrix of normal human breast and benign human mammary tumours (Gomm et al., 1991) and suggests that in the human, as in the rat (Barraclough et al., 1990), bFGF may become sequestered in the extracellular matrix in the region of the myoepithelial cell/basement membrane of the human mammary parenchyma. From the results obtained with the rat mammary cell lines, it has been suggested that the remodelling of the basement membrane during normal growth and development of the ductal tree of the mammary gland may lead to the release of sequestered bFGF, which may then act on the cells of the developing terminal ductal structures (Barraclough et al., 1990). A similar situation may exist during the local uncontrolled growth of the parenchymal cells in benign breast lesions.

In conclusion, the production of bFGF and its mRNAs in human cell lines derived from SV40-transformed normal mammary glands and a spontaneously transformed benign mammary lesion is considerably higher in the myoepithelial-like compared with the epithelial cells. Since the myoepithelial-like cell lines have been derived from the epithelial cell lines, these results support the suggestion that differentiation towards the myoepithelial phenotype in culture is associated with the production of bFGF, and that bFGF may play a major role in normal mammary development.

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