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IMPLICATIONS OF CYTOKERATIN 8/18 FILAMENT FORMATION IN STRATIFIED EPITHELIAL CELLS: INDUCTION OF TRANSFORMED PHENOTYPE

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The cytokeratin (CK) pair 8 and 18 is normally expressed in all simple epithelia. This pair is not normally seen in stratified epithelial cells. Squamous cell carcinomas derived from stratified epithelia show anomalous expression of this CK pair. It is not known whether CKs 8 and 18 in any way contribute to the malignant phenotype of these cells. We used an immortalised, nontransformed human foetal buccal mucosa (FBM) cell line that expresses significantly higher amounts of CK18 compared to CK8. FBM cells were transfected with the full-length *CK8* gene to study the role of CKs 8 and 18 in malignant transformation. Clones with higher expression of CK8 compared to untransfected FBM cells were studied for changes in their phenotypic characteristics. Immunofluorescence studies using antibodies to CKs 8 and 18 revealed well-decorated filaments throughout the cytoplasm in CK8 gene–transfected cells *vs.* untransfected FBM cells. Transmission images showed that FBM cells were isolated while transfected cells were in groups of well-spread cells with cellular projections. Transfected cells were independent of growth supplement requirements and showed anchorage-independent growth in soft agar assay and significantly reduced doubling time compared to nontransfected FBM cells. DNA flow-cytometric studies revealed increased DNA content and prolonged S phase in transfected clones *vs.* FBM cells. Injection of cells s.c. obtained from soft agar colonies developed from 2 of the clones resulted in tumour formation at the site of injection. In both cases, lung metastasis was also seen. Thus, in conclusion, it appears that increased expression of CK8 in some way changes the phenotypic characteristics of stratified epithelial cells, resulting in malignant transformation.
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Key words: *cytokeratin 8; cytokeratin 18; stratified epithelium; transfection; transformation*

CKs are IF proteins specifically expressed by epithelial cells. They occur as a cytoplasmic network of 10–12 nm wide IFs. These proteins are encoded by a large multigene family that includes more than 50 individual members.[1] These are expressed in a tissue-specific and differentiation-dependent manner.[2–6] Moll *et al.*[2] in 1982 catalogued CKs from different human epithelia according to their molecular weights and isoelectric points and numbered them 1–19. Over the years, 4 more members of the acidic CK family have been detected, increasing the number to 23.[7] For filament formation, expression of at least one member of each subfamily is essential.[2,8] Their tissue-specific expression is well illustrated by the fact that simple epithelia like liver and pancreas express CKs 8 and 18,[2,9] while all stratified epithelia express CKs 5 and 14.[2,5,10,11] Nonkeratinising stratified epithelia like buccal mucosa and alveolar mucosa express CKs 4 and 13 along with CKs 5 and 14, while keratinising stratified epithelia like tongue, palate and skin express CKs 1 and 10 along with CKs 5 and 14.[2,5,10,11] Because of their differentiation-dependent and tissue-specific expression, CKs are being used as markers in the diagnosis of various epithelial disorders, including cancer.[12–14]

CK8/18 is the first pair expressed in the process of embryogenesis.[15] CK8/18 is unique in many ways. All type I *CK* genes are located on chromosome 17, while all type II *CK* genes are situated on chromosome 12. Although CKs 8 and 18 belong to the type II and type I subfamilies, respectively, both are found on chromosome 12.[16] Expression of CK18 is regulated by transcription factors belonging to the AP1 and ETS families, which also regulate expression of the oncogenes of the *ras* pathway.[16] Forced expression of this pair in mouse fibroblasts along with vimentin resulted in increased resistance to various chemotherapeutic drugs acting by different mechanisms.[17] Melanomas expressing this CK pair along with vimentin are more invasive than those expressing only vimentin.[18] Some regulatory proteins, *e.g.,* those belonging to the *14-3-3* family, bind to *CK18* in a phosphorylation-dependent and cell cycle–regulated manner.[19] Heat shock proteins like hsp70 bind to CK18 in a phosphorylation-dependent manner under stress.[19] CKs 8 and 18 are normally expressed by simple epithelia, while stratified epithelia do not express this pair. SCCs derived from stratified epithelia show aberrant expression of these CKs.[9,16,20,21] All of these facts suggest that this CK pair, apart from imparting structural integrity to the cells, would also have some regulatory functions. It is not yet known whether anomalous expression of this CK pair contributes to the malignant phenotype of cells.

In the present study, we used an immortalised, nontransformed human FBM cell line, which expresses significantly higher amounts of CK18 compared to CK8. FBM cells were transfected with the full-length *CK8* gene to study the role of CKs 8 and 18 in malignant transformation. Our results show that increased CK8 expression in FBM cells changes the phenotypic characteristics of this cell line towards those of transformed cells.

MATERIAL AND METHODS

Establishment of FBM cell line

A spontaneously aborted 3-month-old fetus was obtained from the maternity ward of Nowrosjee Wadia Hospital (Mumbai, India). It was dissected under aseptic conditions, and the buccal mucosa was incised from the oral cavity and collected in PBS. Tissue was

Abbreviations: CK, cytokeratin; CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole; ECL, enhanced chemiluminescence; FBM, fetal buccal mucosa; IF, intermediate filament; IMDM, Ischove's modified Dulbecco's medium; MAb, monoclonal antibody; PVDF, polyvinylidene difluoride; SCC, squamous cell carcinoma; TBS, TRIS-buffered saline; TBST, TRIS-buffered saline with 0.1% Tween-20.

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rinsed 2–3 times with PBS and cut finely in a watch glass. To the chopped tissue, 1 ml of 400 units/ml collagenase-type I in plain DMEM with antibiotics was added, and the mixture was incubated overnight at 37°C. Enzyme-digested pieces were collected in a tube and rinsed with plain DMEM. The suspension was centrifuged for 10 min at 1,800*g*. The supernatant was removed and the cell pellet suspended in 1.5 ml of complete medium. The suspension was allowed to sediment for 10–15 min. The top 0.5 ml of the suspension was transferred to a 35 mm Petri dish (F1), the middle 0.5 ml to a second dish (F2) and the bottom 0.5 ml, the most cell clump-rich fraction, to a third dish (F3). Cells were allowed to adhere, and after 5–6 hr, an additional 1 ml of medium was added. The next day, nonadherent floating cells from all 3 dishes were collected in a tube and centrifuged at 100*g* for 10 min. Dishes with adherent cells were fed with complete medium. Nonadherent pelleted cells were suspended in complete medium, and the process was repeated. Cultures were washed gently with PBS and fed every second day. F1 and F2 dishes had more fibroblasts with few epithelial patches. Epithelial cells were separated from fibroblasts by partial trypsinisation. The F3 dish had predominantly epithelial cell patches by the fourth day. Complete medium supplemented with CM suppressed differentiation of epithelial cells. Fibroblasts were completely eliminated from culture by 6 weeks. Cells became independent of CM after 5 months, after which CM was deleted from the medium. FBM cells were grown in IMDM containing 10% FCS, penicillin G sodium 100 units/ml, streptomycin sulphate 0.1 mg/ml, amphotericin B 0.25 μg/ml and supplement (transferrin 0.1%, insulin 0.05%, hydrocortisone 4 mg%, BSA 1%, sodium selenate 0.05 mg% and epidermal growth factor 0.05 mg%; all from Sigma, St. Louis, MO).

The FBM cell line is an immortalised, nontransformed line with characteristics typical of epithelial cells, like CK IFs and desmosomal junctions between cells.

Transfections

A *CK8* gene construct (generous gift from Dr. R.G. Oshima, The Burnham Institute, La Jolla, CA) containing the full-length *CK8* gene along with PECE vector and SV40 promoter was used for transfection of FBM cells. Initially, *Escherichia coli* JM109 cells were transformed with the *CK8* gene construct to amplify CK8 plasmid. FBM cells were transfected with CK8 plasmid DNA along with the neomycin-resistant gene *PBKNeo* at a 10:1 ratio using lipofectamine plus reagent (Life Technologies, Gaithersburg, MD). Fifteen G418-resistant clones were obtained.

Isolation of CKs

CKs were isolated from FBM cells using the method described by Achtstaetter *et al.*[22] Briefly, cells collected after trypsinisation were homogenized in detergent buffer [10 mM TRIS-HCl, 140 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100 (pH 7.6)] with 10 μl/ml of protease inhibitor cocktail (Calbiochem, La Jolla, CA) and kept on ice for 30 min with intermittent shaking. Homogenate was centrifuged at 2,800*g* for 10 min at 4°C. High salt buffer [10 mM TRIS-HCl, 140 mM NaCl, 1.5 M KCl, 5 mM EDTA, 0.5% (w/v) Triton X-100 (pH 7.6)] with protease inhibitor cocktail (10 μl/ml) was added to the pellet and centrifuged at 2,800*g* for 10 min at 4°C. An insoluble cytoskeletal pellet was obtained after centrifugation, which was washed thrice with 10 mM TRIS-HCl (pH 7.6) with protease inhibitor cocktail (10 μl/ml).

2-D PAGE of CKs isolated from FBM cells

The insoluble cytoskeletal pellet was dissolved in lysis buffer containing 9.5 M urea. First-dimension isoelectric focusing was performed as described by O'Farrell *et al.*[23] using a combination of Ampholytes (Sigma) with pH ranges of 5–8 and 3–10 in a 2:1 ratio. Gels were loaded onto 10% SDS-PAGE after equilibration in 2 × Laemmli sample buffer. Gels were stained with 0.2% Coomassie brilliant blue.

Western blotting

All samples were solubilized in Laemmli's sample buffer[24] before loading and run on denaturing 10% SDS polyacrylamide gels. Protein bands were transferred to Immobilon PVDF membranes (Millipore, Bedford, MA) using the method described by Towbin *et al.*[25] Membranes were blocked with 1% BSA in TBS for 1 hr. Blots were incubated in MAb (1:1,000) recognising both CKs 8 and 18 (clone C-51; Santa Cruz Biotechnology, Santa Cruz, CA). After 3 washes with TBST, blots were incubated for 1 hr in goat antimouse IgG (40 mU/ml; Amersham, Arlington Heights, IL) in TBS with 0.5% BSA. Blots were washed thrice with TBST. Detection was performed by the commercially available ECL detection system (Amersham). Relative concentrations of CKs 8 and 18 were determined from developed immunoblots by densitometry. All clones were characterised for expression of CK18 and increased expression of CK8. Of 15 clones, 7 showed increased expression of CK8. These clones were labelled C1–C7.

Immunofluorescence

FBM cells and transfected FBM clones were grown on coverslips for 48 hr. Cells were fixed in chilled acetone for 10 min at –20°C and permeabilised in chilled acetone with Triton X-100 (0.3% w/v, Sigma) for 3 min. Nonspecific binding was blocked with 5% BSA in PBS for 30 min at room temperature. FBM cells were incubated in MAb specific to CK4 (clone 6B10) and CK14 (clone CKB1, both from Sigma) for 1 hr in a moist chamber. Both FBM and transfected cells were incubated in MAb specific to CKs 8 (clone M20) and 18 (clone CY90, both from Sigma) for 1 hr in a moist chamber. Excess antibody was washed in PBS, and cells were then incubated with a FITC-conjugated goat antimouse IgG (Sigma) diluted in PBS for 1 hr at room temperature in the dark in a moist chamber. Excess antibody was washed with PBS, and coverslips were mounted in antiquenching mounting agent (Pelco International, Redding, CA) and examined with a laser confocal microscope (Bio-Rad, Richmond, CA).

Soft agar assay

All clones showing high CK8 expression were tested for anchorage-independent growth using a soft agar colony-forming assay. One millilitre of the basal layer of 0.5% agar in complete IMDM was prepared in 30 mm Petri plates. Cells (1×10^4) in complete medium containing 0.3% agar were seeded over the basal layer. Plates were incubated at 37°C in a CO_2 incubator for 15 days and observed microscopically for opaque, dense colonies. Colonies were counted on day 15. The assay was repeated thrice with 3 replicates each time.

Growth supplement requirement of transfected cells

Clones showing higher expression of CK8 than normal FBM cells were grown in IMDM containing 10% FCS but deficient in supplement, to study their dependence on growth supplement. FBM cells were also grown in medium without supplement.

Cell doubling time

Cell doubling time was determined by counting viable cells from freshly trypsinised monolayers. Three 30 mm Petri dishes, each with 2×10^5 cells, were used for each time point (0, 24, 48 and 72 hr). Throughout the procedure, cell viability was determined by the erythrocin B exclusion method. The experiment was repeated thrice. Doubling time was calculated by plotting the graph of cell count *vs.* time using the third-degree polynomial.

DNA flow cytometry

For flow cytometry, cells were trypsinised, washed with PBS, fixed in 70% ice-cold ethanol and stored overnight at 4°C. Fixed cells were stained with 0.01% DAPI (Sigma) for measurement of DNA content. Fluorescence was analysed by flow cytometry using the Becton Dickinson (Mountain View, CA) flow cytometer. Only

cells exhibiting a light scatter typical of intact cells were analysed, and debris was excluded from the study by electronic gating.

Electron microscopy

For ultrastructural studies, cells grown for 24 hr were fixed with 3% glutaraldehyde and 1% osmium tetraoxide (Pelco). After dehydration, cells were embedded in resin (Spurr's Kit, Pelco) and polymerised. Ultrathin sections were cut, mounted on copper grids, contrasted with uranyl acetate and lead citrate and examined under a transmission electron microscope (Zeiss-109; Zeiss, Thornwood, NY) at 50 kV.

Injections in nude mice

NMRI nude mice (6–8 weeks old) were procured from the National Institute of Virology (NIV) (Pune, India). They were maintained in positive pressure isolators (Harlan, Bicester, UK) under standard conditions, *i.e.,* 22 ± 2°C, 55 ± 5% relative humidity and a 12 hr dark/12 hr light cycle each day. All animals were fed a standard in-house pelleted diet and drinking water *ad libitum.*

Animals were injected s.c. with 4×10^6 cells in 300 μl PBS and observed for 4 months for tumour formation. In another experiment, clones C3 and C6 were grown in soft agar as described earlier. Cells (1×10^6) obtained from soft agar colonies were suspended in 300 μl PBS injected s.c. in nude mice and observed for tumour formation. When the tumour achieved a certain size at the end of 2 months, animals were killed by CO_2 anaesthesia and the tumour was exposed from the skin. The tumour piece along with the skin part were incised and cut into pieces. Tumour pieces along with other vital organs like lung, liver, kidneys, spleen and pancreas were collected in 10% formalin solution for histopathology. Samples were processed routinely and embedded in paraffin, and 6-μ-thick sections were stained with haematoxylin and eosin and observed under an optical microscope.

RESULTS

The immortalized nontransformed cell line FBM was established from normal human FBM in our laboratory. The cell line did not show anchorage-independent growth and did not produce tumours in nude mice. FBM cells were characterized for CK expression using 1- and 2-D SDS-PAGE and Western blotting with polyclonal antiserum to CK raised in rabbits in our laboratory. FBM expresses CKs 4, 6, 8, 14 and 18. The 2-D gel electrophoresis pattern of CK expressed by FBM cells is shown in Figure 1. Immunofluorescence staining using MAb to stratified epithelial CK4 (clone 6B10) (Sigma) and CK14 (Sigma) (clone CKB1) showed cytoplasmic filamentous staining (Fig. 2*a,b*). CKs 8 and 18 constitute 35% of the total CKs in FBM.

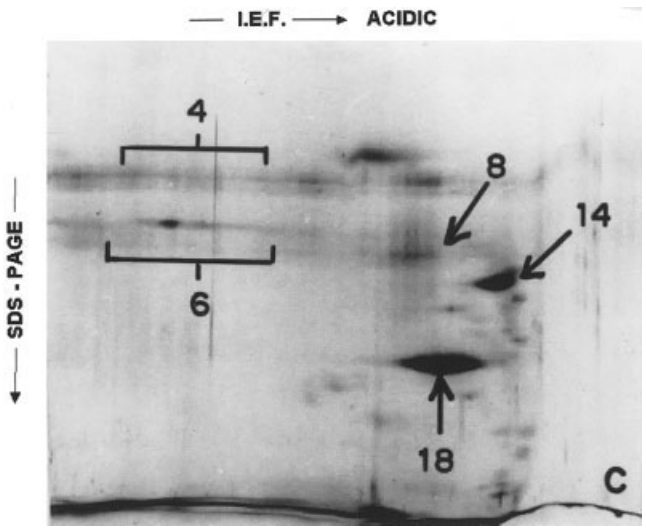

Figure 1 – 2-D gel electrophoresis pattern of CK in FBM cells. Numerals indicate CK numbers.

Status of CKs 8 and 18 in FBM cells

The FBM cell line showed higher expression of CK18 than of CK8 when tested by Western blotting using antibody specific to both CKs. Expression of CKs 8 and 18 in FBM cells was studied by immunofluorescence. In FBM cells, MAb specific to CK8 (clone M20) showed diffuse staining, while filamentous staining was seen with antibody specific to CK18 (clone CY90). (Fig. 3*a,b*). For proper filament formation of CK8/18, it is essential to have equimolar expression of the 2 CKs.[8] Hence, the *CK8* gene was transfected in FBM cells to increase CK8 expression.

Status of CKs 8 and 18 in transfected clones

CK8 gene constructs were transfected in FBM cells using lipofectamine plus reagent. Empty vector was used as a negative control. Fifteen clones of CK8 gene–transfected FBM cells were obtained using G418 selection agent. All 15 clones were characterised for CK8/18 expression using Western blotting. Relative concentrations of CKs 8 and 18 were determined by densitometry of Western blots. In 7 of 15 clones analysed, CK8:CK18 ratios were higher than in untransfected FBM cells and comparable to

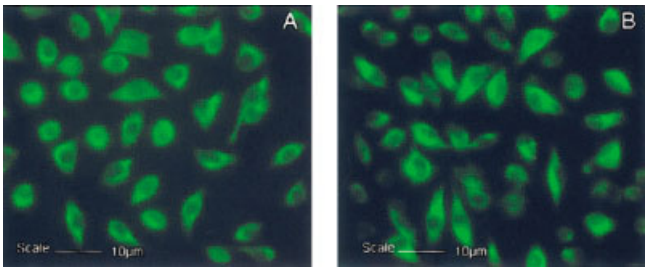

Figure 2 – Immunofluorescence staining of CK4 *(a)* and CK14 *(b)* in FBM cells. Images were captured on a metasystems imaging workstation. Bar = 10 μm.

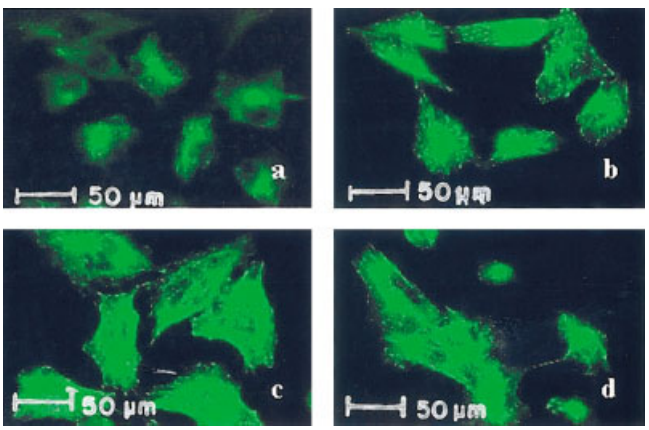

Figure 3 – Immunofluorescence staining of CKs 8 and 18 in FBM cells and CK8 gene–transfected FBM clone C2 using a laser confocal microscope. All confocal images were cut into several focal planes and stacked. *(a)* CK8 staining in FBM cells using MAb to CK8 (clone M20). *(b)* CK18 staining in FBM cells using MAb to CK18 (clone CY-90). *(c)* CK8 staining in CK8 gene–transfected FBM clone C2 using MAb to CK8 (clone M20). *(d)* CK18 staining in CK8 gene–transfected FBM clone C2 using MAb to CK18 (clone CY-90). Bars = 50 μm.

those in HeLa cells. The HeLa cell line, derived from cervical carcinoma, was used as a positive control. Cervical epithelium is a simple epithelium in which CKs 8 and 18 are expressed normally. The 7 clones showing higher CK8:CK18 ratios were labelled C1–C7. CK18 was used as an internal control. The representative Western blot demonstrating CK8 and CK18 expression in untransfected FBM, transfected FBM clones and HeLa cells is shown in Figure 4. In FBM cells, the CK8:CK18 ratio was 0.32, while in HeLa cells, the ratio was 0.85. In empty vector transfectants, the ratio was similar to that in FBM cells. In CK8-transfected FBM clones, the ratio ranged 0.84–1.26. (Table I).

Immunofluorescence staining of CK8-transfected FBM clones using MAb specific to CK8 (clone M20) and CK18 (clone CY-90) showed well-decorated cytoplasmic filaments (Fig. 3*c,d*). The staining pattern of cells transfected with empty vector was similar to that in FBM cells. Filamentous staining was seen in all transfected clones compared to the diffuse staining seen in FBM cells, indicating that increased CK8 expression resulted in CK8/18 filament formation in the clones.

Phenotypic characterisation of CK8-transfected FBM clones

The 7 transfected clones showing higher CK8 expression were studied further for their phenotypic characteristics.

Transfected cells do not require supplement

FBM cells were grown in IMDM supplemented with growth supplements (Fig. 5*a*). Morphologic changes with degeneration were observed in FBM cells grown without supplement (Fig. 5*b*). Transfected clones grew and proliferated without any morphologic changes in the absence of supplement (Fig. 5*c,d*), thus showing supplement independence. Growth of empty vector transfectants was dependent on supplement.

Anchorage-independent growth of transfected FBM cells

All 7 clones were tested for anchorage-independent growth in soft agar assay. All clones consistently showed considerably higher colony counts compared to untransfected FBM cells. Figure 6 illustrates the degenerating cells of FBM in soft agar (Fig. 6*a*) and the soft agar colonies of clone C2 (Fig. 6*b*). Also, in empty vector transfectants, cells degenerated. Average colony counts of clones and FBM cells are given in Table II. The lowest number of colonies (77) was seen in clone C6, while the highest colony number (200) was obtained with clone C4.

Reduction in doubling time of transfected FBM cells

Doubling time was calculated for untransfected FBM cells as a control and for CK8-transfected clones. Average doubling times of the 7 transfected FBM clones from 3 experiments are shown in Table III. In 3 clones, the doubling time was nearly half that of FBM cells. Clone C3 showed the lowest doubling time (20.22 hr), while the highest doubling time (32.09 hr) was found in clone C5. The doubling time of FBM cells was 46.03 hr, while in empty vector transfectants it was 45.05 hr. Thus, significant reduction in

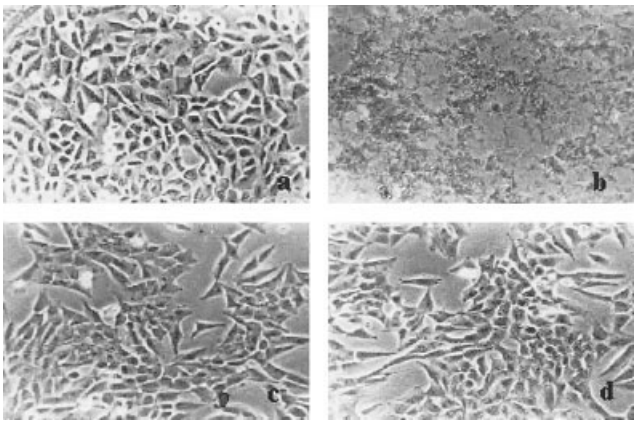

FIGURE 5 – Growth supplement requirement of FBM cells and CK8 gene–transfected FBM cells. *(a)* FBM cells grown in IMDM containing 10% FCS and supplement. *(b)* Degenerating FBM cells in IMDM containing 10% FCS but deficient in supplement. *(c)* CK8 gene–transfected FBM cells (clone C2) grown in IMDM containing 10% FCS and supplement. *(d)* CK8 gene–transfected FBM cells (clone C2) showing no change in morphology when grown in IMDM containing 10% FCS but deficient in supplement.

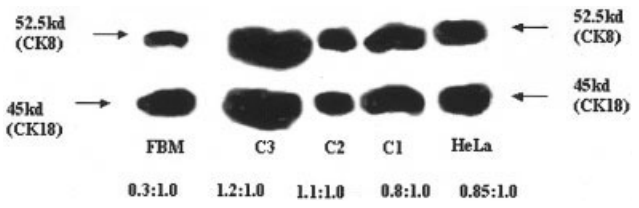

FIGURE 4 – Western blot of FBM cells, CK8 gene–transfected FBM clones C1–C3 and HeLa cells using MAb specific to both CKs 8 and 18 (c-51).

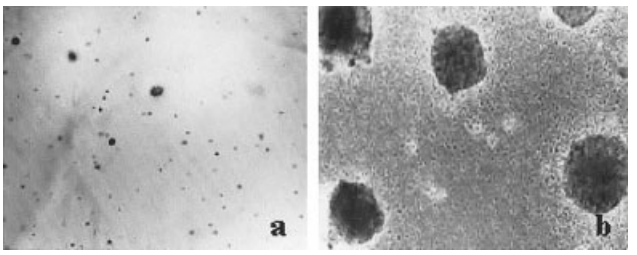

FIGURE 6 – Soft agar assay. *(a)* Degenerating cells of FBM in soft agar. *(b)* Colonies of clone C2 in soft agar.

TABLE I – EXPRESSION OF CK8 AND CK18 IN UNTRANSFECTED AND TRANSFECTED FBM CELLS

Clone	CK8:CK18 ratio
C1	0.84
C2	1.12
C3	1.26
C4	1.14
C5	1.15
C6	0.97
C7	1.18
FBM	0.32
HeLa	0.85
Empty vector transfectant	0.39

TABLE II – SOFT AGAR ASSAY OF UNTRANSFECTED AND TRANSFECTED FBM CELLS

Clone	Average number of colonies
C1	110
C2	190
C3	152
C4	200
C5	79
C6	77
C7	122
FBM	1
HeLa	>500
Empty vector transfectant	1

TABLE III – DOUBLING TIME OF UNTRANSFECTED AND TRANSFECTED FBM CELLS

Clone	Doubling time (hr)
C1	31.77
C2	25.43
C3	20.22
C4	23.04
C5	32.09
C6	30.89
C7	23.74
FBM	46.03
Empty vector transfectant	45.03

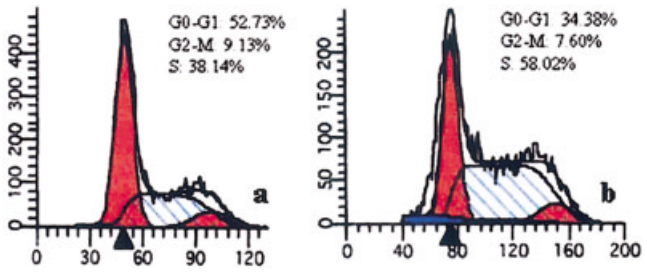

FIGURE 7 – DNA content in *(a)* FBM cells and *(b)* CK8 gene–transfected FBM clone (C2). Clone C2 shows a higher DNA content and longer S phase than untransfected FBM cells.

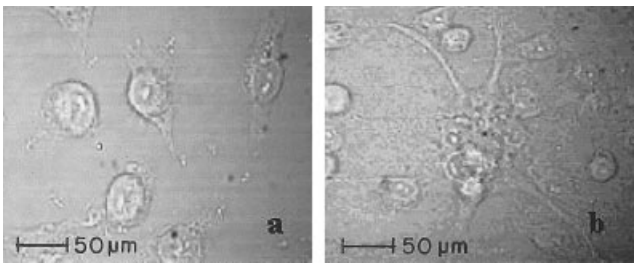

FIGURE 8 – Transmission images of *(a)* FBM cells isolated with no cellular projections and *(b)* CK8 gene–transfected FBM cells (clone C2) in groups showing cellular projections under a laser confocal microscope. Bars = 50 µm.

doubling time was observed in transfected clones compared to untransfected FBM cells.

Increased DNA synthesis in transfected FBM clones

DNA flow-cytometric studies indicated higher DNA content in transfected clones of FBM than normal FBM cells. S phase in the cell cycle of clones was also longer than in FBM cells (Fig. 7). Empty vector transfectants showed DNA content similar to that of FBM cells.

Optical microscopy of transfected and untransfected cells

Transmission images under the laser confocal microscope showed that FBM cells were isolated (Fig. 8*a*), while transfected cells were seen in groups of well-spread cells. Cellular projections typical of the transformed phenotype were also observed in transfected cells (Fig. 8*b*).

Ultrastructural studies

Ultrastructural observations showed that FBM cells were isolated while transfected clones were in tightly packed groups (Fig. 9*a,b*). The nucleus:cytoplasm ratio was changed in transfected cells compared to untransfected cells (Fig. 9*c,d*). The number of vacuoles and activated mitochondria was increased in transfected cells compared to FBM cells.

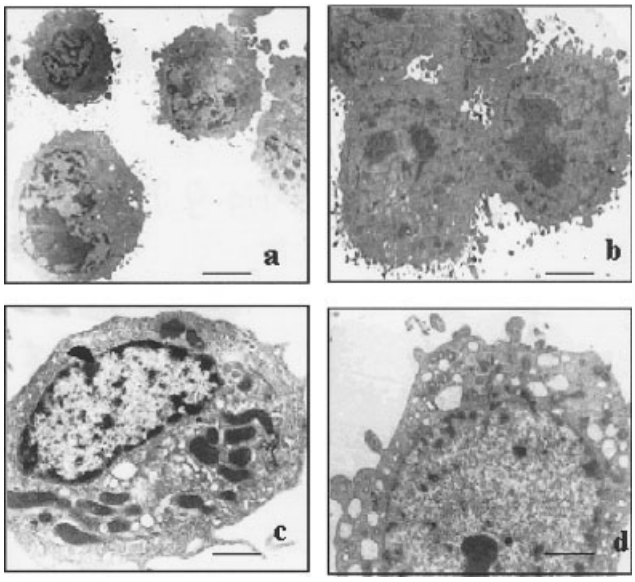

FIGURE 9 – Ultrastructural studies of FBM cells and CK8 gene–transfected FBM clones. *(a)* Isolated FBM cells. *(b)* Tightly packed cells of transfected clone (C2). *(c)* Single FBM cell. *(d)* Single cell of transfected clone C2 showing altered cytoplasm:nucleus ratio and increased number of mitochondria. Bars = 3.3 µm*(a,b)*, 2.2 µm *(c,d)*.

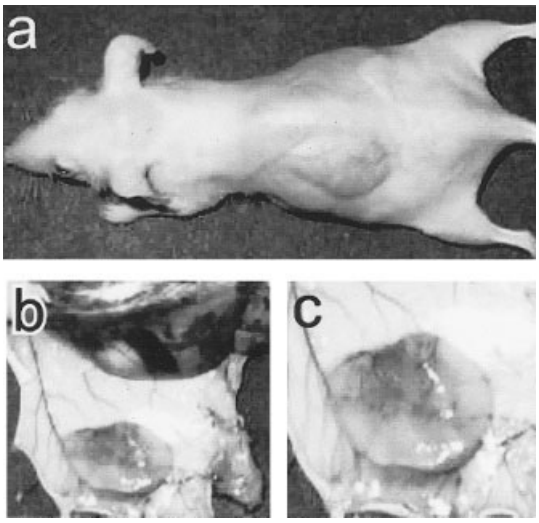

FIGURE 10 – *(a)* Nude mouse with s.c. growth of tumour at paralumbar fossa. *(b)* Tumour growth (s.c.) on opening the mouse. *(c)* Higher magnification of *(b)*.

Injections in nude mice

Injections of both FBM and transfected clones in nude mice did not produce any tumours for over 4 months. Cells of a human oral tumour cell line established from SCC of the tongue (AW13516)[26] were used as positive controls. Animals were killed after 4 months. In another experiment, cells from soft agar colonies developed from 2 of the clones were injected s.c. in nude mice. These animals developed 10 × 4 mm tumours at the end of 2 months (Fig. 10*a*). Upon exposing the tumour from the skin, new growth of capillaries was seen inside the tumour (Fig. 10*b*). The tumour was mucinous and sticky to cut. In one of the mice, histologic reading of the tumour with skin section revealed total necrosis of tumour cells, which left behind eosinophilic debris. However, the fibrous capsule was intact (Fig. 11*a*). Another mouse showed a healthy

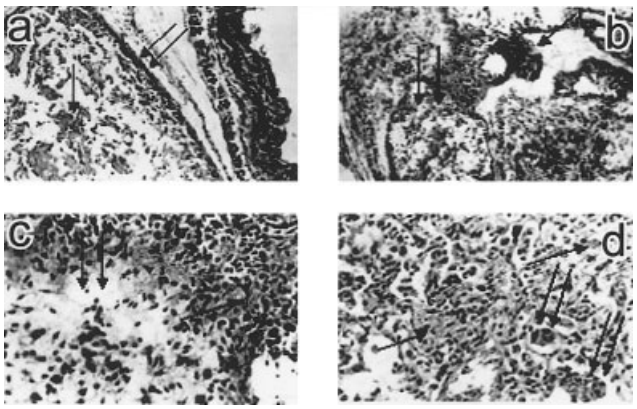

FIGURE 11 – *(a)* Section of skin with necrosed tumour (arrow) surrounded by fibrous capsule (double arrow, ×96). *(b)* Section of skin showing growth of healthy tumour cells (arrow) with foamy cell islands (double arrow, ×96). *(c)* Higher magnification of *(b)* (×250). *(d)* Section of lungs showing metastatic tumour cells (double arrow) in alveoli and accumulation of eosinophilic debris (arrow) in alveoli (×250).

tumour encapsulated by fibrous tissue capsule. This section showed foamy cell islands in between healthy tumour islands (Fig. 11*b,c*).

Lung sections in both mice revealed metastasis of the primary tumour in lung alveoli, accumulation of eosinophilic debris and acute inflammatory cell infiltration in the alveoli. The eosinophilic debris in the alveoli could be necrosed tumour cells or the mucin produced by tumour cells (Fig. 11*d*). Sections of liver, kidney, spleen and pancreas did not reveal any histologic changes.

Taken together, these results indicate that increased CK8 expression induced changes in the phenotypic characteristics of FBM cells indicative of malignant transformation.

DISCUSSION

CKs 8 and 18, normally expressed in simple or single-layer epithelial tissues of the body, are the most common and characteristic members of the IF gene family.[2,9,16] Although the CK8/18 pair imparts characteristics like invasiveness, metastasis and drug resistance to carcinoma cells,[9,17] it is not known whether this pair contributes to the malignant phenotype of cells. It has recently been shown that oncogenes that activate ras signal-transduction pathways stimulate expression of *CK8* and *CK18* genes through transcription factors including members of the AP1 (Jun, Fos) and ETS families.[16] The persistent expression of CKs 8 and 18 may reflect integrated transcriptional activation of such transcription factors. It is not known whether CK8/18 expression is maintained in carcinomas only because normal regulation of the *CK8* and *CK18* genes utilises oncogene-activated transcription factors.

FBM is a cell line derived in our laboratory from human fetal buccal mucosa. The CK profile of this immortalised but nontransformed cell line showed expression of both CKs 8 and 18, though CK8 was expressed to a much lower extent. This observation correlates well with our previous studies on CK expression in human fetal oral tissues.[27]

Immunofluorescence of FBM cells using MAb specific to CK8 showed diffuse staining, while that with MAb specific to CK18 showed filamentous staining. Both members of a CK pair are required in equimolar quantities for proper filament formation.[8] Since CK8 expression is significantly lower in FBM cells, CK18 must pair with other type II CKs, like CK4 or CK6, to form filaments. However, such filaments are functionally inefficient.[28] Induced expression of CK8 in FBM cells should result in proper CK8/18 pairing and filament formation and enable us to understand the phenotypic changes that occur as a result of anomalous CK8/18 expression in stratified epithelial cells.

CK8 gene–transfected clones showed an approximately 3- to 4-fold increase in CK8 expression. Immunofluorescence staining with MAb to CK8 showed well-decorated filaments in these cells, thus indicating formation of CK8/18 filaments.

We looked at the phenotypic changes that occur in FBM cells as a result of increased CK8 expression and CK8/18 filament formation. DNA flow cytometry of transfected cells revealed increased DNA content and longer S phase in these clones compared to parental FBM cells. Expression of CK8/18 has been shown to resist Fas- and TNF-α-mediated apoptosis in hepatocytes, which are simple epithelial cells.[29,30] Gilbert *et al.*[29] found that in the absence of CK8/18 filaments proapoptotic receptors appear on the cell surface and bind to their ligands, initiating the apoptotic machinery. Thus, although in FBM cells both CKs 8 and 18 were present, they were unable to form filaments because of lower expression of CK8. Increased CK8 expression resulted in proper CK8/18 filament formation. Our observation of increased DNA synthesis in transfected clones could be the result of evasion of receptor-mediated apoptosis. Further work is necessary to understand the role of CK8/18 in evasion of apoptosis in the present system.

Parental FBM cells were unable to grow in the absence of additional growth supplements like hydrocortisone and epidermal growth factor, while transfected cells became independent of these growth supplements. These clones also showed anchorage-independent growth in soft agar assay and reduced doubling time. Cellular projections typical of transformed cells were observed in transfected cells. Ultrastructural changes indicative of cell transformation were also observed in these cells. Thus, normal FBM cells after transfection with the *CK8* gene expressed phenotypic characteristics typical of transformed cells.

CK filaments were considered to be inert structures having only a structural role, such as maintenance of cell shape and morphology. The role of CKs in the structural integrity of epithelial cells was underlined by the fact that some severe skin disorders were the result of single point mutations in the rod domain of CK.[31–33] Apart from having a structural function, CKs are believed to be involved in some other regulatory functions, including signal transduction.[9,16] Transfection of a dominant negative mutant form of CK18 into an unusually high metastatic melanoma cell line, which ectopically expressed CKs 8 and 18, resulted in disruption of CK8/18 filaments and decreased invasive ability.[34] Furthermore, transfection of CKs 8 and 18 into a human melanoma cell line resulted in a 2- to 3-fold increase in invasion through the basement membrane matrix and migration through gelatin *in vitro*.[18] Mouse L-cell fibroblasts transfected with CKs 8 and 18 showed higher migratory and invasive ability compared to cells not expressing CKs 8 and 18 or cells expressing only one or the other.[35] L cells or NIH3T3 cells expressing CKs 8 and 18 acquire increased resistance to various chemotherapeutic drugs.[17] The ability to coexpress vimentin and CK may confer a selective advantage to tumour cells in their interpretation of and response to signalling cues from adjacent cells or extracellular matrix. Thus, our present results along with these findings strongly point towards a role of CK8/18 filaments in signalling. IFs, on the one hand, are connected to cell membrane proteins and, on the other hand, are connected to the cell nucleus.[36] IFs can act as signal transducers, relaying information from the extracellular milieu to the nucleus. Thus, any change in the IF assembly of the cell would probably result in changes in their interactions with membrane proteins, which in turn may result in altered signalling. This possibility is being investigated in our laboratory.

Injections of cells obtained from soft agar colonies developed from 2 of the transfected clones produced tumours at the site of injection in nude mice. In both cases, lung metastasis was also seen. This observation indicates that transfected cells not only are tumorigenic but also have invasive potential. Thus, it appears that CK8/18 filament formation leads to transformation of cells, though

the mechanism involved remains unclear. In summary, increased CK8 expression in FBM cells resulted in CK8/18 filament formation, which in turn resulted in changes in the phenotypic characteristics of the cells. Our results suggest that CK8/18, apart from having a structural function, may have a regulatory role and in some way contribute in the malignant transformation of stratified epithelial cells.

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