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The effect of 3T3 fibroblasts on the expression of anchorage independence and cornification of oral keratinocytes

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Summary. This study examined the effect of 3T3 fibroblasts on the expression of anchorage independence and the degree of cornification in early cultures of three carcinoma-derived epithelial cell lines (R59, R63a, R63b) and in one cell line derived from non-malignant dysplastic epithelium where there was no evidence of invasion (R66a). The epithelial cell lines originated from the palatal (R63a, R66a) and the lingual (R59, R63b) mucosa of rats that had been painted with the carcinogen 4-nitroquinoline N-oxide. In the absence of 3T3 fibroblasts, progressive culture resulted in an increase in the colony forming efficiency (CFE) of R63a, R63b and R59 and a decrease in the percentage of cornified cells in all cell lines. 3T3 fibroblasts caused a decrease in the CFE and the degree of cornification in the 3T3-dependent cell line (R63a), particularly at the lower passages, but these parameters remained essentially unchanged by 3T3 fibroblasts in the 3T3-independent cell lines (R59, R63b). 3T3 fibroblasts did not influence the cornification of R66a and this cell line remained anchorage dependent throughout the study. The results suggest that in malignant cell lines characterised by being independent of 3T3 fibroblasts (R63b, R59) the CFE was inversely correlated to the degree of cornification. However, in the malignant cell line showing a greater dependence on support (R63a) the relationship between CFE and cornification was unclear because these parameters may have been modulated by the presence of 3T3 fibroblasts. The cell line from dysplastic non-invasive tissue (R66a) differed from its malignant counterparts in the fact that CFE and cornification were unaffected by 3T3 fibroblasts despite previous studies showing a dependence on mesenchymal support.

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Key words: 3T3 fibroblasts – Anchorage independence – Cornification – Oral keratinocytes

Introduction

The inter-relationship between cell proliferation and differentiation in health and disease has been studied in the past using epidermal cell cultures (Holbrook and Hennings 1983; Fusenig 1986). Homeostasis in such cultures is maintained by a balance between cell division in the basal layer and the loss of terminally differentiated cells from the more superficial layers (Green 1980). The final product of keratinocyte terminal differentiation is the cornified envelope which forms beneath the plasma membrane (Sun and Green 1976) and is the product of a cytoplasmic transglutaminase that catalyses isodipeptide cross-linking of specific keratinocyte cytoplasmic proteins (Rice and Green 1977). The clonal growth of normal mammalian keratinocytes in vitro is closely dependent on lethally irradiated mesenchymal 3T3 fibroblasts (Rheinwald and Green 1975) which exert a permissive effect for keratinocyte proliferation (Rheinwald and Green 1977; Green et al. 1977). The nature by which 3T3 fibroblasts exert their effect remains an enigma but it has been suggested that they act to modify the substratum, reduce inhibitory molecules in the culture media and/or secrete so-called "growth factors" to promote keratinocyte growth (Rheinwald 1980; Citron et al. 1986). Whether 3T3 fibroblasts also directly influence keratinocyte differentiation, however, is currently unknown.

Using a rat model of oral carcinogenesis (Wallenius and Lekholm 1973; Prime et al. 1987), we have developed from oral mucosa painted with 4-

nitroquinoline N-oxide (4NQO) epithelial cell lines that express several in vitro and in vivo characteristics associated with the malignant phenotype (Crane et al. 1986), though their dependence on 3T3 fibroblast support (Crane et al. 1986) and their degree of anchorage independence in agarose gels (Luker et al. 1988) is variable. Similar findings have been reported in human malignant keratinocytes (Rheinwald and Beckett 1981; Easty et al. 1981; Cowley et al. 1983; Virolainen et al. 1983; Rupniak et al. 1985; Boukamp et al. 1985). It is possible, therefore, that the expression of anchorage independence by malignant keratinocytes may reflect their dependence on external mesenchymal support, particularly as there is a growing body of evidence to show that fibroblasts influence the growth in semi-solid medium of a wide variety of cell lines from human solid tumours (Laboisse et al. 1981; Kirk et al. 1981; Brittain et al. 1982; Citron et al. 1986).

The purpose of this study, therefore, was to examine the effect of 3T3 fibroblast feeder cells on the expression of anchorage independence by rat oral keratinocytes and to determine whether the changes in the proliferative capacity of keratinocytes in a semi-solid medium were accompanied by changes in the degree of epithelial differentiation.

Materials and methods

Cell culture. Four epithelial cell lines, from the tongues and palates of Sprague Dawley male rats that had been painted orally three times weekly with the carcinogen 0.5% (w/v) 4NQO (Sigma) in propylene glycol for approximately 7–8 months, were selected because of a variable dependence on 3T3 fibroblast support (Table 1) (Crane et al. 1986). The lingual and palatal mucosa of untreated rats was used to establish control cultures of normal keratinocytes and fibroblasts.

Cells were grown in complete medium which consisted of Dulbecco's modified Eagles' medium (DMEM) containing 20% (v/v) foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 0.075% (v/v) additional sodium bicarbonate, 0.6 mg/ml additional L-glutamine, 0.5 μg/ml hydrocortisone and 10 ng/ml cholera toxin, as described previously (Crane et al. 1986). All cultures were incubated in a humidified atmosphere at 5% CO_2/95% air at 37° C and the medium was changed twice weekly. Primary cultures were established on mitomycin C-treated Swiss embryo 3T3 fibroblasts (Rheinwald and Green 1975) but, after the third passage, separate cultures of all cell lines were grown either in the presence or absence of mitomycin C-treated 3T3 feeder cells. Host contaminating fibroblasts and degenerating 3T3 cells were selectively removed by treatment with 0.02% EDTA at 37° C for approximately 30 s. The effect of repeated EDTA treatment on the 3T3-absent cultures was controlled by carrying out similar procedures on the 3T3-present cultures and then subsequently replacing the 3T3 feeder cells.

Passage of near confluent epithelial cultures was carried out by dissociating the remaining cells with 0.025% trypsin and 0.01% EDTA in phosphate buffered saline (calcium and magnesium free; PBS^-) at 37° C for 30–40 min, resuspending the cells in complete medium and seeding 2×10^5 epithelial cells into 60 mm tissue culture dishes. All cultures were examined with a Leitz Diavert inverted phase contrast microscope.

Table 1. 3T3 fibroblast dependence of epithelial cell lines originating from 4NQO-induced oral carcinogenesis (Crane et al. 1986). Tissues were derived from squamous cell carcinomas (SCC; R59, R63b, R63a) and dysplastic non-invasive epithelium (R66a). (−, complete independence of 3T3 fibroblasts; ++, partial independence of 3T3 fibroblasts, with a slower growth rate of keratinocytes in the absence compared to the presence of 3T3 fibroblasts)

Cell line	Origin	3T3 fibroblast dependence
R59	Lingual SCC	−
R63b	Lingual SCC	−
R63a	Palatal SCC	++
R66a	Non-invasive, dysplastic palatal epithelium	++

Cultures were routinely stained for mycoplasma contamination using the Hoescht stain (Chen 1977). Cell viability prior to gel culture was assessed using trypan blue dye exclusion. Keratinocytes in all cultures showed positive reactivity to a polyclonal anti-human keratin antibody (1:10 dilution in PBS; Dakopatts) which was visualised using a rhodamine-conjugated swine anti-rabbit immunoglobulin (1:10 dilution in PBS; Dakopatts) and narrow band green fluorescent microscopy with a Leitz Dialux 22 microscope. All tissue culture reagents were purchased from either Flow laboratories (Irvine, Scotland) or Gibco (Paisley, Scotland).

Anchorage independence in gel culture. 3T3 fibroblasts were grown to confluence in 75 cm^2 plastic Falcon flasks (Sterilin) containing 30 ml DMEM plus 10% (v/v) newborn calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.075% (v/v) sodium bicarbonate and 0.6 mg/ml L-glutamine. 3T3-conditioned media from confluent cultures was collected at 3-day intervals, filtered through Nalgene vacuum filters (0.2 μm pore size) and stored in 20 ml aliquots at −20° C until further use.

Agarose gel cultures (Luker et al. 1988) incorporating 3T3 fibroblasts/conditioned media were prepared accordingly. 2×10^5 mitomycin C-treated 3T3 fibroblasts were seeded into 60 mm tissue culture petri dishes containing 5 ml complete medium and incubated under standard conditions for 24 h. The media was decanted and the gel underlayer consisting of 6 ml 0.5% (v/v) agarose in complete media at 44° C was gently pipetted over the adherent 3T3 feeder cells. After solidification of the gel underlayer, suspensions of 1×10^5 keratinocytes at passages 8, 12 and 16 were added to 1.5 ml solutions of 0.33% (v/v) agarose (Seaplaque) in DMEM containing 10% (v/v) foetal bovine serum and supplements, of which 20% (v/v) was conditioned media from viable 3T3 cultures; this mixture was poured over the solidified gel underlayers at approximately 44° C. Cultures were examined initially to ensure a single cell suspension and after 14 days the number of colonies, consisting of > 20 cells, was counted in 10 random microscopic fields. The percentage of cells expressing anchorage independence (colony forming efficiency) was calculated as described previously (Luker et al. 1988). The colony forming efficiency (CFE) of each cell line at each passage was assayed in triplicate on at least three separate occasions.

Cornification. In parallel with the studies of colony forming efficiency, the percentage of cornified keratinocytes in each cell line was assayed at passages 8, 12 and 16 using a modification of the technique described by Sun and Green (1976) and Boyce and Ham (1983). Briefly, keratinocytes were cultured until confluence either in the presence or absence of 3T3 fibroblast support. After 3 days, the media was decanted, the degenerating 3T3 feeder cells and host fibroblasts were selectively removed, and the epithelial cells harvested and added to the decanted medium. After washing in PBS^-, 1×10^6 keratinocytes were resuspended in 1 ml of 5% (w/v) sodium dodecyl sulphate (BDH) and 20 mM dithiothreitol (Sigma) in PBS^- and the cell suspension heated at 90° C for 10 min. The number of hollow refractile spheres, representing the resistant envelopes of the cornified cells, were counted in a counting chamber. The degree of cornification was expressed as the percentage of cornified envelopes per original 1×10^6 keratinocytes. Each assay was carried out in triplicate. Controls included 3 day confluent cultures of untreated normal palatal and lingual keratinocytes grown in the presence of 3T3 fibroblast feeder cells.

Controls. Control gels included the use of palatal and lingual keratinocytes (3rd passage) from untreated animals, fibroblasts from untreated animals and mitomycin C-treated 3T3 fibroblasts. Three day confluent cultures of untreated normal palatal and lingual keratinocytes grown in the presence of 3T3 fibroblast feeder cells were also tested in the cornification assay.

Pilot studies using 3T3 conditioned media (decanted from confluent cultures of 3T3 fibroblasts after 3 days) caused a decrease of both the cornification and the CFE of R63a passage 8; the effect was less marked than the use of conditioned media in conjunction with mitomycin C-treated 3T3 fibroblasts. 3T3 conditioned media had no effect on the cornification and CFE of R63b passage 8.

Statistical analysis. All significance testing was carried out using a two-tailed Student *t*-test for unpaired samples, with $p < 0.05$ taken as statistically significant.

Results

Cell morphology

The morphology of the keratinocyte cell lines grown in the presence or absence of 3T3 fibroblasts is shown in Fig. 1. Three of the four cell lines (R59, R63b, R66a) showed no consistent change in appearance, but R63a, particularly in the later passages (15°), was composed of large polygonal cells with granular cytoplasm in cultures where 3T3 feeders were present but much smaller stellate-like keratinocytes were evident in cultures lacking 3T3 fibroblasts.

Anchorage independence

The CFE of the keratinocyte cell lines cultured in the presence or absence of 3T3 fibroblasts/conditioned media is shown in Fig. 2. In the absence of 3T3 cells/conditioned media, R59, R63b and R63a exhibited a significant increase ($p < 0.05$) in CFE between passages 8 and 16. The CFE of R59 and R63b cultured in the presence of 3T3 fibroblasts/conditioned media increased with progressive passage of the cells and closely paralleled the CFE of cells assayed in the absence of 3T3 feeder cell support in these cultures. The CFE of R63a cultured in the presence of 3T3 fibroblasts/conditioned medium was significantly less ($p < 0.05$) at passages 8 and 16 than in cultures assayed in the absence of 3T3 feeder support, but this trend was not significant at passage 12, possibly because of the non-uniform expression of CFE of R63a cultured in the absence of 3T3 fibroblasts/conditioned media. The R66a cell line remained anchorage dependent with increasing passage in the presence or absence of 3T3 fibroblasts/conditioned medium.

Control gels using normal oral keratinocytes, fibroblasts from untreated animals or mitomycin C-treated 3T3 feeder fibroblasts showed no evidence of colony formation.

Cornification

The percentage of cornified cells present in the confluent keratinocyte cell lines cultured in the presence and absence of 3T3 fibroblasts is shown in Fig. 3. In the absence of 3T3 cells, the percentage of cornified cells in all cell lines decreased between passages 8 and 16, though only in R63b was this change statistically significant ($p < 0.05$). In the presence of 3T3 feeders, the number of cornified keratinocytes in all cell lines decreased with progressive culture passage and, in fact, the percentage of cornified cells in R66a and R63b was essentially the same whether grown in the presence or absence of 3T3 fibroblasts. Although significantly ($p < 0.05$) more cornified cells were formed when R59 was grown in the presence compared to the absence of 3T3 fibroblasts/conditioned media at passage 8, this trend was reversed at the later passages. In all culture passages of R63a, the presence of 3T3 feeders resulted in the formation of fewer cornified cells compared to cultures lacking 3T3 fibroblasts and this trend was significant ($p < 0.05$) at passage 8. In the presence of 3T3 feeders, the percentage of cornified cells in R63a remained essentially constant between passages 8 and 16.

Significantly ($p < 0.05$) more cornified cells were formed in cultures of untreated normal palatal keratinocytes (7.29%) than untreated normal lingual keratinocytes (1.18%).

Discussion

It is a widely accepted principle in tumour biology that a higher malignant potential often coincides

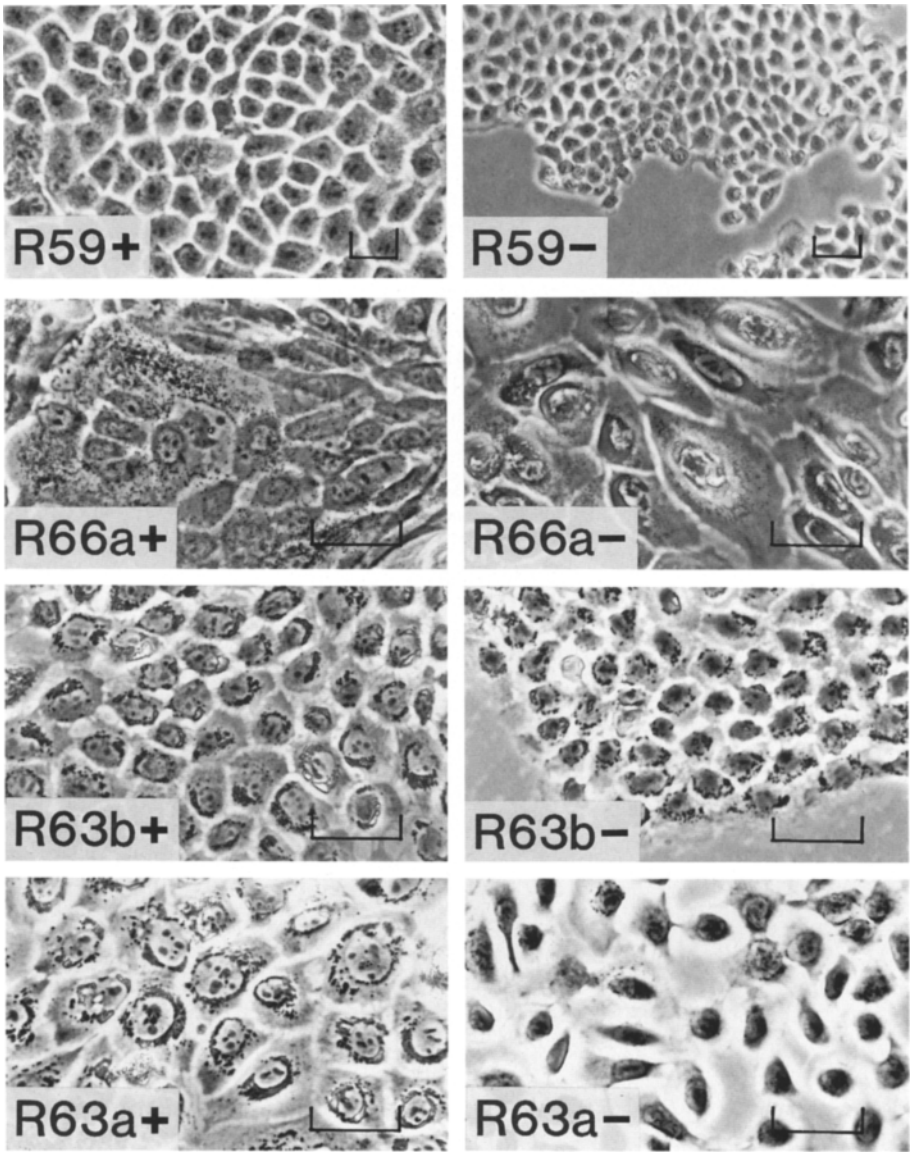

Fig. 1. The morphology of monolayer cultures of rat oral keratinocyte cell lines maintained in the presence (+) and absence (−) of mitomycin C-treated 3T3 fibroblast feeder cells. The keratinocyte cell lines originated from two lingual squamous cell carcinomas (R59, R63b), a palatal squamous cell carcinoma (R63a) and a palatal dysplastic lesion (R66a). Bar = 50 μm

with a decrease or total loss in the potential for differentiation (Foulds 1964; Speers 1982). Assays to examine malignancy and differentiation in keratinocytes are well established; the expression of anchorage independence in vitro closely reflects the transformed phenotype in rodent (Skehan and Friedman 1981) but not human cells (Easty et al. 1981; Boukamp et al. 1985) and the process of differentiation is associated with cornified envelope formation in both rodent and human cells (Rice and Green 1977). Previous findings (Crane et al. 1986) demonstrated that the cell lines used in the present study that were of lingual origin (R59, R63b) were 3T3 fibroblast independent in contrast to those of palatal origin (R63a, R66a) which showed a greater dependence on 3T3 fibroblast support. These findings may reflect a site specific variation in normal epithelial differentiation, particularly as more cornified cells were formed in cultures of untreated palatal keratinocytes than untreated lingual keratinocytes. The progressive passage of R59 and R63b in this study, two cell lines previously shown to be independent of 3T3 fibroblasts (Crane et al. 1986), resulted in an increase in the degree of anchorage independence and in the decrease in the percentage of cornified cells; these changes were essentially independent of the presence or absence of 3T3 fibroblasts. The findings of the present study suggest that in those cell lines where 3T3 fibroblasts are not a prerequisite for the growth of malignant epithelial cells (R59 and R63b), progressive culture leads to an

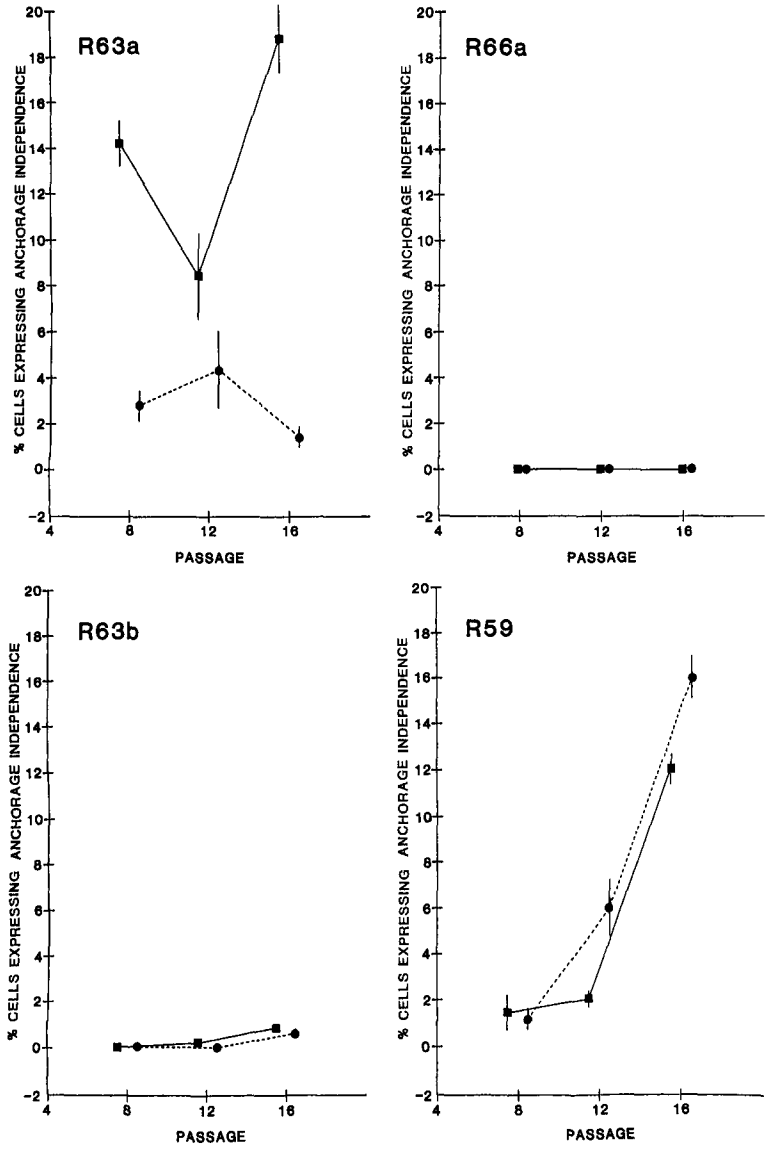

Fig. 2. The colony forming efficiency of R63a, R66a, R63b and R59 at passages 8, 12 and 16 grown in the presence (●---●) or absence (■—■) of mitomycin C-treated 3T3 fibroblasts and conditioned media. Bar = standard error of the mean

increase in malignant potential and a coincidental decrease in the capacity to differentiate, probably as a result of selection in vitro based on malignant characteristics other than a dependence on 3T3 fibroblasts.

The situation was different in the carcinoma cell line (R63a) that had previously been shown to be dependent on 3T3 fibroblasts in cell culture (Crane et al. 1986). In the presence of 3T3's, the degree of anchorage independence was much lower than in cultures where 3T3 fibroblasts were absent and the CFE did not increase with culture passage. Interestingly, several reports have shown that 3T3 fibroblasts can enhance anchorage independence, but these studies utilised human malignant cell lines of colonic (Laboise et al. 1981; Brittain et al. 1982) and prostatic (Kirk et al. 1981) origin and a human tumour clonogenic assay involving the disaggregation of primary biopsy specimens (Citron et al. 1986). In the present study, it is suggested that the presence of 3T3 fibroblasts in cultures of R63a provided less stringent and selective in vitro conditions. That 3T3 fibroblasts caused a decrease in the CFE of R63a at passages 8, 12 and 16 compared to 3T3-free cultures, therefore, may be a reflection of the continued growth of less malignant cells in these cultures. The role of 3T3-induced selective conditions in cultures of R59 and R63b, however, is likely to have been minimal because of the independence of these cell lines from 3T3 fibroblast support.

The effect of 3T3 fibroblasts on R63a was to

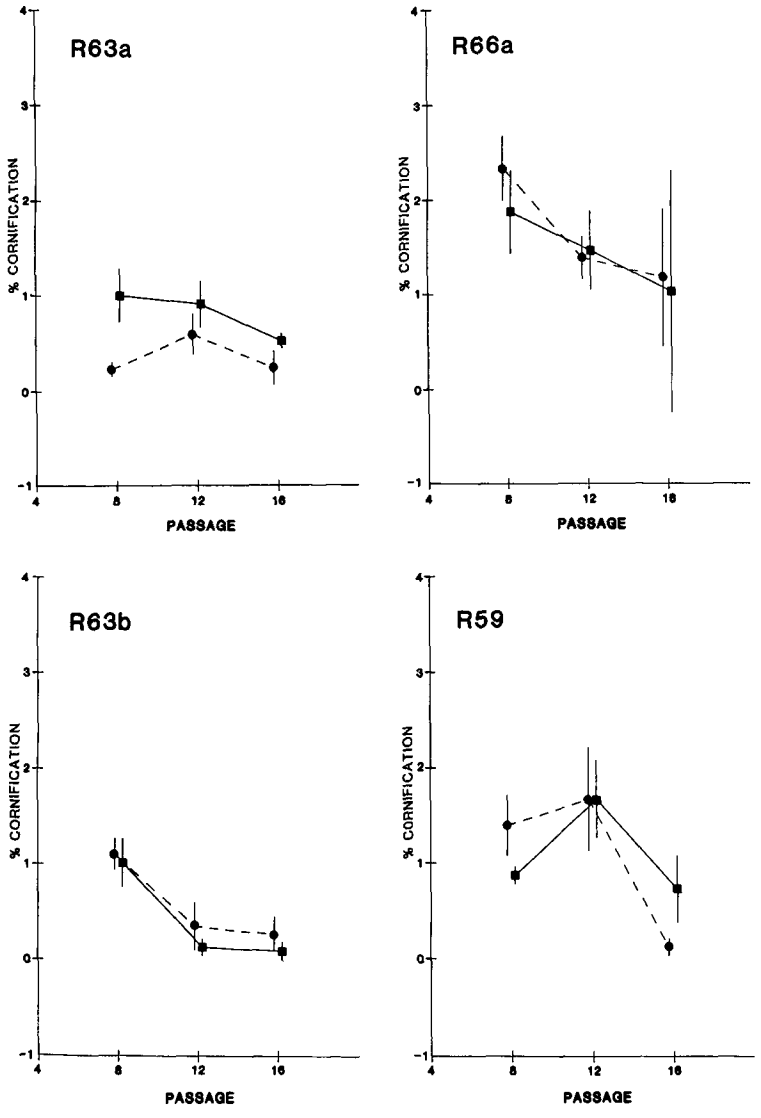

Fig. 3. The percentage of cornified cells in confluent cultures of R63a, R66a, R63b and R59 at passages 8, 12 and 16 grown in the presence (●---●) or absence (■—■) of mitomycin C-treated 3T3 fibroblasts. Bar = standard error of the mean

decrease not only the degree of anchorage independence but also the percentage of cornified cells particularly at the lower passage. The findings suggest that malignancy may not always be inversely correlated to differentiation. This proposal is consistent with the recent findings of Dover and Watt (1987) who demonstrated DNA synthesis in keratinocytes undergoing Ca^{2+}-induced differentiation. It seems unlikely that the 3T3 fibroblasts actively utilised the metabolites available for the expression of anchorage independence and cornification by R63a; the effect was not observed in cultures of R59, R63a and R63b and all fibroblasts in this study were non-dividing following treatment with mitomycin C.

The present study demonstrated that 3T3 fibroblasts failed to induce anchorage independence in the cell line derived from dysplastic palatal epithelium (R66a). These findings support the concept that a genetic change (Bouick and Head 1985; Bouick et al. 1986) is responsible for the ability of cells to divide without anchorage, rather than there being a simple response to external stimulation. The ability of certain growth factors to induce anchorage independence in a number of non-tumourigenic cell types (La Rocca and Rheinwald 1985; Anzano et al. 1986; Kuratomi et al. 1987), therefore, may reflect pre-existing genotypic changes in these well-established cell lines. It was interesting to note in the present study that the presence of 3T3 fibroblasts did not influence the cornification of R66a, despite previous studies indicating that the routine growth of this cell line was dependent upon 3T3 fibroblasts (Crane et al. 1986). By contrast, the behaviour of the cell lines from malignant tissue in this study was consistent with pre-

vious findings in that their cornification and colony forming efficiency was either 3T3 dependent (R63a) or 3T3 independent (R63b, R59). Pera and Gorman (1984), using a similar in vivo-in vitro approach to examine multi-stage carcinogenesis in mice, noted a considerable variation between cell lines from premalignant and malignant tissue with respect to their ability to form colonies in culture in response to 3T3 fibroblast support.

There is much evidence to show that malignant transformation of keratinocytes is associated with defective terminal differentiation (Rheinwald and Beckett 1980; Parkinson 1985). This proposal is partly confirmed by the results of the present study where significantly ($p<0.01$) more cornified cells were present in cultures of untreated normal palatal keratinocytes than either of the palatally-derived cell lines (R66a and R63a). There were no significant differences, however, between the percentage of cornified cells in the lingually derived cell lines (R59 and R63b) and the untreated normal lingual keratinocytes. Cultures of normal lingual keratinocytes are more frequently contaminated by host fibroblasts than palatal cultures and the possibility exists, therefore, that the enzymic process used in the removal of these fibroblasts may have resulted in removal of some cornified keratinocytes in cultures of the normal lingual tissues.

In conclusion, this study demonstrates the complex inter-relationship between the expression of the malignant phenotype and the degree of differentiation in keratinocyte cell lines. Mesenchymal factors appear to have the capacity to alter this relationship but their effect is dependent upon the nature of the individual cell lines.

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