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Growth of malignant oral epithelial stem cells after seeding into organotypical cultures of normal mucosa

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BACKGROUND: Oral squamous cell carcinoma (OSCC) is associated both with the local expansion of clones of malignant cells and with their further migration to regional and distant sites. The interactions that occur between normal and malignant cells during these events are not well modelled by standard culture conditions, but organotypical cultures, in which epithelial cells are grown on a matrix containing fibroblasts, provide a suitable environment for such investigations.

METHODS: Cells from five cell lines, each derived from OSCC and marked by retroviral transduction with alkaline phosphatase, were incorporated as small subpopulations (0.1–5%) in uniformly differentiating organotypical cultures constructed from normal oral mucosal cells. The patterns of growth of the malignant cells within the normal epithelium were examined for 3 weeks.

RESULTS: There was variation between the different cell lines in their rates and patterns of growth, but all cell lines produced clusters of malignant cells that had expanded within 3 weeks to replace the normal epithelium. The appearance and spacing of these clusters suggested that each was derived from a single progenitor cell. The number of malignant cells initially present within a given area of organotypical epithelium was much greater than the number of expanding cell clusters subsequently formed. Cluster-forming cells thus represented only a subpopulation of the tumour cells.

CONCLUSIONS: The organotypical model allows examination of interactions occurring between cells derived from OSCC and normal epithelia. The three-dimensional nature of organotypical cultures, together with their more normal patterns of differentiation, provides an environment that more closely mimics the *in vivo* environment in which tumours develop. The finding that only a subpopulation of tumour cells forms expanding tumour colonies suggests a range of growth potentials within a tumour population and may provide preliminary evidence for some form of stem and amplifying cell pattern.

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Introduction

Despite recent advances in understanding the molecular mechanisms associated with the development of oral cancer, the 5-year survival rate for those afflicted with this disease remains disappointingly low (1, 2), and distant metastasis or loco-regional recurrence, associated with minimal residual cancer or field cancerization, occurs with an unfortunate frequency (1, 3, 4). The aggressiveness of oral tumours is influenced by the behaviour of cells at the invasive tumour front, the site of interaction between the tumour and its surrounding normal tissues (5), and newer immunological and molecular methods for the identification of occult tumour cells have indicated a greater than formerly suspected degree of dissemination of tumour cells into both local and distant tissues (1, 6, 7) and point to an urgent need for better information about interactions that occur between normal and altered cells at the tumour margin, and about the mechanisms of expansion and movement of tumour cells through the adjacent tissues.

The continuous renewal of normal epithelia depends ultimately on a subpopulation of epithelial stem cells, the only cells capable of essentially unlimited proliferation (8). When epithelial stem cells divide, they renew themselves and also produce cells that enter the differentiation pathway and undergo several rounds of division to amplify the cell population before they differentiate (8, 9). Stem cells give rise to cell lineages that result in clonal clusters of cells associated with units of epithelial structure (10), and this basic epithelial pattern presumably underlies the clonal patterns developed by tumours (11). Stem cells, as the only cells permanently resident in the tissue, appear to form the initial target for the actions of carcinogens (12, 13), and the development of epithelial tumours is thought to involve

initial genetic changes in the stem cells themselves, growth selection among altered cell clones developing from them, and then further genetic alteration and progressive competition among the lineages so established (11). It is interesting that clones of epidermal cells containing mutated *p53* expand in a way that suggests persistence of stem cell units, at least during early alteration towards malignancy (14). Continued tumour expansion appears to depend on the production of new stem cells, and development of metastases presumably depends on the dissemination of cells that have properties of continued growth (15).

The patterns of behaviour of tumour cells are not easily studied *in vivo*, and cell interactions are not well modelled by standard *in vitro* conditions as these fail to re-establish *in vivo*-like patterns of normal epithelial growth. However, advances in tissue culture techniques have allowed the development of *in vitro* 'organotypical' models that regenerate patterns of cell behaviour and differentiation similar to those found *in vivo* (16, 17). These cultures are created by plating epithelial cells onto collagen matrices populated with fibroblasts and then maintaining the cultures at the air/medium interface to allow differentiation (18). Using such culture systems, Garlick and co-workers (19, 20) have incorporated cells of an *in vitro* transformed cell line into populations of untransformed keratinocytes and demonstrated an important role for cell interactions in the growth and survival of the transformed cells. We have now extended the use of this type of system to investigate the behaviour of cells derived from naturally occurring tumours. Several malignant epithelial cell lines, isolated from specimens of oral squamous cell carcinoma, have been included in organotypical cultures constructed from normal oral keratinocytes and fibroblasts. The patterns of growth and clonal behaviour of these malignant cells indicates an interaction with the normal cells and also provide some evidence for the persistence of stem and amplifying subpopulations in overtly malignant tumours.

Methods

Isolation and amplification of normal keratinocytes and fibroblasts

Specimens of normal human gingival or palatal mucosa, removed in the course of therapeutic surgical procedures and collected with informed consent, were used to generate cultures of mucosal keratinocytes and fibroblasts. The procedures used for cell isolation have been described previously in detail (21). In brief, samples of normal mucosa were separated into their epithelial and connective components by overnight incubation in 0.1% trypsin at 4°C, the epithelium was then further dissociated into a keratinocyte suspension by brief treatment with 0.1% trypsin/EDTA at 37°C and the keratinocytes were plated for amplification into T-75 flasks containing 0.7×10^{-6} Mitomycin-C-treated 3T3 feeder cells and fed with a compound medium, termed FAD (21), consisting of a 3 : 1 mixture of epidermal growth factor (DMEM) and Ham's F12 medium supplemented with epidermal growth factor (EGF) (10 ng/ml), penicillin/streptomycin (100 IU/ml), 10% foetal bovine serum (FBS), adenine (0.089 mM), hydrocortisone (400 ng/ml), insulin (5 ng/ml) and cholera toxin (10^{-10}M). After initial colony formation and expansion towards confluence, keratinocytes were passaged at a 1 : 10 dilution ratio for amplification in T75 flasks. Fibroblast cultures were generated from fragments of the superficial connective tissue that were suspended in a collagen gel for fibroblast outgrowth. After 10–14 days, fibroblasts were harvested, plated for amplification in T-75 flasks and fed with DMEM supplemented with 10% FBS. Stocks of cells for generation of organotypical cultures were frozen down for storage after the second or third passage.

Isolation of cells from malignant tissues

To generate cell lines for these experiments, cultures of malignant keratinocytes were initiated, as outlined above, from samples of squamous cell carcinoma of the oro-pharyngeal region collected with informed consent. Tumour tissues did not separate as readily into their epithelial and connective tissue components as normal tissues, but after overnight incubation of specimens in trypsin, samples consisting largely of epithelium or of connective tissue were prepared and further treated, as described above. The flasks to receive malignant keratinocytes were pre-treated by coating with Type IV collagen as this appeared to aid cell plating. Two additional established oral carcinoma cell lines, UM-SCC5 and UM-SCC-5PT, gifts of Dr Thomas Carey (University of Michigan), were also examined. To confirm their epithelial nature, all cell lines were stained for cytokeratins (K) 6, 8, 14, 18 and 19 using antibodies and methods as described previously in detail (21).

Transduction of epithelial cells

Each of the carcinoma cell lines was transduced with genes for placental alkaline phosphatase and *Neo* using a replication-deficient retroviral vector termed LAPSN (22), a gift from Dr A. D. Miller. The vector was received in the PA317 packaging line, and to collect retrovirus, PA317 cells at 50–70% confluence were fed with DMEM, the medium collected after 48 h, and stored frozen at −70°C. The retrovirus-containing DMEM supernatant was used in place of fresh DMEM to make up complete FAD medium, as described above. This medium, with the addition of 4 μg/ml of polybrene (Sigma-Aldrich Inc., Milwaukee, WI, USA), was added to actively proliferating subconfluent keratinocytes for 24 h. After a further 48 h of growth in standard FAD, transduced cells were selected by growth in FAD medium containing 0.75 mg/ml G418 (Sigma-Aldrich Inc.) for 7–10 days. Keratinocytes were then returned to FAD medium without G418, amplified and cloned by limiting dilution, and clones were examined by staining for alkaline phosphatase (see below). Some samples of normal oral keratinocytes were transduced in the same way for comparison of their behaviour with that of malignant cells.

Method for construction of compound cultures

The methods used for preparation of organotypical cultures were similar to those described by Parenteau et al. (16), but the collagen matrices were plated with keratinocytes prior to contraction in order to facilitate uniformity of plating (18). Matrices containing approximately 2×10^5 fibroblasts/ml were prepared by addition of 1 part of 10× concentrated DMEM and 1 part of FBS to 9 parts of collagen (0.1% acetic

acid digest of rat tail tendon at 4 mg/ml), followed by neutralization with 1 M NaOH, and addition of 2.4×10^6 fibroblasts suspended in one part of DMEM + 10% FBS. This mixture, which was prepared on ice, was poured into the wells of 12-well tissue culture plates(1.2 ml/well) and allowed to gel at 37°C. Gels were then covered with DMEM + 10% FBS, and after overnight incubation, the medium was removed and the gels were plated with 8×10^5 epithelial cells/well in FAD medium. Forty-eight hours later, the matrix, together with its adherent epithelial cells, was released from the dish and placed onto a nylon mesh that was coated with a thin layer of collagen and supported on a stainless steel grid in a culture dish. Medium was added just to touch the underside of the mesh and was changed every 2–3 days.

Preparation of organotypical cultures containing malignant cells

For each malignant cell line, matrices containing normal oral fibroblasts were plated as above with transduced malignant keratinocytes to examine their patterns of growth as pure cell populations in organotypical cultures. All other experiments aimed to observe interactions between malignant and normal keratinocytes, and these cultures consisted of matrices containing normal oral fibroblasts that were plated with a mixture of normal oral keratinocytes containing 0.1–5% of transduced malignant cells. The malignant cells used consisted of three newly isolated cell lines (G26, G28 and G29) and the pre-established lines UM-SCC5 and UM-SCC-5PT (gifts from Dr Thomas Carey, University of Michigan). Care was taken to dissociate the tumour cells into a single cell suspension that was dispersed evenly within the epithelial mixture during plating. All cultures were set up in FAD medium, and after raising on grids to the air/medium interface, the medium was renewed every 3 days. Specimens were harvested after periods of growth of up to 21 days. For the initial experiments, half of each specimen was frozen for cryotomy and sections cut at 5 μM were collected on poly-lysine-coated slides. The other half of each specimen was separated into epithelial and connective tissue sheets by incubation for 2 h in 20 mM EDTA (23). Buffered 4% formalin was used to fix both sections and epithelial sheets. These were washed in Tris buffered saline, processed to display alkaline phosphatase activity, and mounted in glycerine jelly. For most of the later experiments, specimens were prepared only as sheet preparations. The results reported are based on examination of specimens from 18 separate experiments, each consisting of 12–24 separate cultures.

Results

Isolation of malignant cell lines

Cells isolated from tumours initially provided actively proliferating cultures of both keratinocytes and fibroblasts but, despite the presence of antibiotics in the culture medium, about one-third of the cell isolates developed low-grade bacterial or fungal infections that led to death of the culture. Seven tumours provided infection-free cultures, and of these, three were successfully transduced, selected and cloned. Each of these cell lines grew well on plastic tissue culture flasks in FAD medium without feeder cells. Initially, most isolates of malignant cells were contaminated with fibroblasts, but these either disappeared after a few passages or were eliminated during cloning. When plated at low densities, the various lines of malignant keratinocytes that were used displayed a consistent range of recognizably different cell and colony morphologies. Individual cells had an appearance that varied from the cobble stone appearance of normal epithelial cells, to elongated fibroblast-like cells, to small cells with an oval outline (Fig. 1A–D). Grown on tissue culture plastic, some cell lines formed compact cell colonies with an appearance similar to that of normal keratinocytes, whereas other lines seemed to lack cohesiveness and formed loose colonies of spaced cells. All cell lines stained positively for cytokeratins, with G26, G28 and UM-SCC5 expressing K6, 14, 15 and 18, G29 expressing K6, 8, 15 and 18, and UM-SCC5PT expressing K6, 14, 18 and 19.

Growth of pure populations of tumour cell lines in organotypical culture

When grown as organotypical cultures constructed with normal fibroblasts, each of the tumour cell lines reformed an epithelium showing some degree of change towards restoration of epithelial structure. Some differences were apparent between different cell lines, but these were not as marked as when they were grown on plastic (Fig. 1E–H). In organotypical culture, all of the tumours stratified and most showed some differences between basal and suprabasal cells. However, whereas some showed quite orderly patterns of stratification, others were disorderly with a patchy appearance. None of the cell lines showed much evidence of terminal differentiation or formed a distinct surface stratum corneum.

Growth of subpopulations of tumour cells incorporated into organotypical cultures of normal mucosal cells

When organotypical cultures were constructed with normal keratinocytes that had been seeded with a small subpopulation of transduced normal or malignant cells, marked differences between the behaviours of the normal and malignant keratinocytes were apparent. As the cultures containing transduced normal cells stratified and differentiated, the transduced cells contributed to reformation of the epithelium: some remained in the basal region but the majority entered into the differentiation pathway, flattening as they ascended into the stratum corneum (Fig. 1I). Tumour cells, on the other hand, tended to remain basal, and those cells that did leave the basal region failed to flatten like the surrounding normal keratinocytes but typically remained small and pycnotic. At later time points, clusters of basally positioned tumour cells were seen (Fig. 1K). These differing patterns of behaviour of normal and tumour cells were also apparent when cultures were examined as epithelial sheets, removed intact from the collagen matrix once a stratified epithelia structure had formed about 4–5 days after plating. In sheet preparations, transduced normal epithelial cells were found at various stages of differentiation from small basal to larger flattened cells in the superficial layers; in contrast, transduced tumour cells were found scattered throughout the basal region of the epithelium, with no apparent participation in the process of epithelial differentiation (Fig. 1J,L).

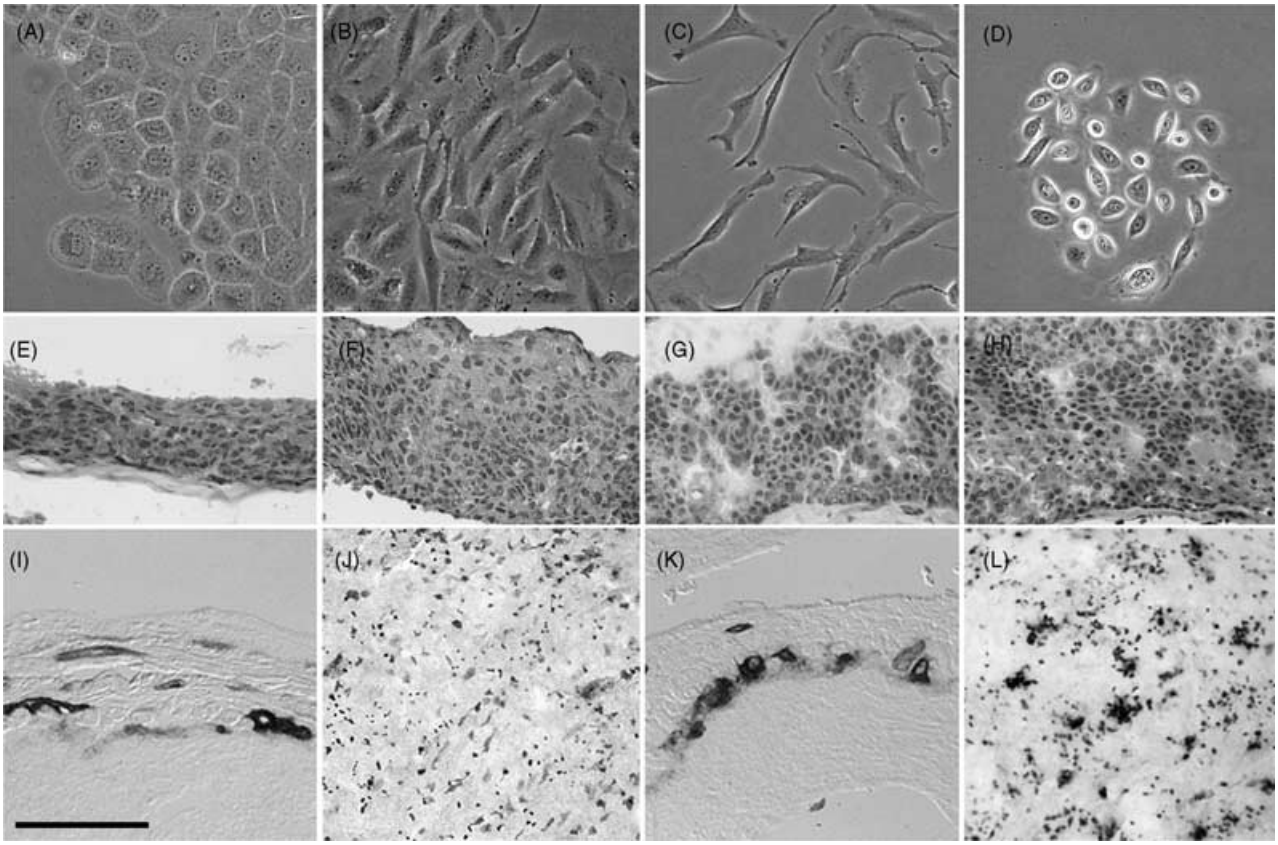

Figure 1 Panels (A–D) show phase contrast photomicrographs of the tumour cell lines, G26, G28, G29 and UM-SCC5PT, respectively, grown on plastic tissue culture flasks in FAD medium. These illustrate the wide range of appearances shown by the different cell lines and the marked differences in cell morphology, in refractility of cells, and in the density of cell packing within colonies. Panels (E–H) show haematoxylin and eosin-stained sections of organotypical cultures formed by plating matrices containing palatal fibroblasts with pure populations of tumour lines G26 or UM-SCC5PT. By 7 days after plating (E), the G26 line has stratified to form 8–10 cell layers, and after a further growth for 7 days, (F) has more than doubled its thickness. At both time periods, a gradient of increase in cell size towards the surface is apparent but there is no sign of normal differentiation. The UM-SCC5PT line also shows stratification at both 7 (G) and 14 (H) days but regular stratification is disturbed, apparently by intraepithelial foci of partial differentiation. Panels (I–L) show organotypical cultures constructed using suspensions of normal keratinocytes to which had been added 5% of either normal or tumour cells (G28) transduced with alkaline phosphatase as a marker. The samples shown were collected 7 days after plating. Sections of cultures containing marked normal cells (I) show that some cells remain basal and others undergo normal cell flattening as they ascend through the epithelial strata. Viewed as a sheet preparation (J), specimens containing transduced normal cells show a uniform distribution of labelled cells and the presence of both small round basal cells and larger flattened suprabasal cells. Sections of cultures containing marked tumour cells (K) show that almost all of these cells remain basal and the few that do leave the basal layer do not flatten normally. Sheet preparations containing tumour cells (L) show unflattened cells that are beginning to be positioned as clusters. Scale bar for panels (A–D), 110 μm; for (E–H), 150 μm; for (I,K), 50 μm; and for (J,L), 300 μm.

Interestingly, even at early stages, tumour cells were found in clusters within the normal epithelium. These appeared to have formed by focal proliferation of malignant cells, and the number of clusters formed indicated that only a subpopulation of the tumour cells had participated in the formation of expanding clones. Between these clusters, the epithelium appeared to contain fewer tumour cells than would have been initially present (Figs. 1L and 2A,B), and this discrepancy became clearer with further growth. For example, the majority of individual tumor cells of the G26 line had disappeared by 10–14 days after plating, leaving only a few expanding and presumably clonal clusters of tumour cells (Fig. 2B). This pattern was more marked for some cell lines than others: individual cells of the G29 tumour line, for example, remained within the epithelium between the expanding cell clusters, but these cells did not seem to actively proliferate (Figs. 1L and 3B). By 2–3 weeks after plating, all organotypical cultures seeded with malignant cells showed clusters of tumour cells that had expanded to replace almost all of the normal epithelium (Fig. 2C). The transduced cells of the UM-SCC5PT cell line showed differing levels of alkaline phosphatase expression, and in the cultures made from these cells, the outlines of individual clones could be discerned even as they became confluent (Fig. 2C).

Although each of the tumour cell lines displayed a similar basic pattern of behaviour in organotypical culture, several differences in rates or patterns of growth were found between the various cell lines. First, even when using organotypical cultures constructed from the same strains of normal fibroblasts and keratinocytes, addition of the same number of cells from different lines produced more expanding cell clones per unit area for some cell lines than for others. Second, once individual cell clusters had become established, their degree of expansion indicated that some types of tumour cells had a more rapid rate of growth.

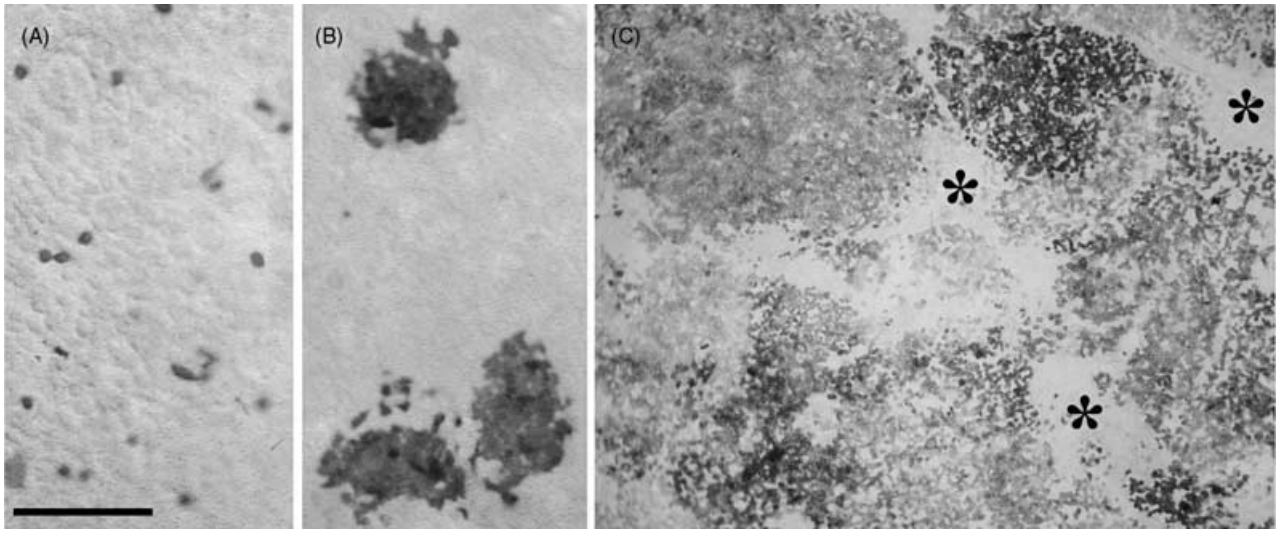

Figure 2 Sheet preparations of organotypical cultures constructed with the inclusion of a 5% subpopulation of marked malignant cells. Panels (A,B) show cultures constructed to contain 5% of the G26 cell line. At day 7, tumour cells are regularly dispersed within the normal epithelium (A). The presence of pairs of cells perhaps indicates some proliferation, although this is less than that had occurred in the 7-day specimen shown in Fig. 1(L). By day 14, further proliferation has produced three large clusters of cells with spacing that suggests independent clonal origins (B). The tumour cells that would have been initially present in the epithelium between these large clusters are no longer present. These appearances allow estimates to be made of the fraction of tumour cells that formed expanding colonies. For example, the epithelial sheet shown in (A) was collected 7 days after plating, and within the area shown, there are approximately 600 basal cells. As the number of tumour cells initially added was 5%, the number of tumour cells expected to be present with the area shown would be about 30. However, only 20 tumour cells are present. Some of these cells lie in pairs, suggesting that division has occurred since plating and that perhaps as many as half of the cells initially present has been lost. (B) shows a specimen from the same experimental series collected 7 days later. This contains only three cell clusters, and if each has arisen from a single precursor cell, as suggested by their spacing, only about 10% of the malignant cells initially present have survived. Panel (C) shows a culture constructed with the inclusion of a 5% subpopulation of line UM-SCC5PT and collected 20 days after plating. Cells of this line showed different levels of expression of alkaline phosphatase, and this enabled visualization of the outlines of cell clusters originating from individual cells. Large cell clusters that appear to be of clonal origin have expanded to displace most of the normal epithelium, leaving only small areas (indicated by asterisks) free of tumour cells. Scale bar for panels (A,B), 110 μm; for (C), 300 μm.

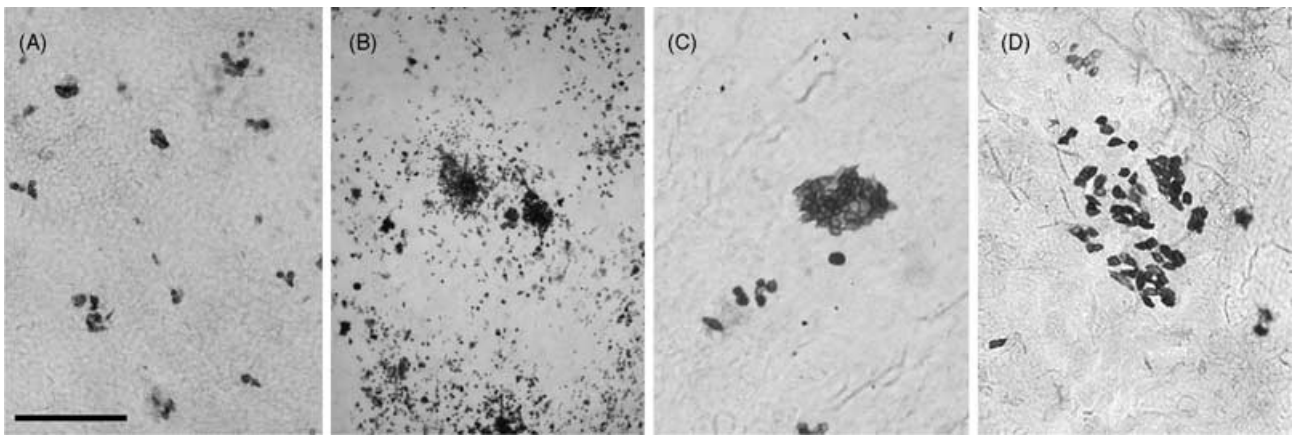

Figure 3 Sheet preparations of organotypical cultures showing variation in the rates and patterns of growth of malignant cells. Panel (A) shows a specimen constructed with the inclusion of a 5% subpopulation of marked G29 cells and collected 7 days after plating. The cell spacing suggests that there has been some loss of cells initially present but the remaining cells have proliferated to form clusters containing four to eight cells. Comparison of this panel with panels (L) in Fig. 1 and panel (A) in Fig. 2 illustrates the differing degrees of proliferation occurring for different tumours during the first 7 days after plating. Panel (B) shows a specimen constructed with the inclusion of a 5% subpopulation of marked UM-SCC5 cells and collected 14 days after plating. As for G28 (shown in panel (L) in Fig. 1), it appears that quite a large number of the cells initially plated remain. However, they are patchily distributed and only a few cells have formed expanding colonies. Panel (C) shows an organotypical culture of UM-SCC5PT collected 10 days after plating and demonstrates the typically tightly packed cell colonies produced by this line. A similar culture of G28 (panel D) shows formation of a less cohesive colony. Scale bar for panels (A), 200 μm; for (B), 400 μm; and for (C,D), 100 μm.

Finally, the morphology of the expanding tumour cell clusters varied, and in some cultures, the cell clusters were tightly packed whereas in others the clusters were more loosely dispersed (Fig. 3C,D).

Discussion

The results of the present study are in keeping with those of previous studies in showing that cells isolated from human oral squamous cell carcinoma can be grown and manipulated *in vitro* to provide information about the behaviour of tumour cells (24, 25). Using a standard method for isolation and growth of normal oral keratinocytes (21), we found that it was possible to isolate and subsequently generate expanding cell colonies for the majority of tumour samples collected. The main cause of failure to establish cell lines from these colonies was inability to suppress the growth of contaminating organisms. However, once established, all cell lines grew well in plastic tissue culture flasks in FAD medium and could be cloned using limiting dilution methods without feeder cells. The cell lines that were used for these experiments varied quite markedly in their individual *in vitro* appearances and behaviours: these properties appeared stable and were typically maintained even after re-cloning and multiple passage.

Pure populations of tumour cells grown on a collagen matrix populated with fibroblasts, i.e. in an organotypical environment, behave somewhat like normal cells in that they stratify and organize an epithelial-like structure (26, 27). Each of the tumour lines examined in the present study showed this behaviour and, to a limited extent, maintained some of the morphological differences that were apparent between individual tumours when grown on plastic. However, even in organotypical culture, the tumour lines failed to form a well-differentiating epithelium similar to that formed by normal oral keratinocytes under these conditions (17, 18). Marked differences were also observed between the behaviours of malignant and normal keratinocytes when they were incorporated as subpopulations within 'host' organotypical cultures generated from normal keratinocytes. In this situation, labelled normal cells could be seen to flatten and participate in the reformation of a stratified epithelium whereas tumour cells failed to do so and either remained in the basal layer or were desquamated without fully differentiating.

The phenotypic patterns expressed *in vivo* by tumour cells and their surrounding normal cells are presumed to be important to cell–cell interactions that may influence the survival and growth of the malignant component. Under standard *in vitro* methods of growth on plastic, neither normal nor malignant cells develop *in vivo*-like patterns of cell differentiation and such culture conditions thus provide a less than ideal cell context for studies. This deficiency can be partially overcome by the ability of organotypical cultures to generate patterns of cell differentiation similar to those expressed *in vivo* and thus mimic pathologically relevant behaviour of primary or metastatic tumour cells more closely. The studies of the fate of transformed genetically marked cells within an organotypical system by Garlick and co-workers (19, 20) demonstrate the advantages of this method for examining how clones of altered cells survive and expand within a relatively normal environment. The present study has used similar methods to examine the effects of interactions occurring between normal oral keratinocytes and cells isolated from human Oral squamous cell carcinoma (OSCC). The results demonstrate the feasibility of using organotypical culture for these more immediately relevant cell types and indicate that it is possible to follow the growth of malignant cells that have been labelled by retroviral transduction. They also indicate that interactions occurring between normal and malignant cells may have a different outcome from that seen in the HaCaT model.

The model used by Garlick and co-workers (19, 20) employed two versions of the HaCaT cell line: (i) the original line that was derived from a spontaneous *in vitro* change in human epidermal keratinocytes; and (ii) a line with malignant behaviour that was derived from it by transduction with the c-Harvey-*ras* oncogene (28). Each of these cell types, when plated to form organotypical cultures, produces a stratified epithelial structure. However, when a mixed population of keratinocytes was plated, differential survival of each cell type was seen. A striking feature of the behaviour of the malignant HaCaT cells was that their survival was critically dependent on the number of malignant cells incorporated initially into the cultures (19). When a greater than 1 : 2 proportion of malignant to normal cells was plated, malignant cells survived and proliferated to form 'nests' or clusters of cells that expanded at the expense of the surrounding non-malignant cells. Below this proportion, irrespective of whether the cultures were maintained *in vitro* or were transplanted to immuno-deficient mice, the entire malignant population was lost. The early stages of growth and neoplastic progression of the *ras*-transformed HaCaT cells thus appeared to be, in some way, suppressed by the presence of the untransformed HaCaT cell population. An ability of normal cells to suppress the growth of altered or malignant cells has been reported in some previous studies, but no clear mechanism of this effect has emerged (29, 30). Possible mechanisms for the survival of malignant cells when plated at high numbers in the HaCaT system include groups of malignant cells being protected from suppressive effects of either direct cell-to-cell contact with normal cells or from diffusible paracrine-acting substances produced by them.

The present study found that malignant cells isolated from OSCC were also able to proliferate within organotypical cultures to form clusters of cells that expanded at the expense of the normal epithelium. However, a major difference between the behaviours of the OSCC and the HaCaT cells was that the survival of OSCC cells was not dependent on the number of cells initially added to the cultures. Cells of each of the OSCC lines were found to survive and proliferate even when the fraction of malignant cells present in the initial plating mixture was as low as 0.1%. Growth of OSCC cells at very low plating densities indicates that their survival does not depend on the sort of density-dependent mechanisms required by HaCaT cells, but it is unclear whether an influence of cell density on the survival of transformed HaCaT cells may be a property of the HaCaT model itself or it is a requirement of cells at early stages of progression to malignancy. The OSCC cells used in the present study were

all derived from well-established malignant tumours, and cells at such later stages of malignancy may have a greater ability for independent cell survival and growth. This does not, however, provide an explanation of why over 90% of the OSCC cells that were initially plated to form the cultures failed to survive and participate in the formation of the cell clusters present 14 days later. Further studies of the initial phases of cell proliferation and death are needed to rule out trivial reasons, such as a differential ability to attach to the matrix at the time of plating, but an interesting alternative explanation is that malignant cell populations are intrinsically heterogenous and, like normal keratinocyte populations, contain only a small fraction of cells with the ability to act as clonogens (31).

It was suggested, nearly 20 years ago, that the presence of stem cells in malignant tumours could be reasonably assumed from the fact that stem cell systems are present in the tissues from which they arise (32). However, what the particular properties of malignant stem cell systems might be remains a controversial issue. Kummermehr, who has discussed this problem in detail (15, 33), has pointed out that the ability of primary tumours or metastases to develop from single cells indicates that some malignant cells possess at least one stem cell property: the capacity for unlimited proliferation. The proliferative hierarchy of stem and amplifying cells present in normal epithelia results in keratinocytes, both *in vitro* and *in vivo*, having a range of proliferative potentials and showing cell kinetic heterogeneity (8). Tumours consistently show patterns of kinetic heterogeneity (15, 31), and the different clonogenic potentials of cells in haematological malignancies have been suggested to reflect the persistence of stem cell hierarchies (31, 34, 35). Estimates for the number of cells acting as clonogens suggest fractions as low as 0.01–1% of the total population (35), but it is recognized that the identity of clonogenic cells with stem cells is questionable (32). Estimates for an experimental murine carcinoma suggest that cells with clonogenic ability may represent about 5% of the tumour cells (15), a figure similar to the percentage of OSCC cells found to survive in the organotypical cultures used in the present study.

The presence within epithelial tumours of stem and amplifying cells with different behavioral properties would require modification of several prevailing concepts of tumour growth and therapy. For example, the dissemination of malignant cells from OSCC to local and distant sites appears higher than the rate of formation of secondary lesions (1). But if malignant stem cells form only a small fraction of the population and have less migratory properties than amplifying cells have, it may be that most of the disseminated cells lack clonogenic properties. Concerning therapy, it is not yet known how stem and amplifying cells differ in their responses to drugs and radiation, but if therapies were to act mainly on the amplifying cell fractions, they could greatly reduce tumour mass without eliminating the cells from which the tumour actually regenerates. Therapies selectively targeting stem cells would therefore be advantageous. Further considerations of this kind require the development of better experimental models for investigating the nature of tumour cells, and it appears that the inclusion of malignant cells within organotypical cultures of normal epithelium may provide a type of functional clonogenic assay that will be useful in unravelling the problems of tumour stem cell behaviour.

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