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Differentiation of a squamous carcinoma cell line in culture and tumourigenicity in immunologically incompetent mice

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Summary. The process of differentiation in keratinocytes is accompanied by specific membrane and cytoplasmic changes. Using simple tissue culture techniques a well differentiated squamous carcinoma cell line is shown to exhibit in vitro keratinization with the formation of a multilayered structure and shedding of cells with a cornified envelope. The cell line produces tumours when xenografted into mice which are well differentiated and indistinguishable at the light and electron microscope level from the original surgical biopsy. It is concluded that the tumour will provide a suitable model for detailed in vitro and in vivo studies to compare both biological and pathological features of normal keratinocytes and their malignant counterparts.

Key words: Squamous carcinoma – Keratinocytes – Differentiation – Basement membranes

Introduction

Squamous carcinomas are an important group of tumours which arise from both skin and mucous membranes. They show varying degrees of differentiation ranging from the very well differentiated verrucous carcinomas that are often difficult to distinguish from benign proliferative lesions through to the highly anaplastic tumours that can be misinterpreted as sarcomas. The majority of the tumours that arise in the oropharynx are moderately to well-differentiated carcinomas and it was our intention to characterise a tumour that was established in culture which retained the ability to exhibit differentiated characteristics and could thus be used for comparative studies with normal keratinocytes. Recently Easty et al. (1981) have described ten squamous carcinoma cell lines. These were screened to identify the line which most closely fulfilled the criteria for keratinization in vitro in a manner similar to that reported previously for keratinocytes derived from both skin

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(Friedman-Kien et al. 1966; Karasek 1966; Matoltsy 1960; Rheinwald and Green 1975) and oral mucosa (Flaxman et al. 1967; Gusterson and Monaghan 1979). The results are discussed in relation to cell lines reported by other workers. The potential usefulness of this cell line to compare the in vitro and in vivo characteristics of normal and malignant keratinocytes is considered together with the application of methods to isolate defined cell populations in different stages of differentiation.

Materials and methods

Tissue culture. The cell line LICR HN-5 was derived from a surgical biopsy of a moderately to well differentiated squamous cell carcinoma of the tongue (Easty et al. 1981). The cells were grown in 25 cm^2 tissue culture flasks (NUNC) containing Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum; benzyl penicillin 10^5 units/l; streptomycin 100 mg/l; kamamycin 100 mg/l and amphoteracin β 2.5 mg/l. Each flask was gased with 10% CO_2 in air to obtain a pH optimum of 7.4. Cultures were incubated at 37° C. Feeder layers were not required but the cells were seeded at a high density (equivalent to 2.5×10^5 cells/25 cm^2 flask). Cultures were routinely sub-cultured at confluence (i.e. every 7 days) but in some experiments cells were left for up to four weeks when they became hyperconfluent. Subcultures were performed using a 0.1% solution of typsin-versene. Cultures were examined at intervals using a WILD M40 phase contrast microscope. Cultures used for electron microscopy were treated in the same way as those used for phase contrast viewing with the exception that the cells were grown on Permanox cover slips.

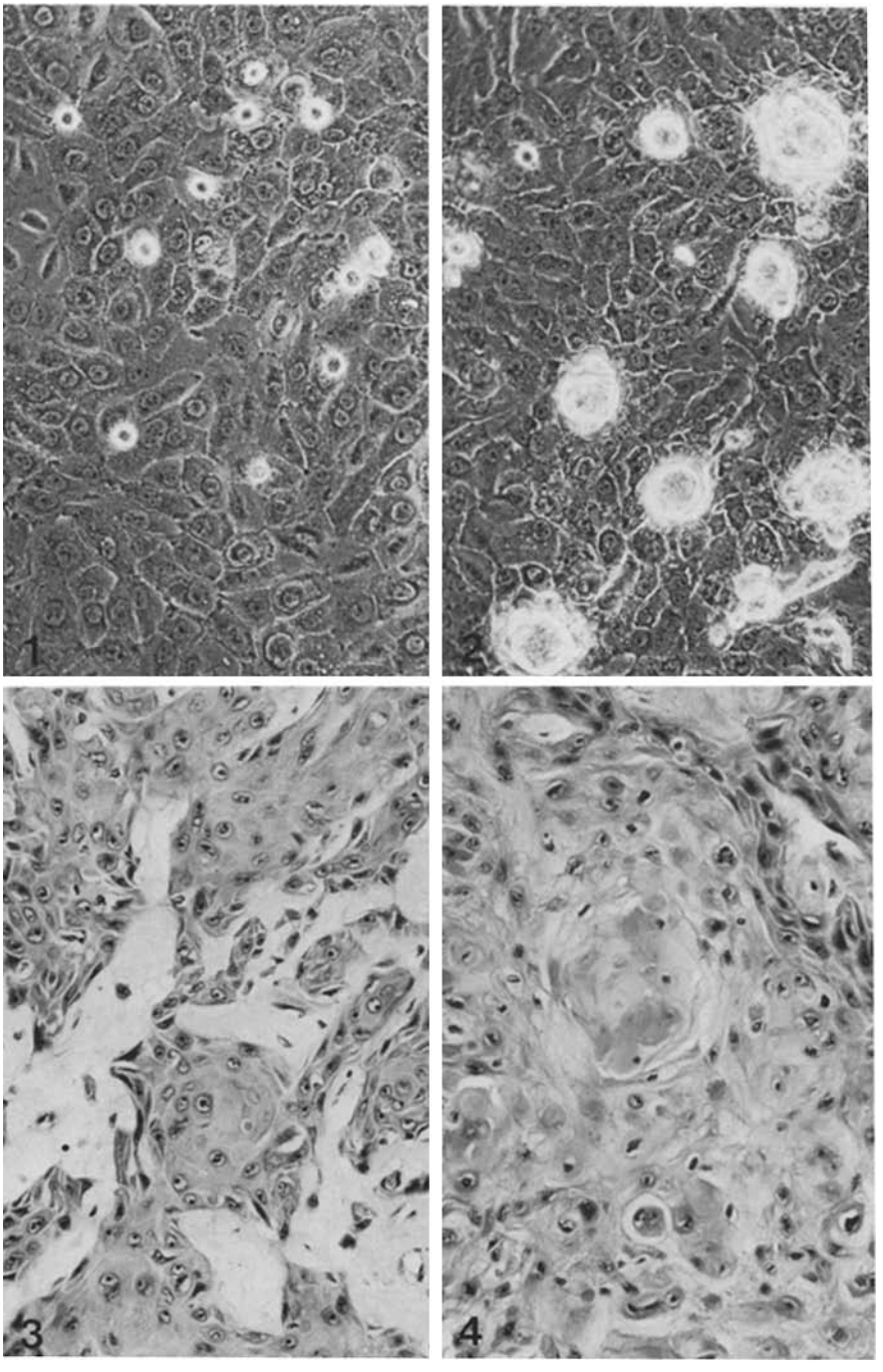
Xenografts. Two methods were used to establish the cell line as a xenograft. 5×10^6 cells in 0.2 ml of medium 199 were injected into both nude and CBA/LAC immunosuppressed female mice (Miller et al. 1963). Alternatively cells were grown on absorbable gelatin sponge and the fragments implanted subcutaneously into immunosuppressed mice. Both techniques produced tumours which were then repassaged into either nude or immunosuppressed animals. Material taken for histology at each passage was fixed in neutral formal saline and embedded in paraffin wax. Sections were cut at 4 μ and stained with haematoxylin and eosin.

Electron microscopy. After 14 days of culture the tissues maintained on Permanox were processed for electron microscopy. The culture media were poured off and the tissues fixed for 1 h at 4° C in 2% glutaraldehyde, and post-fixed for 2 h in 1% osmium tetroxide, also at 4° C. Both fixatives were phosphate buffered (0.05 M, pH 7.2–7.4) and the osmotic pressure adjusted to 350 milliosomoles with sucrose. The tissue was dehydrated with ethanol and embedded via propylene oxide in Epon/Araldite (Mollenhauer 1964). Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined using a Phillips EM400. Material from the original surgical biopsy and from the xenografted tumours was treated in the same way as the cultured material.

Results

Cultural characteristics

When seeded at low density (2.5×10^5 cells/25 cm^2 flask) the cells migrated together to form epithelial islands within 48 h of plating. These monolayer colonies proliferated until the cells formed a confluent sheet. Under the phase contrast microscope the cells at confluence formed an epithelial pavement with some cellular heterogeneity (Fig. 1) and abundant mitotic figures, some of which were tripolar. If seeded at hyperconfluence or if cultured



Figs. 1, 2. Seven day (**Fig. 1**) and fourteen day (**Fig. 2**) cultures of LICR HN-5 passage 32 demonstrating epithelial monolayer at confluence and nodules formed in hyperconfluent cultures. Magnification ×180

Figs. 3, 4. Squamous carcinoma of the tongue. Original surgical biopsy (**Fig. 3**) showing a moderately differentiated region with keratin pearl formation. Passage 6 of the xenografted tumour (**Fig. 4**) established from the cell line LICR HN-5 with a similar degree of keratinization to that seen in the surgical material. Magnification ×240

beyond confluence the cultures became multilayered with focal solid nodules (Fig. 2). These nodules were eventually connected by dense bands of compacted cells. As the cells reach confluence there is shedding of epithelial 'squames' from the surface. The desquamation is best seen after two weeks in culture when cells are continuously lost as the cells undergo terminal differentiation.

At the electron microscope level, the presence of a multilayered structure is confirmed (Fig. 5). The cells adherent to the substrate, are less flattened than the cells nearer the culture medium, and contain less bundles of tonofilaments. These cells which have many of the ultrastructural characteristics seen in basal cells are joined by interdigatating cell processes, which become less prominent in the more flattened cells in the upper layers of the culture.

There were characteristic changes in the plasma membrane according to a cell's position in the multilayer. In general, although desmosomes could be identified at all levels, they were most numerous in the intermediate layers (Fig. 6). In the superficial cells desmosomes were quite sparse, but residual desmosomal plaques could be identified. The 'desquamating' cells and the most superficial cells were characterised by a thickened plasma membrane.

This thickening was due to condensation of an electron dense material on the cytoplasmic side of the tri-laminar membrane (Fig. 7). The cells were also characterised by numerous bundles of tonofilaments with marked desmosome-filament complexes. The most superficial cells had the typical tonofilament arrangement of cells in the stratum corneum with a breakdown of tonofilaments to form a dense network of amorphous filaments. Within these superficial cells there were very few cytoplasmic organelles. Keratohyalin granules were very occasionally seen, and membrane coating granules were not identified.

In the "nodules" formed at hyperconfluence the majority of the cells were packed with tonofilaments and were similar to those seen in the upper layers of the multilayer. Mitotic figures and basaloid cells were not seen in the nodules.

Xenograft

The tumours formed were identical in both nude and in immunosuppressed mice. They formed well circumscribed nodules with central keratinization. The tumours were well to moderately differentiated squamous cell carcinomas with keratin pearl formation (Fig. 4) similar to that seen in the original surgical biopsy (Fig. 3). Some cells had the ultrastructural features of basal cells with a few bundles of tonofilaments, numerous interdigitating cell process and hemi-desmosomes which inserted into a well formed basement membrane (Fig. 8). Typical spinous cells (Fig. 9) and cells containing keratohyalin granules (Fig. 10) were also clearly defined. The tumour is now in its twentieth passage having been maintained for 2 years and the morphology is unaltered.

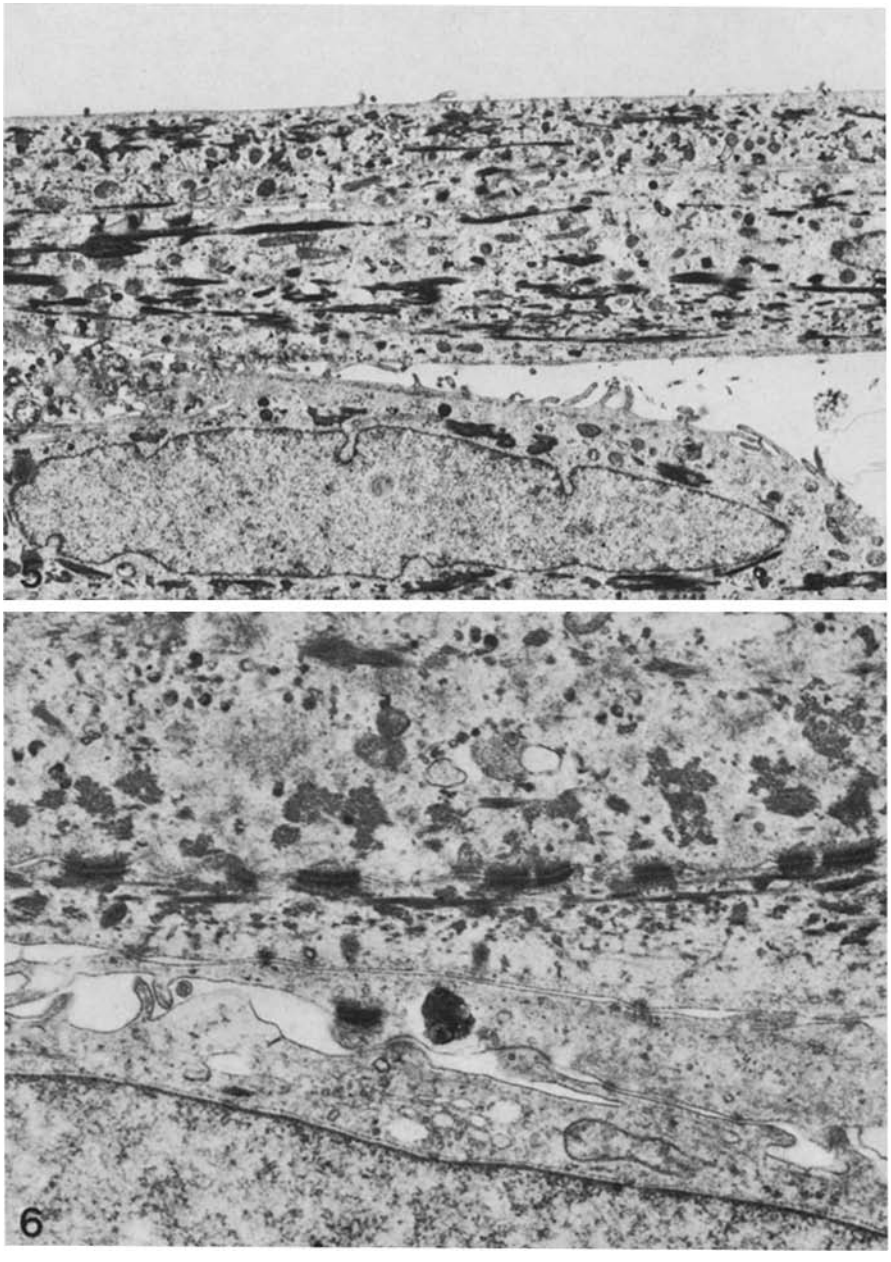


Fig. 5. Electron micrograph showing multilayering in a fourteen day culture of LICR HN-5 passage 31 with flattening of cells in the superficial layers and an increase in tonofilaments. Magnification × 5,500

Fig. 6. Electron micrograph from cells of intermediate position in the multilayer with a "basaloid" cell at the bottom and numerous desmosomes joining the cells mid-way in the multilayer. Passage 31 of LICR HN-5. Magnification × 20,000

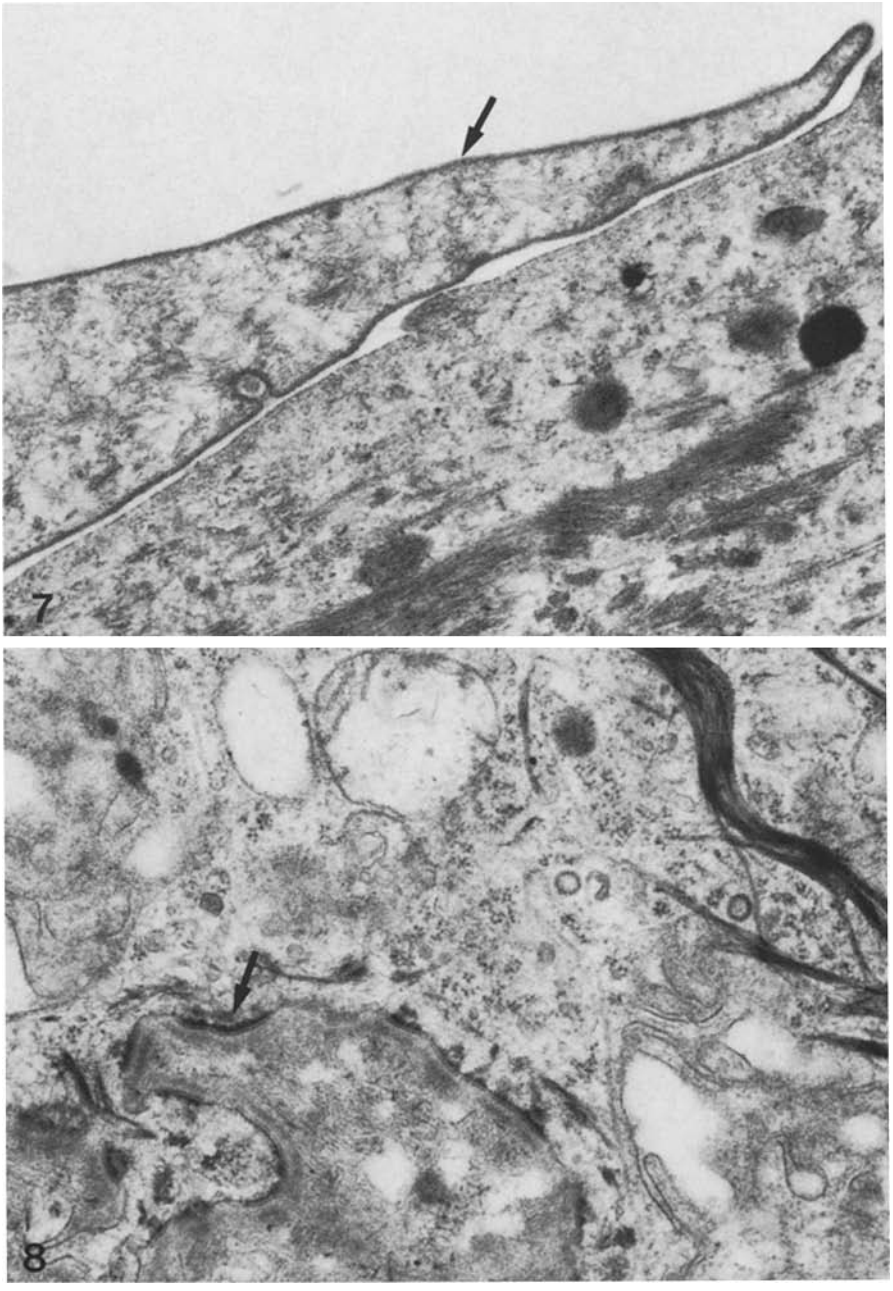
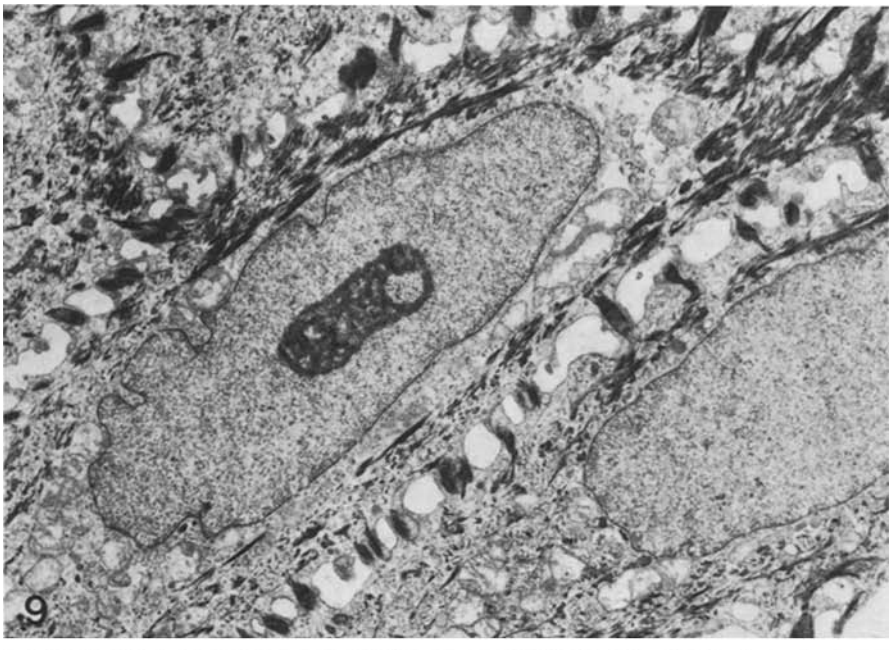


Fig. 7. Electron micrograph of superficial cells from the same culture as Fig. 6, demonstrating thickening of the plasma membranes (*arrow*) loss of cellular junctions and early "desquamation". Magnification ×48,000

Fig. 8. Xenografted LICR HN-5. Electron micrograph of a basal cell with basal lamina production and hemidesmosomes (*arrow*). Magnification ×30,000



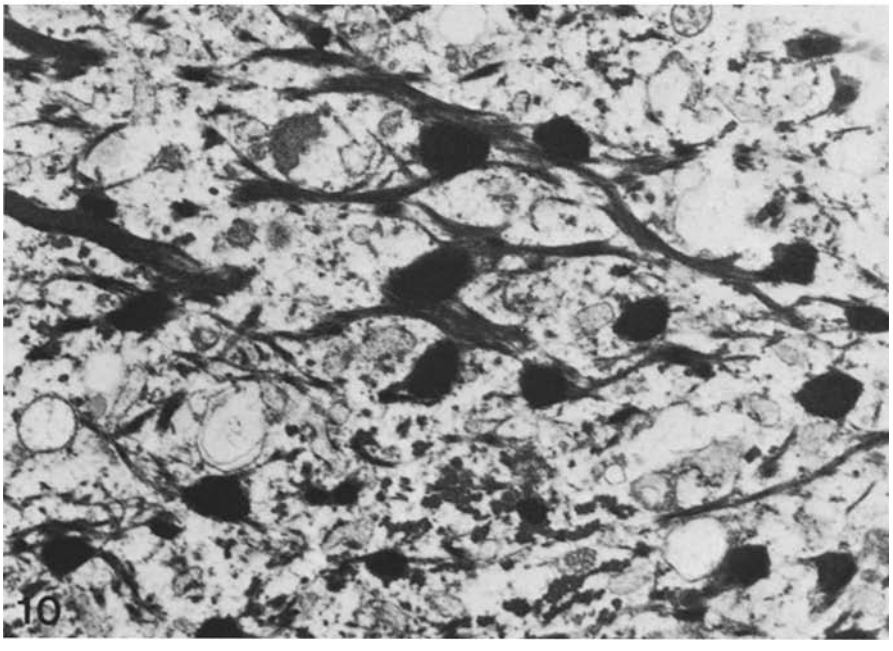


Fig. 9. Xenografted LICR HN-5. Electron micrograph of a typical spinous cell with numerous desmosomes and tonofilament bundles. Magnification × 7,500

Fig. 10. Xenografted LICR HN-5. Electron micrograph showing keratohyalin granule formation. Magnification × 18,000

Discussion

The advances produced by the ability to serially culture keratinocytes from normal human tissues (Rheinwald and Green 1975) and to reimplant these cells into immune deprived animals (Doran et al. 1980) has laid the foundations for detailed studies into the mechanisms controlling keratinocyte differentiation. The establishment of cell lines from squamous carcinomas will provide the material for both morphological and biochemical comparisons between normal keratinocytes and their malignant counterparts. These cell lines with their ability for continuous growth and the production of tumours in animals will also make it possible to obtain reproducible defined cell populations that can be utilized to examine the kinetic parameters that control the proportions of cells in different stages of the differentiation process.

This squamous carcinoma cell line unlike those reported by Rheinwald and Beckett (1981) forms a well organised multilayered structure in culture with ultrastructural evidence of keratinization similar to that reported for normal keratinocytes (Gusterson et al. 1979). The cell line is not dependent upon fibroblast support nor has it any special requirements for epidermal growth factor or other additives. The cells will not however survive seeding at low density and like those of Rheinwald and Beckett are anchorage dependent and will not form colonies in agar (Easty et al. 1981). The tumour cell line has now been transferred beyond passage 90 and the morphology is unchanged. Similarly the xenografted tumour is still maintained as a moderately to well differentiated squamous cell carcinoma after 20 passages.

The formation of well defined basal cells with hemidesmosomes and the production of basement membrane material suggests that this tumour will provide a good model for the investigation of basement membrane production by squamous cells. The well differentiated nature of the tumour with typical squamous cells and keratohyalin production also suggests that the tumour may be a suitable model to investigate the mechanisms controlling keratinocyte differentiation in malignant keratinocytes. The similarity between the differentiation in this tumour and in normal keratinocytes is also supported by the lack of staining with the intermediate filament antibody LE61 (a gift from Dr. B. Lane, Imperial Cancer Research Fund, London) (Gusterson et al. unpublished observation); a filament absent in normal keratinocyts in culture, but present in the SV40 transformed cells (Taylor-Papadimitriou et al. 1982).

Results recently published on the separation of keratinocyte population, with defined biological functions, from normal human skin using velocity sedimentation (Morhenn et al. 1982) suggests that by applying similar technology to this cell line it should be possible to obtain unlimited supplies of cells in different stages of differentiation for kinetic, biological and morphological studies.

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