CELL LINES OF HUMAN ORAL SQUAMOUS-CELL CARCINOMAS RETAINING THEIR DIFFERENTIATED PHENOTYPE

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Two cell lines from head-and-neck squamous-cell carcinomas (SCC) have been established and characterized. Cell line R105 was derived from a xenografted SCC of the floor of the mouth and cell line T87/rc from a SCC of the epiglottis. Identification of individual cytokeratins 4, 5, 7, 8, 10, 13, 14, 18 and 19 led to the conclusion that both cell types had squamous characteristics and that keratinization occurred in xenograft Ultrastruclurally, junctions were observed in both cell lines. Characteristic marker chromosomes were found and although both cell lines were derived from male patients, the Y chromosome was missing from all examined cells. The basic biological parameters of both cell lines were modal chromosome numbers of 59 (R105) and 60 (T87/rc), a doubling time of 60 (R105) and 45 hr (T87/rc) and a DNA index of 1.54 (R105) and 1.31 (T87/rc). The tumorigenicity of the 2 cell lines was proved by the ability to form colonies on a plastic substratum, as well as in a soft agar assay. Furthermore, the cells could produce multi-cellular tumour spheroids and formed tumour nodules after subcutaneous inoculation into nude mice. The R105 tumour cells appeared to be better differentiated than the T87/rc as observed by histology and immuno( histo)chemistry. Both cell lines appear to retain SCC differentiation after being xenografted into nude mice, cultured for more than 40 passages in vitro and thereafter again xenografted into nude mice.

A substantial number of cell lines from squamous-cell carcinoma (SCC) of the head and neck region have been reported (Easty et al., 1981; Rapaport et al., 1983; Krause et al., 1984). Only a few of these cell lines are fully characterized, while most of them are used in early passages. SCC-derived cell lines are useful model systems for investigating defective terminal differentiation of malignant keratinocytes (Rexhaind and Beckett, 1980), invasive behaviour (Boukamp et al., 1982), and cytogenetic characteristics of SCC cells (Boukamp et al., 1982; Easty et al., 1981; Tilgen et al., 1983). Most established SCC cell lines of the head and neck region are, however, not useful for in vitro irradiation studies because of their limited radiosensitivity and their poor plating efficiency in soft agar. Only a few reports described the possibility of producing multicellular tumour spheroids with SCC cell lines (Schwachöfer et al., 1989). Cultures from primary human head and neck SCC are difficult to obtain, be it from primary human specimens or from xenografts in nude mice (Bijman et al., 1987). Furthermore, it is essential to confirm the SCC nature of established cell lines. We now report the establishment and full characterization of 2 new human SCC cell lines of the head and neck region. Both lines retain a certain degree of SCC differentiation, as concluded from their cytokeratin profile.

MATERIAL AND METHODS

Establishment of squamous-cell carcinoma cultures

All studies with tissue cultures were performed in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 100 units penicillin/ml, 100 units streptomycin/ml and 2 mm L-glutamine (Gibco, Paisley, Scotland).

The tumour xenografts were chopped into small fragments and incubated in Tyrode's buffer (Ca²⁺ and Mg²⁺-free) with 0.025% collagenase, type IA, (Sigma, St. Louis, MO) and 0.004% deoxyribonuclease 1 (Sigma), and incubated for 30 min at 37°C. After filtering through a 50-μm nylon mesh, the tumour cells were seeded into 25-cm² tissue culture flasks (Nunc, Roskilde, Denmark). The flasks were stored at 37°C in a humidified incubator in 7.5% CO₂. Further culturing was performed as described by Mungyer et al. (1987). Calculation of the doubling time, flow cytometric analysis of the DNA content and cell cycle distribution were performed as described by Van Niekerk et al. (1988).

Light and electron microscopy

Cells grown on Multisiot slides (Flow, Irvine, Scotland) were fixed for 5 min in methanol (−20°C) and routinely stained with May-Grünwald/Giemsa stain. Paraffin sections from formalin-fixed tissues of nude mouse xenografts were stained with haematoxylin and eosin. For electron microscopy, monolayers of tumour cells and subcutaneously growing tumour nodules were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 420 mOsm) at 4°C, and further processed according to standard procedures (Mungyer et al. 1987).

Immunohistochecmistry

The indirect immunofluorescence technique was used to test the activity of monoclonal antibodies (MAbs) (described below) on 5- to 7-μm thick cryostat sections of the xenografts according to Van Niekerk et al. (1988). Cultured tumour cells grown on Multisiot slides (Flow) were fixed in methanol (−20°C) for 1-2 min, and processed in the same way. This short and gently used fixation procedure did not influence MAb reactivity. The following MAbs were used:

A. Mouse MAbs against intermediate filament proteins: cytokeratin 5 and 8: RCK102 (Broers et al., 1988); cytokeratin 4: 6810 (Broers et al., 1988); cytokeratin 7: RCK105 (Broers et al., 1988) and OV-13/20 (Van Niekerk et al., 1988); cytokeratin 8: M20 (Schaafsma et al., 1989); cytokeratin 10: RKE520 (Broers et al., 1988); cytokeratin 13: IC7 (Broers et al., 1988); cytokeratin 14: LL002 (Parkis et al., in press); cytokeratin 15: RGE53, RCK 106 (Broers et al., 1988); cytokeratin 19: LP2K (Broers et al., 1988); vimentin: RV202 (Broers et al., 1988); desmin: RD301 (Van Niekerk et al., 1988).

B. Mouse MAbs BW431/31 directed against a CEA epitope (Van Niekerk et al., 1988) and BW495/36 directed against a 200-KDa glycoprotein found in epithelial cell types other than mesothelial cells (Van Niekerk et al., 1988).

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Colony formation and multicellular tumour spheroids

Colon formation and plating efficiency were determined in 2 assays: 1. on a plastic substrate by plating of $10^2$–$10^4$ cells per 35-mm diameter dish. The number of colonies consisting of at least 50 cells were scored after an incubation period of 10 days; 2. in soft agar (Courtenay et al., 1978). Multicellular tumour spheroids (MTS) were produced by the liquid overlay method (Schwachfelder et al., 1989).

Cytogenetic analyses

Both cell lines were examined cytogenetically at passage 40 (Van Nickel et al., 1988). Chromosome numbers were counted from 52 (in the case of cell line R105) and 54 (in the case of cell line T87/rc) well-spread metaphases after conventional Giemsa staining. Detailed chromosome analysis was performed on 12 (R105) or 14 (T87/rc) GTG-banded cells, respectively. For both cell lines 10 cells were also studied after QFQ-staining to confirm the absence of Y chromosome. From each cell line 2 cells were fully karyotyped.

**Xenografts in nude mice**

From various subcultures $5 \times 10^3$ to $1 \times 10^6$ cells were

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**TABLE 1 - BASIC BIOLOGICAL PARAMETERS OF CELL LINES R105 AND T87/rc AT PASSAGE 40**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (days)</th>
<th>DNA index</th>
<th>Formation of MTS</th>
<th>PE14 (%)</th>
<th>PE24 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R105^6</td>
<td>40 ± 9</td>
<td>1.54 ± 0.10</td>
<td>+</td>
<td>0.06</td>
<td>9.7</td>
</tr>
<tr>
<td>T87/rc^7</td>
<td>38 ± 3</td>
<td>1.31 ± 0.03</td>
<td>+</td>
<td>0.64</td>
<td>7.3</td>
</tr>
</tbody>
</table>

^1Squamous-cell carcinoma. ^2Flow cytometry. ^3Multicellular tumour spheroids. ^4Plating efficiency in soft agar. ^5Plating efficiency of colonies on plastic substrate. ^6Cell line obtained from xenografted SCC (passage 3) in nude mice. ^7Cell line obtained from a xenografted SCC (passage 6) in nude mice.

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**FIGURE 1** - Light microscopy (LM), phase-contrast microscopy (PhCM) and electron microscopy (EM) of cell lines R105 (a–j) and T87/rc (g–l). (a) Confluent growth pattern of cultured epithelioid tumour cells. Scale bar: 50 μm, PhCM. (b) Xenografted tumour cells with a focus of squamous differentiation pattern. Scale bar: 5 μm, LM. (c) Cultured and suspended tumour cell with microvilli and circumscribed areas of glycogen particles (arrows). Scale bar: 0.5 μm, EM. (d) Projections of cultured tumour cells showing junctional complexes of the fascia adherens type (arrows) (g indicates glycogen). Scale bar: 0.5 μm, EM. (e) Xenograft with a focus of flattened squamous tumour cells arranged in concentric layers. Scale bar: 5 μm, EM. (f) Detail of xenografted and neighbouring tumour cells showing fascia adherens (arrows) (g indicates glycogen particles). Scale bar: 0.5 μm, EM. (g) Confluent growth pattern of cultured epithelioid tumour cells. Scale bar: 50 μm, PhCM. (h) Xenografted tumour cells showing few mitoses. Scale bar: 5 μm, LM. (i) Cultured and suspended tumour cell with mitochondria (arrows); at the same site microvilli projections concur with deep cytoplasmic invaginations. Scale bar: 0.5 μm, EM. (j) Cultured tumour cells connected by fascia adherens (arrow). Scale bar: 0.5 μm, EM. (k) Three closely apposed tumour cells of a xenograft; at x parts of intracellular lumina and at y part of one intercellular lumen. Scale bar: 0.5 μm, EM. (l) Two xenografted tumour cells with microvilli lining an intercellular lumen; arrows point to junctional complexes. Scale bar: 0.5 μm, EM.
efficiency was 9.7% for R105 and 7.3% for T87/rc. It was also possible to produce multicellular tumour spheroids.

The DNA indices of the 2 tumour cell lines were 1.54 for R105 and 1.31 for T87/rc. Human lymphocytes were used as reference with a DNA index = 1.0. For both cell lines the DNA content is thus considered as nearly triploid (pseudo-triploidy).

**Light and electron microscopy of cell lines (Fig. 1)**

After 40 passages both cell lines exhibited a stable morphology. The cells adhered to plastic as well as to glass surfaces, growing as monolayers, multilayered and in clusters. The cells showed an epithelial growth pattern with elongated processes.

Ultrastructurally, the cell lines showed numerous junctional complexes of fascia adherens type. Cell line R105 showed large glycogen areas and randomly distributed organelles, whereas T87/rc contained dispersed glycogen particles with polarized, localized organelles in the junctional regions.

**Light and electron microscopy of xenografts (Fig. 1)**

About 4 weeks after s.c. inoculation of 0.5 to 1 × 10⁷ tumour cells, the xenografts had reached a diameter of at least 1 cm with a take rate of 100%. Subcutaneously, nodules of both cell lines were encapsulated within fibrous connective tissue. Xenografts from R105 consisted of loosely packed, small, round, polyhedral cells and a range of lobulated sheets or anastomosing columns. Generally, epithelial cells appeared pale and showed no scantly cytoplasm. Epidermoid islands were more distinct but without characteristic keratinization patterns. Mitoses were abundant.

T87/rc tumour cells were closely packed and arranged in well-defined clumps or in small foci with epidermoid features surrounded by stromal cells with a spindle-cell morphology. Mitoses were conspicuous. Inter- and intracellular lumen formation was observed.

**Cytogenetic characterization**

In cell line R105 the number of chromosomes per cell varied from 51 to 89 (mean ± SD = 59.0 ± 4.9) with a modal number of 59, while in cell line T87/rc, 44 to 83 (mean ± SD = 57.7 ± 5.3) chromosomes per cell were counted, with a modal number of 60. In both cell lines marker chromosomes were present, and some of these markers were considered to be clonal (Fig. 2). None of the marker chromosomes was recognized as HeLa cell marker.

**Immunohistochemistry of the cell lines (Fig. 3 and Table II)**

Cell line R105 reacted strongly with virtually all MAb's against cytokeratin, with the exception of RKSE60. For RGE53, 50% of the cells were strongly positive while the rest reacted weakly or showed no reaction (<10%). The cells of T87/rc reacted strongly with MAb's RGE53, RCK102, RCK105, OV-TL 12/30 and M20. A moderately positive reaction was shown with MAb's LL002 and 1C7, and a negative reaction with MAb's GB10, RKSE60 and LP2K.

The epithelial character of the 2 cell lines was confirmed by a positive, although heterogeneous reaction with BW495/36. The MAb against desmin RD301 was negative with both lines. T87/rc expressed vimentin as determined by a positive filamentous reaction with MAb RV202. Although several epithelial cell types in culture expressed vimentin, cell line R105 did not. The anti-CEA MAb BW431/31 showed a strong positive reaction with R105 and a very weak to negative reaction with T87/rc.

**Immunohistochemistry of xenografts (Figs. 3 and 4, Table II)**

R105 xenografts showed the same immunohistochemical phenotype as the cultured cells, except for a positive reaction
with CK 10 (RKSE60) and a diminished reaction for CK 18 (RGE53) and BW495/36.

Immunoblotting of cytoskeletal preparations from R105 xenografts (Fig. 4a) confirmed results of immunohistochemical assays. The use of MAb RCK102 in immunoblotting studies revealed the presence of CK 5 in tumour cells. This conclusion could not be drawn on the basis of the immunofluorescence study, since both RCK102 (recognizing CK 5 and 8) and M20 (recognizing CK 8 alone) were positive. In conclusion, in R105 immunohistochemical studies proved the presence of significant amounts of proteins migrating in the position of cyto-keratins 4, 5, 7, 8, 10, 13, 14, 18 and 19. Vimentin could not be detected. Proteins migrating faster in the one-dimensional gels from the individual cyto-keratins most likely represent breakdown products.

Also T87/rc xenografts showed a similar reactivity pattern with the MAbs when compared to the cultured T87/rc cells, except for a positive reaction with LP2K (against CK 19) and a negative reaction with 1C7 (against CK 13) in the xenografts. Immunoblotting of the cytoskeletal preparations of T87/rc xenografts confirmed the results found by immunofluorescence assays, except for a strongly positive reaction with CK 10 (RKSE60). These xenograft preparations could also be shown to contain CK 5 by application of RCK102 MAb to the immunoblots. Figure 4b shows that cytokeratins 4 and 13 are absent from T87/rc xenografts. The vimentin reactivity in the cyto- skeletal preparations must be ascribed to expression of this intermediate filament protein in the human tumour cells. Antibody RV202 does not react with mouse vimentin that is present the host stromal cells.
TABLE II – IMMUNOHISTOCHEMICAL AND IMMUNOBLOTTING RESULTS OBTAINED WITH INTERMEDIATE FILAMENT ANTIBODIES TO EPITHELIAL MARKERS IN CELL CULTURES AND XENOGRAFTS OF R105 AND T87/rc

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Antigen recognized</th>
<th>R105 cultured IF</th>
<th>R105 xenograft IF</th>
<th>T87/rc cultured IF</th>
<th>T87/rc xenograft IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>6B10</td>
<td>CK 4</td>
<td>+/+</td>
<td>+/+ -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RCK102</td>
<td>CK 5 + 8</td>
<td>+</td>
<td>+</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>RCK103</td>
<td>CK 7</td>
<td>+/+</td>
<td>+/+ +/+</td>
<td>+</td>
<td>+/+ +/±</td>
</tr>
<tr>
<td>OV-TL12/30</td>
<td>CK 7</td>
<td>+</td>
<td>+/±</td>
<td>+</td>
<td>+/±</td>
</tr>
<tr>
<td>M20</td>
<td>CK 8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RKSE60^3</td>
<td>CK 10</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>IC7</td>
<td>CK 13</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LL002</td>
<td>CK 14</td>
<td>+</td>
<td>+</td>
<td>+/±</td>
<td>+/±</td>
</tr>
<tr>
<td>RGE53</td>
<td>CK 18</td>
<td>+/+ -</td>
<td>±</td>
<td>+/±</td>
<td>+/±</td>
</tr>
<tr>
<td>LP2K</td>
<td>CK 19</td>
<td>±</td>
<td>+</td>
<td>+/±</td>
<td>+/±</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desmin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EPH5/36</td>
<td>Glycoprotein of 200 kDa</td>
<td>+/+</td>
<td>±</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

^Immunofluorescence results. - Immunoblotting result. - ND, not done. - +, +, Strongly positive reaction; +, Moderately positive reaction; +, weakly positive reaction; - negative reaction. - +/+ - means partly (+/0%) positive reaction. +/+ or +/+ in the case of the immunoblotting results of RCK102 indicates reactivity with CK 5 (before the slash mark) and with CK 8 (after slash mark). - Within this series focal or sporadic positivity occurs.

**FIGURE 4** – Immunoblots of cytoskeletal preparations of R105 (a) and T87/rc (b) xenografts incubated with: lane 1: RCK102, cytokeratin (CK) 5 + 8; lane 2: RCK105, CK 5; lane 7: IC7, CK 13; lane 4: RKSE60, CK 10; lane 3: 6B10, CK 4; lane 6: M20 after 6B10, CK 8 after CK 4; lane 7: LP2K, CK 19; lane 8: LL002 after LP2K, CK 14 after CK 19; lane 9: RGE53, CK 18; lane 10: vimentin RV202 after RGE53 (CK 18).

**ANALYSIS OF ANTIGENS SHED INTO THE CULTURE MEDIUM**

Cell line R105 released hCG and, to a lesser extent, CEA and the SCC-antigen recognized by antibody TA-4. For hCG the amount released increased to a maximum of 754 ng/10^6 cells after 7 days. CEA and the SCC-antigen were only detected after 9 days in very low concentrations, 1.4 µg/l and 0.22 µg/l, respectively. The control values of fresh RPMI with 10% FBS were 1.0 and 0.20 µg/l, respectively. Cell line T87/rc shows levels of hCG, CEA and SCC-antigen below the sensitivity limits.

**DISCUSSION**

We have described establishment and characteristics of the 2 human squamous-cell carcinoma (SCC) cell lines R105 and T87/rc after xenografting in nude mice. No changes in histologic features were found during passages in nude mice of these xenografts, although selection of aneuploid cells occurred (Elprana et al., 1990). The DNA indices of the xenografts were 1.65 for R105 and 1.8 for T87/rc (Elprana et al., 1990). After remaining in culture for more than 40 passages, the DNA indices decreased to 1.54 and 1.31 for cell line R105 and for T87/rc respectively, as measured after different intervals during culturing. This indicated that cell selection has occurred after disaggregation and culturing of the xenografts.

The epithelial nature of the tumour cells was stressed by the presence of junctional complexes between cells and by the presence of cytokeratins. Their squamous type of differentiation was identified by the presence of cytokeratins 5 and 14 as well as of cytokeratins 4 and 13 in the case of R105. The last two proteins suggest a derivation from non-keratinizing squamous epithelium. The extent of keratinization that occurs in the tumours is recognized by the antibody to cytokeratin 10.

The presence of cytokeratins in these xenografts, which are normally not found in squamous-cell carcinomas, or found...
only in low concentrations, i.e., cytokeratins 7, 8 and 18, is not so striking, since earlier reports have shown that SCC cell lines may obtain these types of intermediate filament proteins (Rupniak et al., 1985).

The positive reaction with MAb BW495/36 also confirmed the epithelial character of the 2 cell lines. The absence of desmin eliminated the possibility of muscular-type differentiation. The presence of vimentin, as in T87/rc cells but not in R105 cells, can be considered as an adaptation to cell culture conditions (Franke et al., 1979). Xenografts obtained from cultured cells showed almost the same reaction pattern as cells in culture. For some MAb's the reactivity of cultured cells was weaker than that of the xenografts.

For normal tissue of the floor of the mouth and normal commissure, 4 and 7 cytokeratin proteins were found after electrophoresis and immunoblotting (Clausen et al., 1986). In R105 (derived from a carcinoma of the floor of the mouth) 9 different cytokeratins (CK 4, 5, 10, 13, 14, 18 and 19) were found. In the normal epiglottic epithelium and its tumours 9 (CK 4, 5, 6, 13, 14, 15, 16, 17 and 19) and 10 (CK 4, 5, 6, 8, 14, 15, 16, 17, 18 and 19) different cytokeratins were found by Moll et al. (1982). For T87/rc (derived from a epiglottic carcinoma) 7 different cytokeratins (CK 5, 7, 8, 10, 14, 18 and 19) were identified. Cell-type heterogeneity in epithelial tissue and selection might be an explanation for the difference between cytokeratins from normal tissue, tumours and tumour cell lines of the same origin (Moll et al., 1982).

The plating efficiency of colonies grown on a plastic substratum gave results comparable to those of Rupniak et al. (1985). Both cell lines formed colonies in soft agar with a plating efficiency of 0.06% for R105 and 0.64% for T87/rc which is high when compared to the results of Boukamp et al. (1982), Easty et al. (1981) and of Rheinwald and Beckett (1981). The last-named group found that only 1 out of 6 SCC lines tested had a colony-forming capacity. The plating efficiency of SCC cells in the soft agar assay was very low because of the high anchorage-dependence of proliferation (Rupniak et al., 1985).

As for the chromosomal rearrangements, there appeared to be no similarities between our 2 cell lines. Apart from chromosomal count, the karyotype in both cell lines appeared to be extremely aberrant after GTG-banding and only a few “normal” chromosomes per cell could be analysed. However, several marker chromosomes were detected in all analyzed cells. Strikingly, no Y chromosomes were found although both cell lines were derived from male patients. Even in primary cell cultures of squamous-cell carcinomas of head and neck multiple unrelated chromosome abnormalities exist (Jin et al., 1988). However, chromosome 1 seems to be more involved in chromosomal rearrangements than other chromosomes (Hauser-Urfer and Stauffer, 1985; Jin et al., 1988). In our cell lines, almost every chromosome appeared to be involved in chromosomal rearrangements, including chromosome 1. None of the chromosome markers was recognized as a HeLa marker, excluding the possibility of contamination with HeLa cells (Nelson-Rees et al., 1981).

R105 cells were found to release CEA, hCG and the SCC antigen. The production of CEA as detected by a radioimmunoassay was confirmed with MAb BW431/31 (directed against a CEA-epitope) using immunofluorescence. SCC antigen TA-4 is normally found in concentrations up to 4 ng/ml for SCC of the floor of the mouth and 4.5 ng/ml for tumours of the epiglottis (Eibling et al., 1989). The concentration of hCG shed into the medium increased to a maximum of 754 ng/10^6 cells, which is higher than levels reported by Easty et al. (1981) for hCG production by SCC cell lines.

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SQUAMOUS-CELL CARCINOMA CULTURES


