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Establishment and characterization of four new squamous cell carcinoma cell lines derived from oral tumors

Revati J. Tatake [1], Nirmala Rajaram [1], R. N. Damle [1], Binaifer Balsara [2], A. N. Bhisey [2], and Sudha G. Gangal [1]

[1] Immunology Division and [2] Cell Biology Division, Cancer Research Institute, Tata Memorial Centre, Bombay-400 012, India

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Summary. Four cell lines were established from squamous cell carcinomas (SCC) of the oral cavity. Cell lines AW 13516 and AW 8507 were derived from poorly differentiated SCC and epidermoid carcinoma of the tongue respectively. Cell line AW 10498 was derived from moderately differentiated SCC of the lower alveolus, and AW 9803 grew from a well-differentiated SCC of a retromolar trigone. The cultures showed typical epithelial cell morphology, numerous mitotic figures, occasional multinucleated giant cells, individual cell diskeratosis and nuclear and nucleolar abnormalities. The cell lines AW 13516 and AW 8507 were fast growers with a doubling time of 35.5 h and 31.9 h, respectively, which was independent of the initial seeding density. Cell lines AW 10498 (doubling time 52.2 h) and AW 9803 (doubling time 66 h) showed slower growth and had shorter doubling times at higher seeding densities. The presence of cytokeratins was detected in all the four cell lines by using polyclonal antikeratin antisera in indirect immunofluorescence and in Western blotting. None of the cell lines expressed major histocompatibility complex (MHC) class II antigens. MHC class I antigens were expressed by three cell lines but not by AW 9803. Flow cytometric analysis of DNA content and chromosomal studies suggested the presence of polyploidy and aneuploidy in all the four cell lines. Ultrastructural studies revealed typical epithelial cell features, such as the presence of desmosomes, tonofilaments and keratin bundles.

Key words: Squamous cell carcinoma – Epithelial cell lines – Keratin expression

Introduction

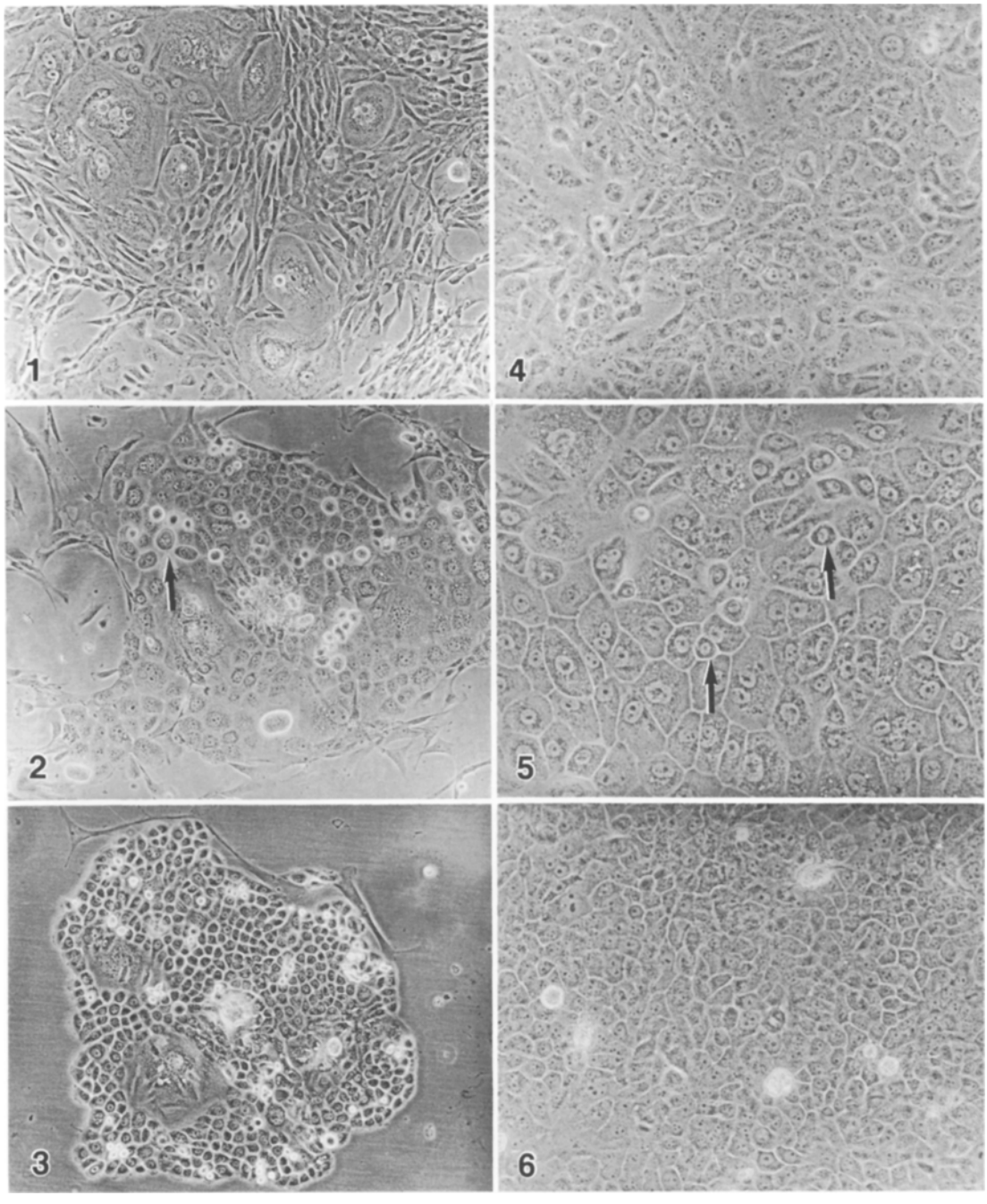
The establishment of continuously growing cell lines from human solid tumors provides a useful tool for research on various facets of tumor cell biology. Human tumor cell lines have been used to characterize the tumor cells (Carey 1985; Easty et al. 1986; Gioanni et al. 1988) and to study integration and expression of the viral genome and oncogenes (Sacks et al. 1988), and they are also used as targets for different autologous and allogeneic cytotoxic effectors (Mukherji and MacAlister 1984; Tatake et al. 1989). Squamous cell carcinoma (SCC) is the most common type of cancer occurring at many sites, perhaps because these cells are more accessible to carcinogenic insults (Carey 1985). Despite the prevalence of this cancer type, relatively little is known about the cell biology and features associated with the progression of SCC, especially the cell variants involved in metastasis. This is because of the low success rate in growing SCC as continuous cultures in vitro compared to malignant melanomas and adenocarcinomas of colon and kidney (Krause et al. 1981). Recently, with improvements in methodology, SCC cell lines, mainly arising from head and neck cancers have been established (Easty et al. 1981; Carey 1985; Gioanni et al. 1988; Sacks et al. 1988). In this communication we report the establishment and characterization of four new SCC lines derived from tumors of the oral cavity.

Abbreviations: SCC, squamous cell carcinoma; PBS, phosphate-buffered saline; MHC, major histocompatibility complex

Offprint requests to: S. G. Gangal

Materials and methods

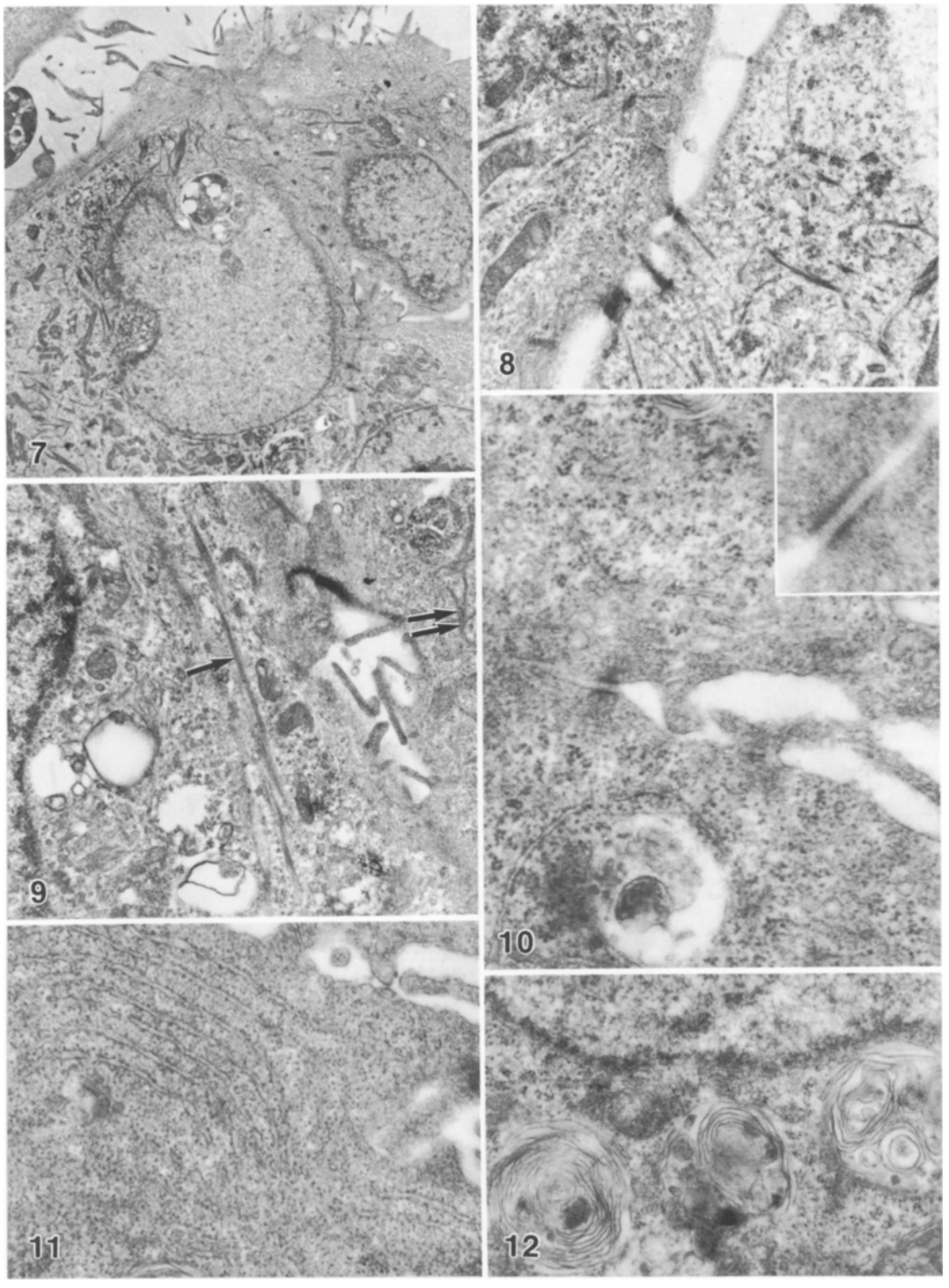
In vitro culture. Surgically ressected tumors were obtained from the operating theatre in sterile Iscove's modified Dulbecco's medium (Gibco, USA) supplemented with 10% human blood group AB serum (culture medium) and antibiotics (penicillin 200 IU/ml, streptomycin 100 μg/ml, gentamycin 80 μg/ml and nystatin 10 U/ml). Tumors were cleaned of the necrotic tissue and blood clots, rinsed two or three times in the medium containing antibiotics and cut finely. Small pieces of the tumor tissue were treated with 0.25% trypsin in phosphate-buffered saline (PBS) for 30 min at 37° C in two cycles. The single-cell suspension obtained was washed with medium and cultured in 25-cm^2 tissue-culture flasks (Nunc, Denmark). Next day, cells that were not attached to the plastic surfaces, were removed. These were mainly tumor-infiltrating lymphocytes and dead tumor cells. Adherent cells were fed with culture medium and within a week the primary cultures showed giant tumor cells sur-



Figs. 1–6. 1 Primary culture of AW 10498 showing giant multinucleated cells surrounded by fibroblasts (75×; live phase contrast). **2** Primary culture of AW 10498 showing a patch of epithelial cells surrounded by fibroblasts (75×; live phase contrast). Cells showing diskeratosis are indicated by the *arrow*. **3** AW 10498 in passage 4 showing growing epithelial cell colony cleaned of fibroblasts by differential trypsinization (75×; live phase contrast). **4** AW 8507 in passage 45 (150×; live phase contrast). **5** AW 9803 in passage 27 showing a typical cobblestone appearance (150×). Cells showing keratinization are indicated by the *arrow*. Many multinucleated cells are also seen (live phase contrast). **6** AW 13516 in passage 45 (150×). Numerous mitotic figures are seen (live phase contrast)

rounded by fibroblasts. Some areas showed patches of epithelial cells (Figs. 1 and 2). In order to obtain pure epithelial cell growth, fibroblasts, which tend to overgrow the epithelial cells, were removed by selective trypsinization.

Removal of fibroblasts from cultures and subculturing. Fibroblasts were removed by selective trypsinization as described by Carey (1985). For this purpose, about 2 ml 0.25% warm trypsin in PBS was added per flask. The flasks were incubated for 2–5 min at 37° C, with intermittent monitoring under the microscope to see the detachment of fibroblasts. The treatment was stopped when fibroblasts, but not the epithelial cells, were detached. This treatment was repeated biweekly until only epithelial cell patches filled the flasks (Fig. 3). The period required for this procedure varied from sample to sample and was about 2–4 weeks. Sufficient epithelial cells grew in another 2–3 weeks and the subculturing was performed. Initially the cultures were split at a 1:2 ratio, and during earlier passages confluent cultures were obtained within 2–4 weeks. After 4 or 5 passages, the cultures were stabilized and could be split into 4 or 5 flasks once a week with one feeding in between. Cultures were initiated



Figs. 7–12. 7 An overview of AW 8507 cells with nuclei showing very little condensed chromatin and deeply indented nuclear membrane. Cytoplasm shows intermediate filament bundles, myelinoid bodies (4300 ×; electron micrograph). **8** Junction between two AW 8507 cells showing desmosomes. The cytoplasm shows intermediate filament bundles and long mitochondria (16 200 ×; electron micrograph). **9** Peripheral cytoplasm of AW 8507 cells. ↓, Bundles of microfilaments; ↓↓, intermediate filaments (15 500 ×; electron micrograph). **10** Cytoplasm of AW 10498 cells showing clusters of ribosomes. Desmosomes are seen between the cells (40 200 ×). *Insert*, high-magnification view of desmosome between two AW 10498 cells (100 500 ×; electron micrograph). **11** Stacks of rough endoplasmic reticulum in the cytoplasm of AW 10498 cells (25 800 ×; electron micrograph). **12** Myelinoid bodies in cytoplasm of AW 10498 cells (25 800 ×; electron micrograph)

Table 1. Clinical features of patients[a]

Patient's no./ cell line	Sex/age (years)	Tumor site and differentiation	TNM classification
AW 8507	M/50	Tongue; poorly differentiated SCC[b]	$T_3N_0M_0$
AW 13516	M/35	Tongue; poorly to moderately differentiated SCC	$T_4N_1M_0$
AW 10498	F/45	Alveolus; moderately differentiated SCC	$T_4N_1M_0$
AW 9803	M/53	Retromolar trigone; well differentiated SCC	$T_4N_1M_0$

[a] Patients from whose tumor tissues cell lines could be developed. Total number of specimens processed for culturing: 10
[b] SCC, squamous cell carcinoma

from ten tumors, of which epithelial tumor cell lines could be established from four tissues (Table 1).

Measurement of doubling time. Passages 23–45 were used to assess the doubling time. Cells were seeded in 96-well flat-bottom microtitre plates at the concentration of 5×10^3 and 1×10^4 cells/well in six replicates in culture medium. Cultures were harvested at 24, 48, 72 and 96 h to determine the cell growth (Tagliaferri et al. 1987). After removing the culture medium the cells were washed once with PBS and stained with 0.05% crystal violet in 20% methanol for 10 min at room temperature. After thorough washing of stained cells with PBS, the dye was eluted with buffer (6.1 ml 0.1 *M* disodium citrate, 3.9 ml 0.1 *M* HCl and 10 ml 95% ethanol) and the absorbance was read at 570 nm.

Flow cytometric analysis of DNA content. For flow cytometric analysis cells were trypsinized, washed and fixed in 70% alcohol. Fixed cells were treated with 0.5% pepsin for 5 min at room temperature. The cells were then stained with 4,6-diphenylindole, 5 μg/ml in PBS for 30 min at 4° C. DNA measurements were carried out on a Partec PAS-II flow cytometer (Partec AG, Switzerland) illuminated with a HBO 100 mercury lamp. The filters used were KG1, UG1, BG38 followed by dichroic mirrors TK 420 and TK 560 and barrier filter GG 435. A total of 2×10^4 cells were analysed with a cell-cycle analysis programme supplied by the manufacturers. Ficoll/Hypaque-separated human peripheral blood lymphocytes were used as the standard for calibration of the instrument adjusted to coefficient of variance of 1.1 for the normal lymphocytes.

Electron microscopy. Cells were grown in 35-mm plastic petri dishes (Nunc, Denmark) for 2 days. Semiconfluent cultures were washed with PBS, fixed in 2.5% glutaraldehyde in phosphate buffer at room temperature and post-fixed in 2% osmium tetroxide. The cultures were embedded in situ in Epon (Brinkley et al. 1968). Ultrathin sections were cut on a Sorvall-MT 5000 ultra-microtome, stained with uranyl acetate and lead citrate and observed under a Zeiss electron microscope 109 E at an accelerating voltage of 80 kV.

Indirect immunofluorescence. Indirect immunofluorescence was used to determine the presence of major histocompatibility complex (MHC) antigens and intracytoplasmic keratins in the cell lines. Expression of MHC class I and class II antigens was determined using monoclonal antibodies W6/32 and L227, respectively, on trypsinized viable cells. For localization of keratins, cells were grown on coverslips, washed with PBS, air dried and fixed in acetone for 10 min at room temperature. Coverslips were incubated with rabbit anti-(human keratin) antibody and then with fluorescein-isothiocyanate-labelled goat anti-(rabbit Ig) antibody. Coverslips were washed and mounted in PBS/glycerol 1 : 9 (v/v) and observed under a Zeiss fluorescence microscope.

Extraction and identification of cytoskeletal proteins (keratins) by Western blotting. Keratins were extracted by the method described by Fisher et al. (1987). Cells were homogenized in 10 m*M* TRIS/HCl buffer, pH 8.0 containing 1.5 *M* KCl, 10 m*M* NaCl, 2 m*M* dithiothreitol, 0.5% Triton X-100 and 0.5 m*M* phenylmethylsulphonyl fluoride. Homogenates were spun in a microfuge at 12000 rpm for 15 min. The insoluble pellet was similarly extracted twice and then dissolved in PBS containing 5% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol. SDS/polyacrylamide gel electrophoresis was performed with a 3% stacking and 10% resolving gel. Gels were electroblotted onto the nitrocellulose paper. Keratins were identified using rabbit anti-(human keratin) antibodies and goat anti-(rabbit Ig) antibody conjugated with horseradish peroxidase.

Results

Morphology of the cell lines

Cells from all the cell lines showed typical epithelial cell morphology with numerous mitotic figures, a large nuclear to cytoplasmic ratio and prominent multiple nucleoli (Figs. 4–6). Cells of AW 8507 and AW 9803 were larger in size, the former being loosely attached to the plastic surface. Cells of AW 9803 showed a typical cobblestone appearance with a number of binucleated cells. The cells of AW 13516 and AW 10498 were smaller and uniform in size. Individual cell diskeratosis was seen in earlier as well as late passages (Figs. 2 and 5). All the four cell lines, however, appeared morphologically distinct from each other.

Ultrastructural features

At the electron microscope level, the cells of all the four cell lines displayed typical epithelial cell characteristics. The distinct features of all the cell lines are given below.

AW 8507. Most of these cells had large nuclei with the nuclear membrane showing deep indentation (Fig. 7). There was very little condensed chromatin. The cytoplasm was extensive and contained a large number of long mitochondria, lysosomes and cytoplasmic organelles (Figs. 8 and 9). While clusters of polyribosomes were plentiful in the peripheral cytoplasm, endoplasmic reticulum was scanty. Some cells showed many residual bodies in the cytoplasm. Bundles of intermediate filaments were frequently seen in the cytoplasm. Microfilament bundles were seen mainly in the submembrane regions (Fig. 9). Long microtubules oriented towards the cell membrane were seen in most large cells. Adjoining cells formed desmosomal junctions with tonofilaments (Fig. 8).

AW 10498. This cell line showed epithelial cells with one or two nucleoli and very little condensed chromatin. The cytoplasm was dense and full of polyribosomes (Fig. 10). Rough endoplasmic reticulum was seen as single cister-

nae or well-organised parallel stacks (Fig. 11). Some cells showed bundles of intermediate filaments typical of epithelial cells. Mitochondria appeared swollen in some cells. All the cells had a large number of mylinoid bodies (Fig. 12). The Golgi vesicles were flat and disc-like. Cytoplasmic microtubules appeared to be few. Bundles of microfilaments were seen in some cortical areas. Under confluent and near-confluent conditions the cells formed a typical epithelium-like growth pattern and desmosomes were frequently seen between adjoining cells (Fig. 10).

AW 13516. These cells had dense cytoplasm and irregularly shaped nuclei showing indentations with very little condensed chromatin. The cytoplasm had large clusters of polyribosomes and small cisternae of endoplasmic reticulum. Golgi vescicles were dilated. Many cells showed active micropinocytosis at the cell periphery. The cell had a large number of cytoplasmic microtubules and scattered bundles of intermediate filaments. Though the membranes of neighbouring cells ran a parallel course, desmosomes were rather infrequent.

AW 9803. These cells had dense cytoplasm packed with clusters of ribosomes. Occasional cisternae of rough endoplasmic reticulum were seen. Some oval mitochondria were seen in the juxtanuclear zone along with residual bodies. The cells had a well-developed cytoplasmic microtubule complex and occasional bundles of intermediate filaments. Desmosomal junctions were not seen between the cells.

Cell growth

The results of the cell growth studies have been expressed as absorbance versus the time in culture (Fig. 13). The doubling time for AW 8507 and AW 13516, which were derived from poorly differentiated tumors, was independent of initial seeding density. On the other hand, for cell lines AW 9803 and AW 10498, which were derived from moderately and well differentiated tumors, the doubling time was shorter with a higher seeding density. In general, cell lines derived from histologically high-grade tumors grew faster than those derived from low-grade tumors.

Flow cytometry

Table 2 and Fig. 14 show the DNA distribution of the cell lines, obtained by flow cytometry. The DNA index was calculated as the ratio of the modal channel of the tumor population to that of normal human peripheral blood lymphocytes (channel 77). For each population the coefficient of variance of the G1 peak was calculated according to Roa et al. (1985). AW 8507 showed a bimodal distribution (Fig. 14). The first peak was made up of hyperdiploid cells at channel 90, while the second peak was at channel 160 and consisted of cycling cells from the first peak and the population from the second peak. It was, therefore, not possible to estimate the percentage of G1,

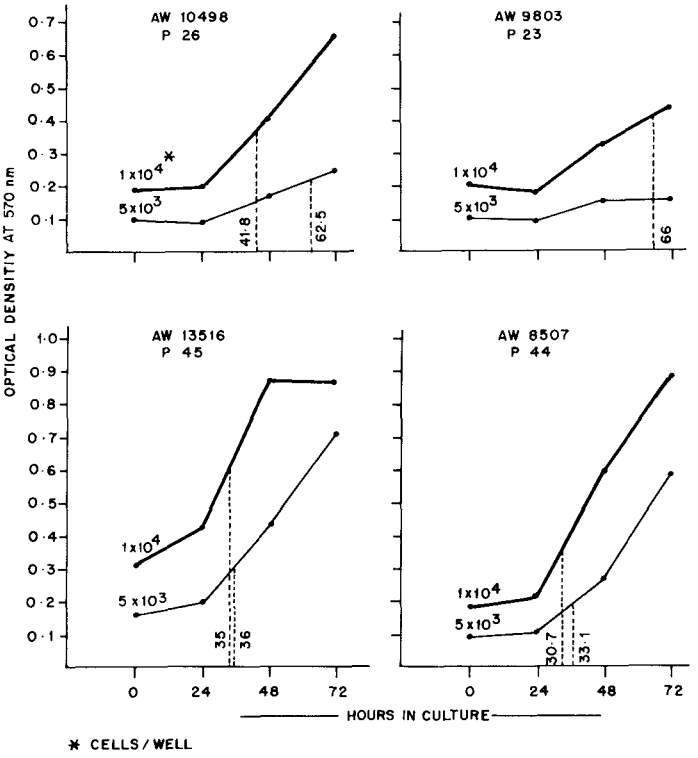


Fig. 13. Growth curves and doubling time for oral cancer cell lines

Table 2. Flow cytometric analysis of DNA index, coefficient of variance (CV) and cell cycle of oral cancer cell lines

Cells/cell lines	DNA index	CV	Cells (%) in		
			G_1	S	G_2
Human PBL[a]	1.0	1.1	100	–	–
AW 8507	2.078	4.7	–	–	–
AW 13516	1.94	5.6	48	49.4	2.8
AW 10498	1.73	6.6	68	28.1	3.1
AW 9803	1.66	4.4	77	18.2	3.0

[a] PBL, peripheral blood lymphocytes

and G2 cells in this cell line. The other three cell lines had their main G1 peak in the triploid/tetraploid region and also showed a defined G2/M population at twice the mean G1 channel number. The broader distribution in the population is shown by the higher coefficient of variance of the G1 peak (4.4%–6.6%, Table 2), while the higher DNA index indicates the occurrence of triploidy. The proliferating fraction (S+G2+M, %) was higher in the fast-growing cultures as compared to the two slow-growing cell lines (Fig. 13).

Expression of MHC antigens and keratins

All the cell lines lacked expression of MHC class II antigens. MHC class I antigens were expressed by all the cell lines except AW 9803.

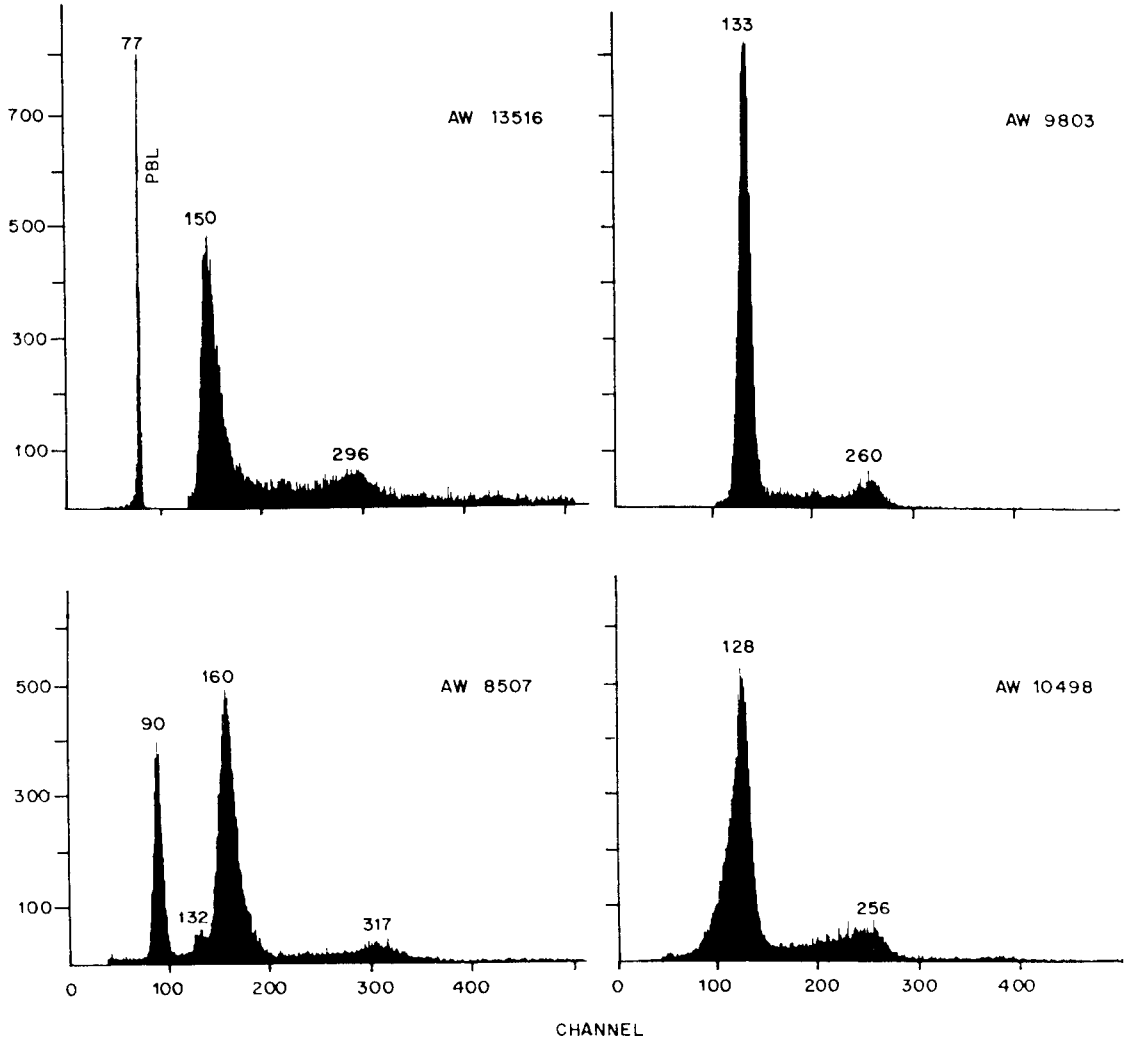


Fig. 14. DNA flow cytometry plots of cell lines. *Abscissa*, Increasing fluorescence intensity in proportion to DNA content (channel no.), *Ordinate*, no. of cells. The mean peak channel no. of each population is shown. In AW 13516, channel 77 shows the mean peak of normal human lymphocytes used as a diploid standard. More cells are seen in the S phase fraction. AW 8507 shows a bimodal profile

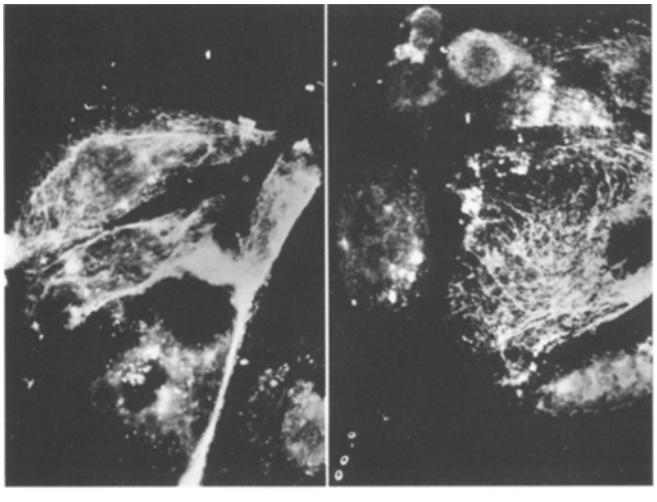


Fig. 15. Indirect immunofluorescence staining of cytokeratins of AW 13516 cells using rabbit anti-(human keratin) polyclonal antibody (1500×)

Table 3. Expression of keratins by oral cancer cell lines

Molecular mass (kDa)			
AW 13516	AW 8507	AW 10498	AW 9803
65	59	56	65
58.7	58	54	59
54	54	52	56
50	50	50	52
48	48	48	48
45	45	45	45

All the four cell lines showed the presence of cytokeratins when indirect immunofluorescence techniques were used (Fig. 15) using rabbit anti-(human keratin) antibodies, confirming the squamous epithelial lineage of the cell lines. The differences in keratins extracted from the cell lines were further confirmed by Western blotting, and the pattern of keratin expression is given in Table 3. Ker-

atins of 45 kDa and 48 kDa were expressed by all the cell lines, whereas, AW 9803, a line derived from well-differentiated tumor, did not express the 50-kDa and 54-kDa keratins that were expressed by the other three cell lines. However, the 65-kDa keratin, characteristic of well-differentiated squamous epithelium, was expressed by AW 9803 (Vaidya et al. 1989).

Discussion

Squamous cell carcinoma (SCC) is the most common type of cancer in human malignancies. In spite of this, relatively few SCC cell lines were available until recently (Easty et al. 1981; Carey 1985; Gioanni et al. 1988; Sacks et al. 1988). We have reported here the establishment of four new SCC cell lines from human oral tumors. Most of the SCC cell lines available so far are derived from head and neck cancers, the common sites being the tongue and larynx (Carey 1985). Two of the cell lines developed by us are also derived from the tongue. In most of the reports the cell lines were initiated with tumor explants (Easty et al. 1981; Okabe et al. 1978), some showing anchorage dependence of a requirement for a feeder (Rheinwald and Beckett 1981; Cobleigh et al. 1984). We have established the cell lines by direct trypsinization of the tissues, and for eliminating the fibroblasts we have used a selective trypsinization procedure as described by others (Carey 1985; Sacks et al. 1988).

All the cell lines showed typical epithelial cell morphology and the presence of keratines, and three cell lines showed desmosomal junctions between cells. Several attempts to karyotype the chromosomes were unsuccessful because of crowding of chromosomes, since the chromosome number was high. The few metaphases analysed showed an almost triploid–tetraploid chromosome number. This observation was further substantiated by flow cytometric analysis of the DNA content, where the DNA index was closer to 2 in all the four cell lines.

The growth rate of the cell lines correlated with the stage of differentiation. Cell lines derived from well differentiated tumors showed a slower growth rate as compared to those derived from poorly differentiated tumors and they also had a lower proliferating fraction, as shown by flow cytometry.

None of our lines developed tumors in nude mice in 6 weeks. This could be because a suboptimal initial cell number was used for transplantation (3×10^6 cells/animal). On the other hand, there are reports suggesting that not all the SCC cell lines develop tumors in nude mice (Baker 1985; Boukamp et al. 1982).

We did not make any attempt to grow cells in semisolid medium since earlier reports (Rheinwald and Beckett 1981; Boukamp et al. 1982; Hu et al. 1984) have indicated poor or no growth of SCC cells in semisolid medium. Nevertheless, many other important characteristics of SCC, such as the presence of keratins, abnormal DNA content, very high ribosomal content, and the presence of microvilli, tonofilaments and desmosomes, confirmed that our cell lines have maintained the characteristics of SCC cells.

There is sufficient evidence to show that our cell lines are not cross-contaminated with each other or with other epithelial cell lines. They differ in their morphology, ultrastructural features, growth pattern and growth rate, expression of MHC antigens, DNA content and pattern of keratins on Western blots. These cell lines have been used as targets for cytotoxic effectors (Tatake et al. 1989; Gangal et al. 1989) and have been shown to express tumor-associated antigens as identified by monoclonal antibodies (Tatake et al. 1990). These cell lines can serve as important biological material for studies on oral cancer.

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