Pergamon

Oral Oncol, Eur J Cancer, Vol. 30B, No. 5, pp. 296–301, 1994

0964–1955/94 $7.00 + 0.00

Tumorigenicity of Cell Lines Established from Oral Squamous Cell Carcinoma and its Metastatic Lymph Nodes

Tokio Osaki, Yukihiro Tatemoto, Kazunori Yoneda and Tetsuya Yamamoto

We investigated the biological and histopathological characteristics of seven human tumour cell lines established from primary tongue squamous cell carcinoma (OSC-1), from metastasised lymph nodes of the gingival carcinoma (OSC-2 and OSC-3) and from tongue carcinoma (remaining four lines). The doubling time ranged from 22 h (OSC-2 and OSC-4) to 55 h (OSC-7), and did not correlate with tumour cell stratification in a collagen gel matrix. An invasive tendency was most prominent in OSC-2 and OSC-4; with the other cell lines, except OSC-6 and OSC-7, only a few sporadic invading cells were found in the tissue culture. In the cell lines established from the metastatised tumours, originally exhibiting grade 3 invasion, the invasion became more sporadic when the tumour cells were xenografted into the tongues of nude mice, while an invasion similar to the original was observed in the cell lines obtained from the original site (OSC-1) and from tumours of Grade 4C invasion. These findings suggest that the biological behaviour of the established tumour cells is markedly different even in tumours of the same tissue origin, and strongly invasive cells may selectively invade, and metastatise to the lymph nodes.

Keywords: oral squamous cell line, tissue culture, xenograft, invasion, metastasis
Oral Oncol, Eur J Cancer, Vol. 30B, No. 5, pp. 296–301, 1994.

INTRODUCTION

RECENTLY, TISSUE culture in a collagen gel matrix containing fibroblasts has been tried in several laboratories [1–5]. Using this technique, for example, development of a substitute for skin has been tried [1, 2], and the characteristics of established human tumour cell lines have been analysed [3–5]. In a collagen gel matrix, tumour cells are expected to multiply and invade in a manner similar to that observed in the original human tissue. It is thought that the original mode of invasion, which is an important marker of the lymph node metastasis in oral squamous cell carcinomas [6, 7], can be reestablished in the gel matrix. If so, tissue culture would be a very useful *in vitro* test for the invasion mode of each tumour.

The transplantability and metastatising activity of established tumour cells are usually tested in nude mice. High transplantability is associated with strong tumorgenicity. Tumour formation rate and tumour growth speed largely depend on the mouse tissue used for tumour cell inoculation [8, 9], and the tongue seems to be suitable for the multiplication of tumour cells.

In the present study, the biological characteristics of seven cell lines, established from oral squamous cell carcinomas and their metastatic lymph nodes, were investigated. In addition, tumour cells cultured on collagen gel matrices and xenografted to nude mice were histopathologically examined. Based on the results, the relationship between the biological and histological characteristics, and also between *in vitro* and *in vivo* findings, is discussed.

Correspondence to T. Osaki.
The authors are at the Department of Oral Surgery, Kochi Medical School, Kohasu, Oko-cho, Nankoku-city, Kochi 783, Japan.
Received 27 July 1993; provisionally accepted 4 Sep. 1993; revised manuscript received 17 Sep. 1993.

MATERIALS AND METHODS

Cell source

Tumour cells were obtained from the untreated metastatic lymph nodes and the primary oral lesion in 6 cases and 1 case, respectively. The TN stage, shown in Table 1, was based on the UICC definition [10]. Histopathological characteristics of the tumours were determined on specimens stained with haematoxylin and eosin which were prepared from biopsied materials before treatment, and the mode of tumour cell invasion was classified as described by Willen *et al.* [11] (Fig. 1a, c).

Cell culture

The metastatic lymph nodes and oral tumour tissues were minced to about 1–2 mm in diameter, and placed on the surface of 60-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 200 U/ml penicillin G,

Table 1. Clinical and histopathological summaries of tumour cell lines established

Cell line	Age, sex of the donor	Primary site and T-stage	Differentiation and mode of invasion	Tissue of cell isolation
OSC-1	54, M	Tongue T3	Moderate Grade 3	Tongue (primary)
OSC-2	68, F	Gingiva T2	Well Grade 3	Submandibular LN (metastatic)
OSC-3	56, M	Gingiva T4	Moderate Grade 4C	Cervical LN (metastatic)
OSC-4	76, M	Tongue T2	Well Grade 3	Submandibular LN (metastatic)
OSC-5	48, F	Tongue T2	Well Grade 3	Submandibular LN (metastatic)
OSC-6	57, M	Tongue T2	Well Grade 4C	Cervical LN (metastatic)
OSC-7	57, M	Branchiogenic T4	Poor Grade 3	Cervical LN (metastatic)

LN = lymph node.

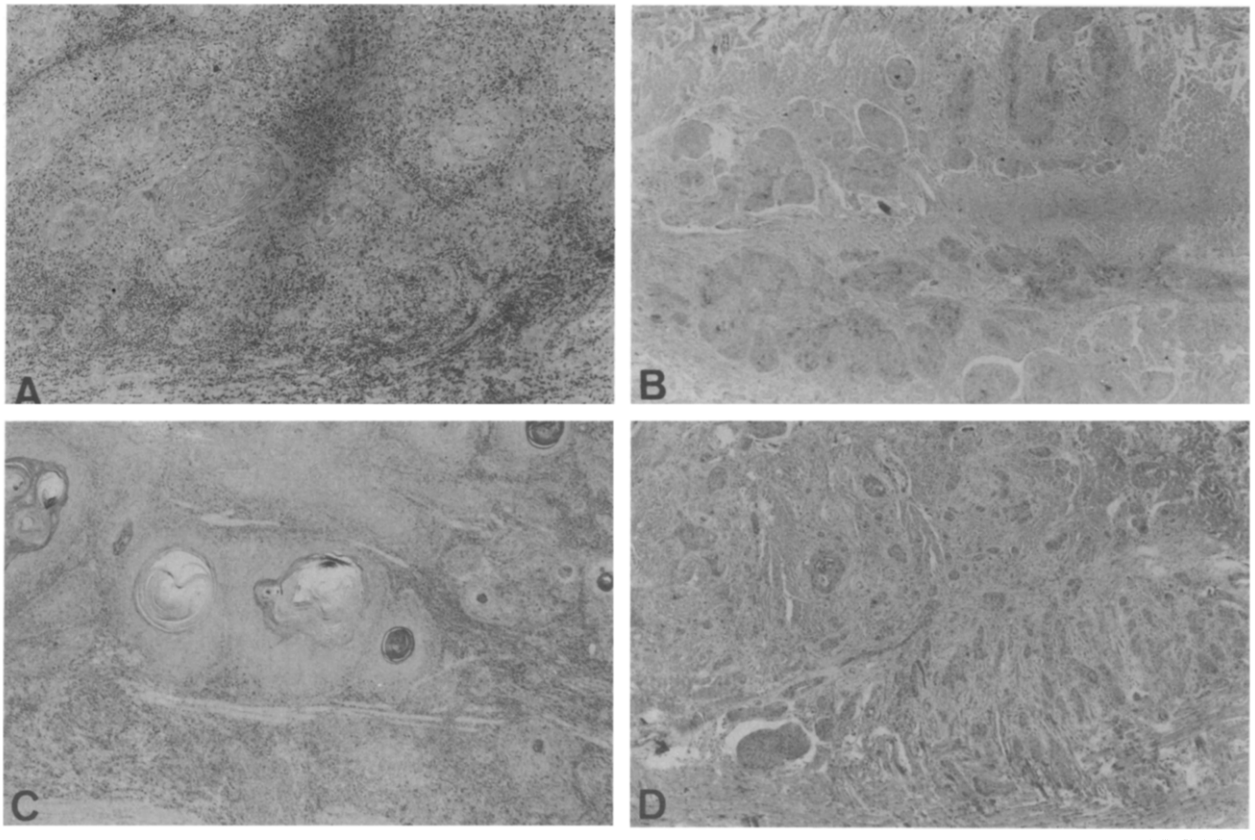

Fig. 1. Microphotographs of cancer cells in the biopsied materials (A: OSC-2, C: OSC-5), and tumour cells after multiplication in the tongue of nude mice (B:OSC-2, 105th passage. D:OSC-5, 48th passage. Four weeks after inoculation in both cell lines. ×60 in all). Well-differentiated tumour cells invaded into the stromal tissue without any distinct borderline exhibiting an invasion mode of grade 3 in both A and C. In the nude mice, OSC-2 formed large islands of tumour nests among the muscular bundles (B). The histological features and invasive mode at the periphery of the tumour are similar to those in (A). In contrast, cord-like or diffusely invasive tumour cells are visible in the xenograted OSC-5 (D). Compared with the biopsied material, the tumour cell invasion is more sporadic.

200 μg/ml streptomycin, 0.075% sodium bicarbonate, 10% fetal calf serum (FCS), and 0.6mg/ml L-glutamine (culture medium). The chopped tumour tissues were cultured in the culture medium at 37°C in a humidified incubator at 95% atmosphere and 5% CO_2, changing the medium twice a week. Initial cell passage was performed when a rapid tumour cell

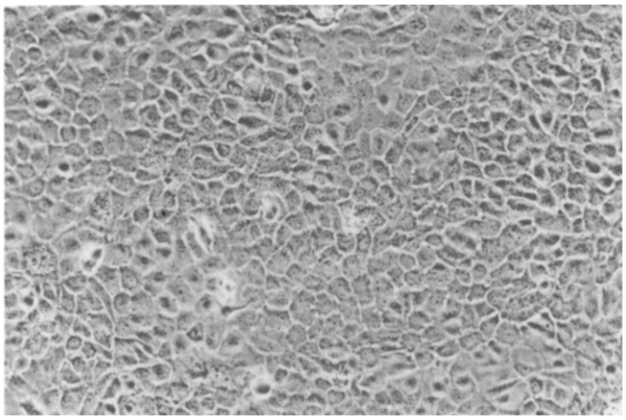

Fig. 2. A phase contrast micrograph of OSC-1 at the late log-phase of the growth (50th passage, × 300). The cells are uniformly squamous.

growth was observed (usually at 3–8 weeks from the start of culture) (Fig. 2). The seven cell lines examined showed constant growth after five passages, and all subsequent experiments were performed using cell lines passaged more than 10 times.

Cell growth

Cells (10^5) of each cell line were seeded in 60-mm dishes filled with 10 ml of culture medium in the presence or absence of the indicated reagents. The cells were trypsinised every 48 h, and viable cells were counted after staining with 0.5% trypan blue. Cell counts were plotted, and the growth curve of each cell line was obtained. The doubling time was determined by the multiplication in the logarithmic phase.

Chromosomal analysis

Chromosome and karyotype were analysed by the modified G-banding technique [12]. Briefly, the tumour cells in the exponential growth phase were treated with colcemid at a final concentration of 0.1μg/ml for 4 h at 37°C, then dispersed using 0.25% trypsin and washed with 0.05 mol/l phosphate-buffered saline (PBS, pH 7.4). The cells were then suspended in a hypotonic solution containing 0.075 mol/l KCl and incubated for 10 min. After centrifugation at 1200 rpm for 5 min, the pellets were fixed twice with a methanol–acetic acid (3:1) mixture. The fixed cells were placed on clean slides and air-dried, then stained with Giemsa solution for 10 min. Modal chromosome distribution and karyotype features were examined by analysing metaphases of each cell line using photomicrographs (× 1000).

Colony-forming efficiency

Five hundred cells of each cell line were poured into 60-mm plastic dishes filled with 6 ml of the culture medium. After incubation for 7 days, colonies consisting of 10 or more cells were microscopically counted after Giemsa staining. Colony formation in soft agar was also examined using the following procedure; 5×10^4 cells were plated on the bottom surface of 35-mm plastic dishes coated with 0.5% agar, and the cells were overlayed with 0.3% agar. After 14 days, clusters larger than 60 μm in diameter were counted in 10 random fields of an inverted microscope (× 50). Four plastic dishes were used for each cell line, and the examination of each cell line was repeated twice. The mean number of colonies or clusters was expressed as the colony forming efficiency (%).

Xenograft of tumour cells

Cells (5×10^5) in 0.05 ml of PBS were subcutaneously inoculated into the tongue of 4-week-old Balb/C-nu/nu mice using 10 animals for each cell line. Four weeks after transplantation, the tumours formed were removed, fixed in Carnoy's solution and histopathologically examined after haematoxylin and eosin staining. Tumour cells (1×10^6 suspended in 0.1 ml of PBS) were also injected into the tail vein of 6-week-old BALB/c-nu/nu mice anaesthesised with subcutaneous pentobarbital using 10 mice for each cell line. Six weeks later, the mice were killed and the lungs were resected. The extirpated lungs were fixed with Carnoy's solution. The pulmonary colonies were microscopically examined in haematoxylin and eosin stained sections.

Tissue culture in collagen gel matrix

Collagen gel matrix was prepared by mixing 0.3% type I-P collagen (pH 3.0, Koken Co. Ltd, Tokyo, Japan) with 5-fold DMEM and a reconstituted buffer solution (0.05 N sodium hydroxide with 2.2% sodium bicarbonate and 200 mol/l Hepes) at a ratio of 7:2:1, and layered in 35-mm dishes. Normal human fibroblasts (6×10^5), isolated from explants of the oral mucosa of a female infant, were poured onto the collagen matrix at 4°C. After 30 min incubation at 37°C and solation of the gel matrix, 1 ml of the culture medium was added on the gel, and the dishes were incubated for 3 days. Cancer cells (5×10^5) suspended in the culture medium were poured onto the gel matrix. After 24 h, the collagen matrix was removed from the dishes and soaked in a mixed culture medium composed of one volume of DMEM containing 10% FCS and two volumes of KG-M medium (Epipak, Sanko Co. Ltd, Tokyo, Japan). The collagen matrices containing the tumour cells and fibroblasts were cultured for 14 days with medium changes at intervals of approximately 48 h. The gel matrices were microscopically observed after fixation with Carnoy's solution for 3 h and staining with haematoxylin and eosin.

RESULTS

Morphological characteristics of the monolayered cell lines

All the established cell lines monolayered on the culture dish surfaces exhibiting characteristics of squamous cell origin. OSC-1, 2, 4, 5 and 6 were well-differentiated with atypism in the cell size (Fig. 2). OSC-3 cells were predominantly a large polygonal shape, and their nuclear–cytoplasmic ratio was the highest of the cell lines examined. Cells in OSC-7 were polygonal to spindle-like in shape, and poorly differentiated.

Cell growth

OSC-2 and OSC-4 became confluent in 8 days, and their doubling times were 22.9 h and 21.2 h, respectively. OSC-3 and OSC-5 multipled slightly more slowly, with a doubling time of 31.3 and 32.5 h, respectively. OSC-1 and OSC-6 exhibited very similar *in vitro* growth, and required more than 10 days to achieve confluency (their doubling times were 38.6

Table 2. Colony-forming efficiency and transplantability of cell lines examined

Cell Line	Doubling time(h)	Passage*	Colony-forming efficiency(%)		Transplantability		Chromosome
			Plastic†	Agar‡	Tongue§	Lung‖	
OSC-1	38.6	72	8.4	0	9/10	0/10	74, XX-Y
OSC-2	22.9	150	19.8	0	10/10	2/10	76, XX
OSC-3	31.3	83	14.2	2.3	10/10	1/10	97, XX-Y
OSC-4	21.2	168	18.2	0.05	10/10	4/10	69, X-Y
OSC-5	32.5	69	12.8	0.05	10/10	ND	69, XX-Y
OSC-6	39.4	35	9.6	0	10/10	ND	70, XXX-Y
OSC-7	54.6	14	4.3	0	9/10	ND	85, XX-Y

*At 30 June 1993.
†Two × 10^2 cells were seeded in 60 mm plastic dishes and incubated for 10 days.
‡Five × 10^4 cells were plated on 0.3% agar, and overlayed with 0.5% agar. Colonies were counted after 3 weeks incubation.
§Five × 10^5 cells were inoculated into the tongue of 4-week-old BALB/C-nu/nu mice, and tumour formation was observed at 4 weeks after inoculation.
‖One × 10^6 cells were injected into the tail vein of 6 week-old BALB/C-nu/nu mice. At 6 weeks after inoculation, colonies formed in the lung were counted microscopically.

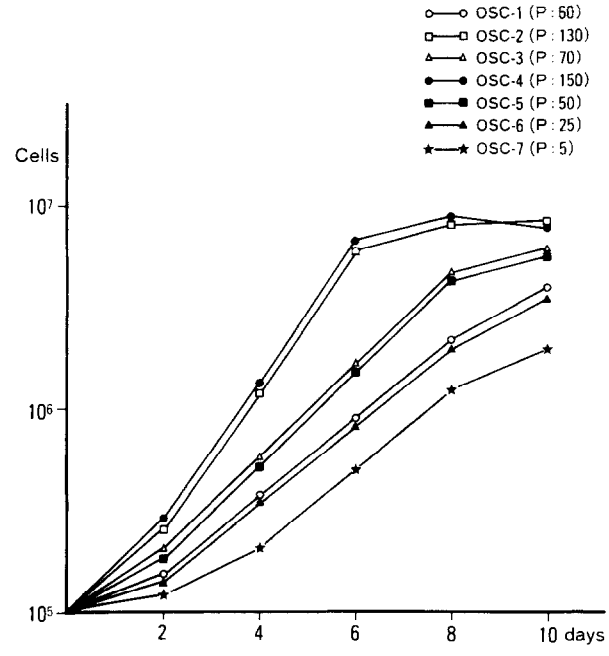

Fig. 3. Growth curves of the tumour cell lines. Each point represents the mean of duplicates.

and 39.4 h, respectively). OSC-7 showed the slowest multiplication, with a doubling time of 54.6 h (Table 2, Fig. 3).

Chromosome analysis

The modal number of chromosomes in metaphase ranged from 69 (OSC-4) to 97 (OSC-3). OSC-3 and OSC-7 appeared to be tetraploid, while the other cell lines were triploid. The range of chromosome number was from 71 to 76 in OSC-1, 70 to 76 in OSC-2, 96 to 100 in OSC-3, 65 to 70 in OSC-4, 68 to 72 in OSC-5, 60 to 71 in OSC-6, and 80 to 87 in OSC-7. Six marker chromosomes were observed in OSC-1, two in OSC-2, 10 in OSC-3, one in OSC-4, three in OSC-5, two in OSC-6, and nine in OSC-7 (Table 2).

Colony-forming efficiency

On the plastic substratum, the average colony-forming efficiency was 8.4% for OSC-1, 19.8% for OSC-2, 14.2% for OSC-3, 18.2% for OSC-4, 12.8% for OSC-5, 9.6% for OSC-6, and 4.3% for OSC-7. The efficiency in the soft agar was very low, being 2.3% for OSC-3, 0.05% for OSC-4 and 5, and 0% for the other cell lines (Table 2).

Xenografted tumour cells

About 4 weeks after inoculation into the tongue, the xenografted cells multiplied to form tumours of about 0.5–0.8 cm in diameter. Tumour formation was observed in all nu/nu mice except for mouse case each for OSC-1 and OSC-7. The histopathological characteristics of the tongue tumours were generally similar to those in the donor's tissues. However, the mode of tumour cell invasion in the tongue was somewhat different from that of the original mode in OSC-2, OSC-4 and OSC-5. These cell lines, which were established from the metastatic lymph nodes, exhibited more sporadic invasion in the xenografted tissue compared to the original mode of invasion (Fig. 1A, D).

Pulmonary lesions were observed after intravenous inoculation in three of four cell lines examined. Of these, OSC-4 showed the most frequent lodging of the intravenously inoculated cells, and tumour formation in the lung was found in four out of 10 mice, compared to two or one mice for OSC-2 and OSC-3, respectively, and no pulmonary lesions were formed in OSC-1 (Table 2). The pulmonary tumour cell differentiation resembled that in the tongue; however vascular invasion was occasionally observed in the lung lesions (Fig. 4A, B).

Tissue culture in collagen gel matrix

OSC-1 stratified to two or three cell layers in the collagen gel matrix, but the tumour cells did not deeply invade (Fig. 5A). The cells of OSC-2, 3, 5, 6 and 7 multiplied to form polylayered sheets with mitosis and dyskeratosis; OSC-2 and OSC-7 formed prominent thick sheets (Fig. 5B, G). All cell lines, except OSC-6 and OSC-7, invaded into the gel matrix; however, the invasive pattern differed with each cell line.

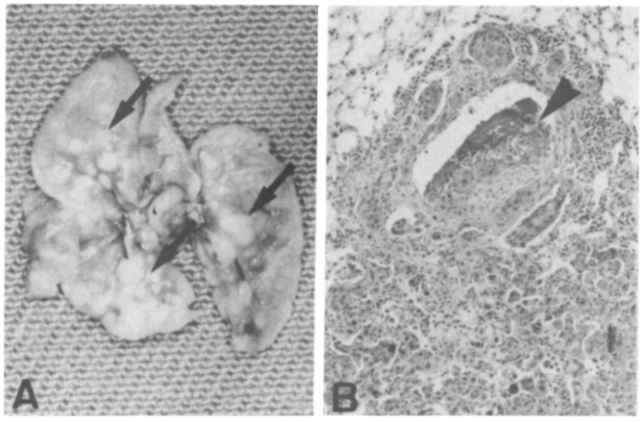

Fig. 4. Pulmonary colonisation of xenografted OSC-4 (120th passage). (A) A view of multifocal tumour nests in the lung (arrows), formed after venous transfusion of the tumour cells. (B) Microscopy of the pulmonary lesion exhibiting marked atypia and vascular invasion (arrow head) with slight cell infiltration (×80).

Invasive tendency was the most strong in OSC-2 and OSC-4 (Fig. 5B, D); and only a small number of OSC-3 and OSC-5 tumour cells invaded deeply (Fig. 5C, E). Compared to OSC-2, in which a cord-like invasion pattern with small nest formation was observed, OSC-4 exhibited a more sporadic invasion pattern (Fig. 5B, D). OSC-6 and OSC-7 were the least invasive with only a few solitary invading cells (Fig. 5F, G).

DISCUSSION

The cell lines in this study, all of which possessed distinctive marker chromosomes, had different doubling times, which correlated with growth in nude mice, but not with multiplication in the gel matrix. Their colony-forming efficiencies were very weak in soft agar. However, all cell lines could be transplanted to nude mice. Because of the low colony-forming efficiencies and high tumour formation in the mouse tongue, no relationship between the two was found.

Compared to monolayer cells on plastic dishes, stratified tumour cells in the collagen gel matrix seem much more suited to the examination of tumorgenicity. It is likely that rapidly multiplying tumour cells (OSC-2 and OSC-4) stratify more than slowly growing tumour cells (OSC-7). However, the stratification in Figs 5 did not correlate with the growth curve (Fig. 3) in OSC-4 and OSC-7. Both OSC-2 and OSC-4 exhibited prominent invasion into the matrix. In addition, after intravenous inoculation, OSC-4 followed by OSC-2, most frequently formed pulmonary lesions. The original mode of invasion of their primary tumour cells in the gingiva and tongue, respectively, was grade 3 for both, while in the collagen matrix they invaded more diffusely. These cell lines were obtained from the metastatic lymph nodes. It is therefore considered that the explanation for this discrepancy is that the more invasive cells in the original tumour cells had disseminated to the lymph nodes, however, there is a possibility that the difference may reflect some form of selection under the tissue culture condition.

The tumour cells xenografted into the tongue of nude mice grew more sporadically and more invaded into the muscular tissue than the primary tumour cells; however, OSC-3 and OSC-6, whose original mode of invasion was grade 4, and

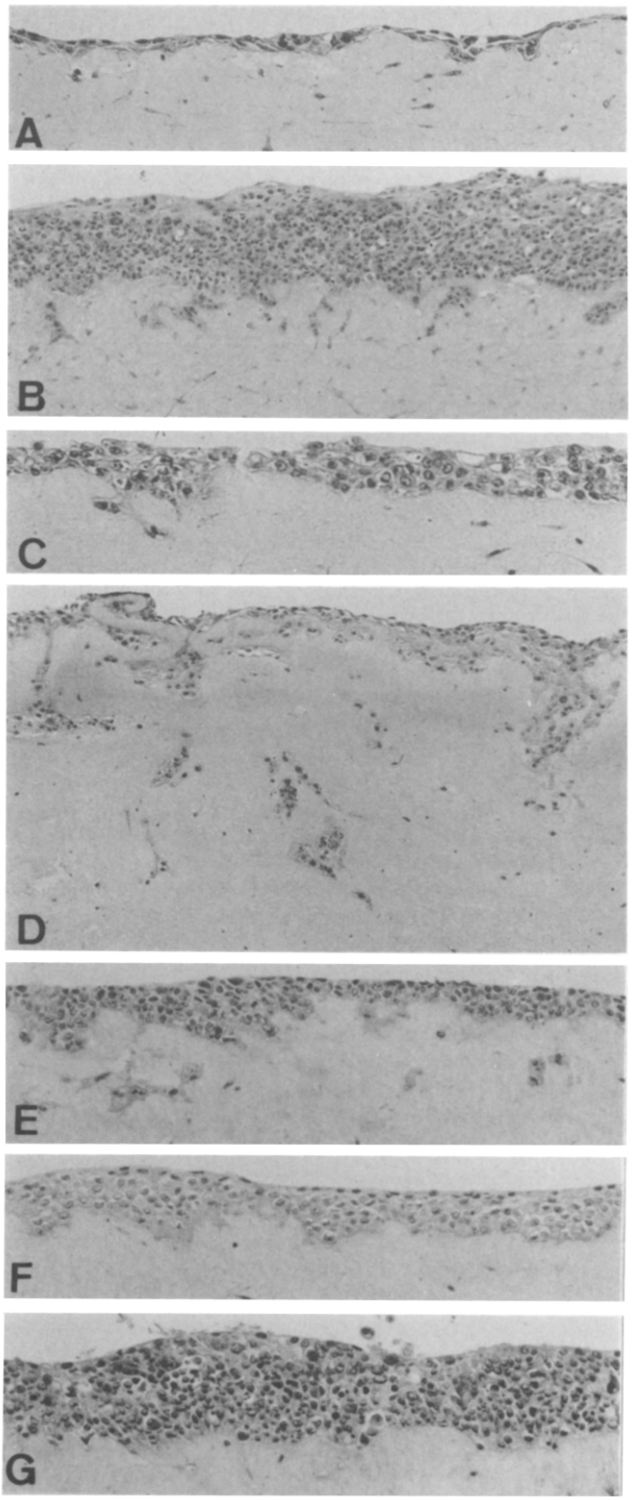

Fig. 5. Typical microphotographs of the tumour cell lines cultured in the collagen matrix for 2 weeks (cross-section, ×160). OSC-1 shows an almost non-invasive proliferation, with formation of only two to three cell layers (A). OSC-2 stratifies moderately and invades very sparsely into the gel matrix (B), while the poorly-differentiated OSC-4 does not stratify and invades diffusely into the matrix exhibiting a cord-like structure (D). OSC-3 (C) and OSC-5 (E) are moderate in stratification, and only a small number of invading cells are visible. Both OSC-6 and OSC-7 are not invasive in the gel matrix (F and G, respectively).

OSC-1, which isolated from the original tissue, retained the same invasion mode as the original. Taking these *in vivo* results together with the *in vitro* findings, the above hypothesis, namely that the more invasive cells among the tumour cells will selectively metastatise to the regional lymph nodes or distant organs, seems to be valid. To confirm this assumption, it would be useful to examine invasion- and metastasis-regulating oncogene expression [13–15], cell surface adhesion molecules [16–18] and metalloproteinase activity [5, 14, 19–21] in the established cell lines.

Further basic studies can be carried out using the cell lines described here, and we plan to use these lines for analysis not only of invasion and metastasis, but also of signal transduction involved in cell differentiation [22, 23] and apoptosis [22–25].

1. Bell E, Ehrlich HP, Buttle DJ, Nakatsuji T. Living tissue formed *in vitro* and accepted as skin-equivalent tissue of full thickness. *Science* 1981, **211**, 1052–1054.
2. Yoshizato K, Nishikawa A, Taira T, *et al.* A reconstitute skin: dermal–epidermal interactions in collagenolysis and cell morphology. *Biomed Res* 1986, **7**, 219–231.
3. Boukamp P, Thomas H, Rupniok R, Fusenig NE. Environmental modulation of the expression of differentiation and malignancy in six human squamous cell carcinoma cell lines. *Cancer Res* 1985, **45**, 5582–5592.
4. Matsumoto K, Horikopshi M, Rikimaru K, Enomoto S. A study of an *in vitro* model for invasion of oral squamous cell carcinoma. *J Oral Pathol Med* 1989, **18**, 498–501.
5. Kawahara E, Okada Y, Nakanishi I, *et al.* The expression of invasive behavior of differentiated squamous carcinoma cell line evaluated by an *in vitro* invasion model. *Jpn J Cancer Res* 1993, **84**, 409–418.
6. Yamamoto E, Miyakawa A, Kohama G. Mode of invasion and lymph node metastasis in squamous cell carcinoma of the oral cavity. *Head Neck* 1984, **6**, 938–947.
7. Osaki T, Yoneda K, Hirota J, Yamamoto T, Ueta E. Immunological investigation on lymph node metastasis in oral squamous cell carcinomas. *J Jpn Soc Cancer Ther* 1990, **25**, 621–631.
8. Naito S, von Eschenbach AC, Giavazzi R, Fidler IJ. Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. *Cancer Res* 1986, **46**, 4109–4115.
9. Morikawa K, Walker SM, Nakajima M, Pathak S, Jessup JM, Fidler IJ. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. *Cancer Res* 1988, **48**, 6863–6871.
10. Hermanek P, Sobin LH. *TNM classification of Malignant Tumours*. 4th ed. International Union Against Cancer, Berlin, Springer, 1987, 16–18.
11. Willen R, Nathanson A, Moberger G, Anneroth G. Squamous cell carcinoma of the gingiva; histological classification and grading of malignancy. *Acta Otolaryngol* 1975, **79**, 146–154.
12. Marina S. A rapid banding technique for human chromosomes. *Lancet* 1971, **II**, 971–972.
13. Nicolson GI. Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. *Cancer Res* 1987, **47**, 1473–1478.
14. Ura H, Bonfil RD, Reich R, *et al.* Expression of type IV collagenase and procollagen genes and its correlation with the tumorigenic, invasive, and metastatic ability of oncogene-transformed human bronchial epithelial cells. *Cancer Res* 1989, **49**, 4615–4621.
15. Lester BR, McCarthy JB. Tumor cell adhesion to the extracellular matrix and signal transduction mechanisms implicated in tumor cell motility, invasion, and metastasis. *Cancer Met Rev* 1992, **11**, 31–44.
16. Mortarini R, Anichini A, Parmiani G. Heterogeneity for intergrin expression and cytokine-mediated VLA modulation can influence the adhesion of human melanoma cells to extracellular matrix proteins. *Int J Cancer* 1991, **47**, 551–559.
17. Takeda K, Hatakeyama K, Tsuchiya Y, Rikiishi H, Kumagai K. A correlation between GM-CSF gene expression and metastasis in murine tumors. *Int J Cancer* 1991, **47**, 413–420.
18. Samuel SK, Hurta RAR, Kondaiah P, *et al.* Autocrine induction of tumor protease production and invasion by metallothionen-regulate TGF-β1 (ser 223, 225). *EMBO J* 1992, **11**, 1599–1605.
19. Kusukawa J, Sasaguri Y, Shima I, Kameyama T, Morimatsu M. Production of matrix metalloproteinase 2 (gelatinase/type IV collagenase) and 3 (stomelysin) by cultured oral squamous cell carcinoma. *Oral Pathol Med* 1992, **21**, 221–224.
20. Sato H, Kida Y, Mai M, Endo Y, Sasaki T, Tanaka J, Seiki M. Expression of genes encoding type IV collagen-degrading metalloproteinsaes and tissue inhibitors of metalloproteinsaes in various human tumor cells. *Oncogene* 1992, **7**, 77–83.
21. Muller D, Wolf C, Abecassis J, *et al.* Increased stromelysin 3 gene is associated with increased local invasiveness in head and neck squamous cell carcinomas. *Cancer Res* 1993, **53**, 165–169.
22. Piacentini M, Fesus L, Farrace MG, Ghibelli L, Piredda L, Melino G. The expression of "tissue" transglutaminase in two human cancer cell lines is related with the programmed cell death (apoptosis). *Eur J Cell Biol* 1991, **54**, 246–254.
23. Jensen LM, Zhang Y, Shooter EM. Steady-state polypeptide modulations associated with nerve growth factor(NGF)-induced terminal differentiation and NGF deprivation-induced apoptosis in human neuroblastoma cells. *J Biol Chem* 1992, **267**, 19325–19333.
24. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B, Costa J. Induction of apoptosis by wild-type p 53 in a human colon tumor-derived cell line. *Proc Natl Acad Sci U.S.A.* 1992, **89**, 4495–4499.
25. Obeid LM, Linardic CM, Karolak LA, Hannum YA. Programmed cell death induced by ceramide. *Science* 1993, **259**, 1769–1771.