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Papers

Development of a New Invasion and Metastasis Model of Human Oral Squamous Cell Carcinomas

S. Kawashiri, S. Kumagai, K. Kojima, H. Harada and E. Yamamoto

A new model was devised in order to establish an *in vivo* model for oral carcinoma that exhibits significant local invasion and metastasis. One hundred and fifty-two nude mice had tumour cells from one of two established oral squamous cell carcinoma (SCC) cell lines (OSC-19 and OSC-20) implanted into the tongue or the oral floor via an intra-oral route and, as a control, the subcutaneous tissue of the back. The back tumours showed an expansive growth pattern, lacking significant invasion of surrounding tissues. In contrast, the tumours implanted into the tongue or the oral floor exhibited invasive growth and the histological appearance was similar to that of the original tumours. Moreover, regional neck lymph node and pulmonary metastases were observed in this model. Regional neck lymph node metastases were detected in 81.0% of mice implanted with OSC-19 cells and in 13.6% of mice implanted with OSC-20 cells. OSC-19 and OSC-20 cells showed pulmonary metastases in 9.5 and 9.1% of mice, respectively. These results suggest that this intra-oral implantation model is valuable in the study of the mechanism of invasion and metastasis of oral SCC.

Keywords: orthotopic implantation, invasion and metastasis model, human oral squamous cell carcinoma cell line

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INTRODUCTION

MANY INVASION and metastasis models have been developed in order to better understand the complex mechanisms of cancer cell invasion and metastasis. These models are classified into two groups: *in vitro* and *in vivo* models. The *in vitro* model [1, 2] has the advantages of enabling the environment around cancer cells to be simplified and the results obtained in a short length of time. However, it does not necessarily represent *in vivo* phenomena. The benefit of the *in vivo* model is that the interaction between the host and the cancer cells can be observed. *In vivo* models have been described in which the cancer cells were implanted into the subcutaneous tissue of the back of nude mice [3–5]. In these models, however, invasion and metastasis were observed only with specific cancer cells. Generally, the tumours implanted into the back of nude mice show a benign growth pattern and lack the active invasion into the surrounding tissues that is observed in the original human cancer. Pulmonary metastasis is observed in the model using intravenous injection into a tail vein [6, 7]. This model is not a true representation of the mechanism of metastasis in the human, as it is in view of the process after removal of cancer cells from a primary site in haematogenous metastasis.

Bresalier *et al.* [8, 9] reported an orthotopic implantation model of human colon carcinoma cells into the caecal wall of nude mice. They observed that human colon carcinoma cells were tumorigenic following subcutaneous implantation, but neither regional mesenteric lymph node nor hepatic metastases were produced. In contrast, intracaecal inoculation of cancer cells resulted in the production of metastases. These results suggest that the interaction of tumour cells with an organ environment is an important factor in tumour invasion and metastasis. Therefore, we tried implanting human oral carcinoma cells into the tongue or the oral floor in nude mice, and as a control, into the subcutaneous tissue of the back. Histological examination of the tumours, regional lymph nodes and lungs in each implantation site was carried out, and results were compared with the original human tumours.

Correspondence to S. Kawashiri.
All authors are at the Department of Oral and Maxillofacial Surgery, School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920, Japan
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MATERIALS AND METHODS

Mice

One hundred and fifty-two female athymic BALB/c-nu/nu nude mice (Charles River Japan, Yokohama, Japan) were used at 6 weeks of age. They were maintained in a laminar flow iso-

rack under specific pathogen-free conditions in the Institute for Experimental Animals of Kanazawa University. The mice were given food (Oriental Koubo, Tokyo, Japan) and water, which were autoclaved. All mice were handled with sterile techniques under a laminar flow hood when removed from their cages.

Cell lines

Two cell lines, OSC-19 and OSC-20 cells [10, 11], derived from human oral squamous cell carcinoma (SCC) were used. OSC-19 cells were established from a metastatic tumour which was found in a cervical lymph node of a 61-year-old Japanese man with well-differentiated SCC of the tongue [10]. OSC-20 cells were established from a metastatic lymph node from the tongue cancer of a 58-year-old Japanese woman. The pathologic study of its origin showed a moderately differentiated SCC [11]. These cells were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Laboratories, New York, U.S.A.) and antibiotics (60 mg/l kanamycin and 2 mg/l amphotericin B), at 37°C in a humidified atmosphere of 5% CO_2 in air.

Implantation of cells

The cells were injected into the tongue or oral floor via an intra-oral approach at 2.0×10^5 viable cells/0.025 ml in cell culture medium per mouse, and into the subcutaneous tissue of the back at 1.0×10^6 cells/mouse, using a tuberculin syringe with a 26-gauge disposable needle (Terumo, Tokyo, Japan). The mice were sacrificed on days 4, 10, 15 and 20 post-implantation, and submitted for autopsy and histological examination. One hundred and fifty-two mice were implanted: six mice each into the tongue for days 4, 10, 15; into the oral floor for days 4, 10, 15; into the subcutaneous tissue of the back for days 10, 15, 20; 11 mice each into the tongue for day 20; into the oral floor for day 20. The same number of animals received OSC-19 and OSC-20 cells.

Histological examination

At autopsy, the abdomen and chest were also opened. The trachea, heart, lungs, liver, pancreas and stomach were examined for any evidence of tumour under an operating microscope, model OMK1 (Olympus Optical Co., Tokyo, Japan). The subcutaneous tumours, the oral (tongue or floor of the mouth) tumours, regional lymph nodes and lungs were removed and fixed in periodate lysine paraformaldehyde solution at 4°C for 24 h. Oral tumours were decalcified employing an 18% ethylenediamine tetraacetic acid solution adjusted to pH 7.5 (Wako Pure Chemical Industries, Osaka, Japan) at 4°C for 72 h. Subsequently, these specimens were embedded in paraffin wax, and cut into 3 μm-thick sections. They were stained with haematoxylin and eosin (HE) for microscopic observation. They were histologically evaluated as to growth, mode of cancer invasion (Table 1) [12], bone invasion, lymph node metastasis and pulmonary metastasis. The χ^2 test was used for statistical comparison of the metastatic ability of OSC-19 and OSC-20 cells.

Table 1. Histological grading of mode of cancer invasion

Grade	Criteria
1	Well-defined borderline
2	Cords, less marked borderline
3	Groups of cells, no distinct borderline
4	Diffuse invasion
	Cord-like type invasion (4C)
	Diffuse type invasion (4D)

The mode of invasion of the oral SCC was graded by the criteria reported by Yamamoto *et al.* [12].

RESULTS

Subcutaneous tumours

Macroscopically, subcutaneous tumours developed at the back of nude mice within a week of implantation. The tumours grew gradually and reached diameters ranging from 3 to 9 mm on day 20 postimplantation. No discernible differences were observed in the size of tumours between the two cell lines. Tumours on the back revealed benign growth and mobile nodular lesions.

Histologically, the subcutaneous tumours of OSC-19 cells showed well-differentiated SCC. Tumour cells were polygonal, and showed marked keratinisation, forming a cancer pearl. They did not show invasive growth beyond the dermis (Fig. 1a). Tumours of OSC-20 cells showed moderately-differentiated SCC. The majority of these tumours revealed central necrosis. They remained encapsulated by fibrous tissue and did not invade neighbouring tissues (Fig. 1b). The parenchyma of these subcutaneous tumours possessed a histological

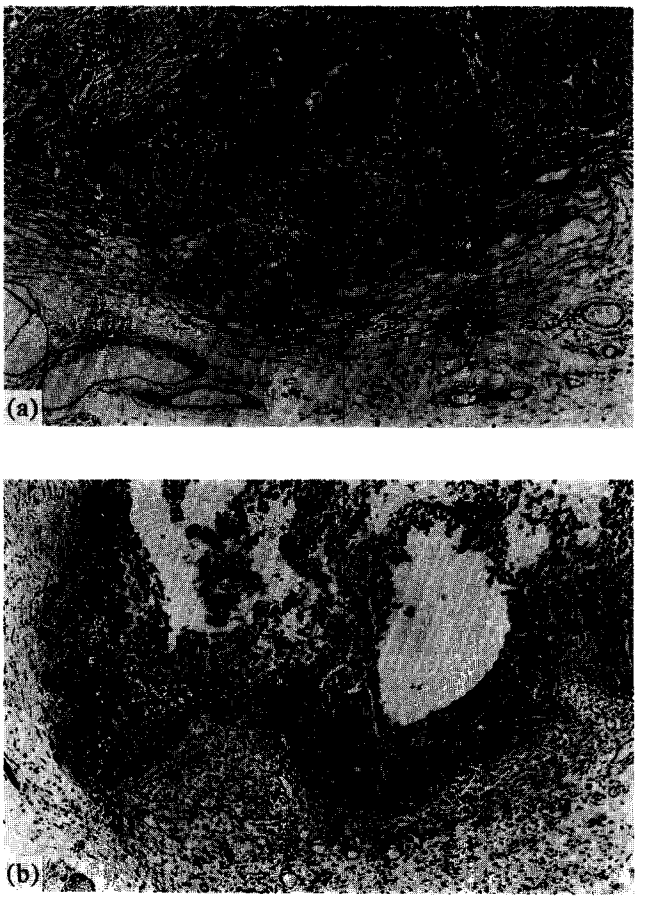
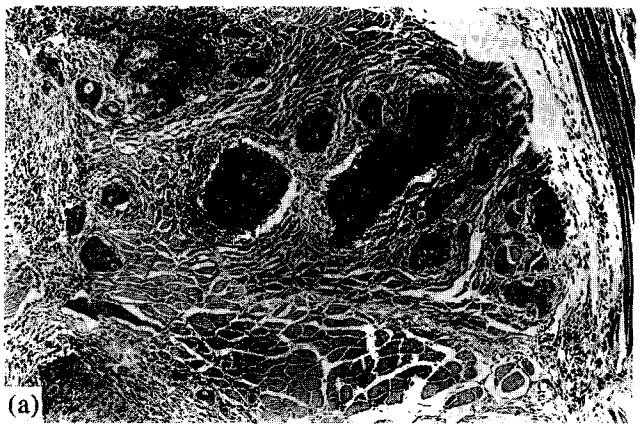


Fig. 1. Photomicrograph of the subcutaneous tumours on day 20 postimplantation: (a) OSC-19 cells; (b) OSC-20 cells.



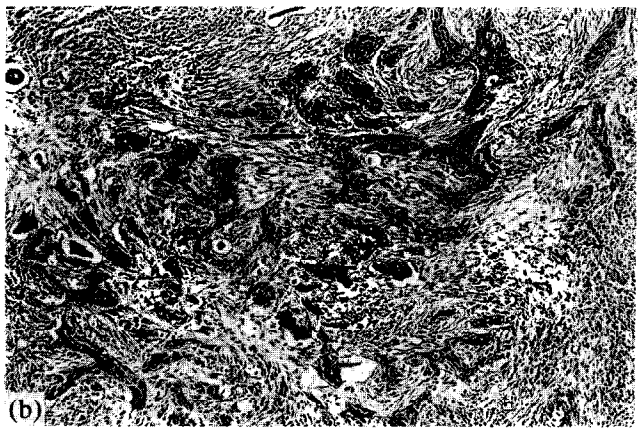
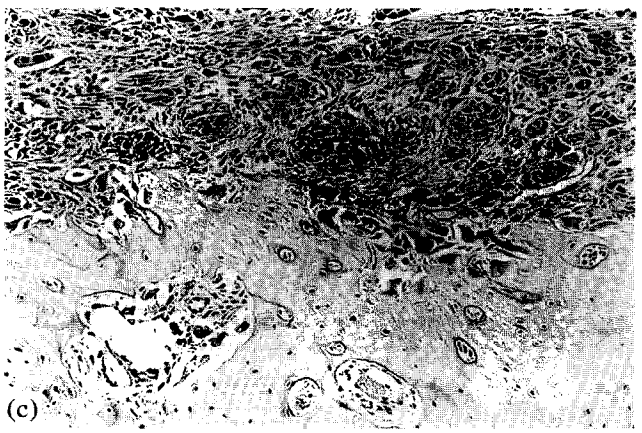


Fig. 3. Photomicrograph of the oral tumours of OSC-20 cells. (a) Tumour of OSC-20 cells showed moderately-differentiated SCC and the invasion of various round-shaped tumour nests on day 20. (b) Original tumour of OSC-20 cells in the patient.



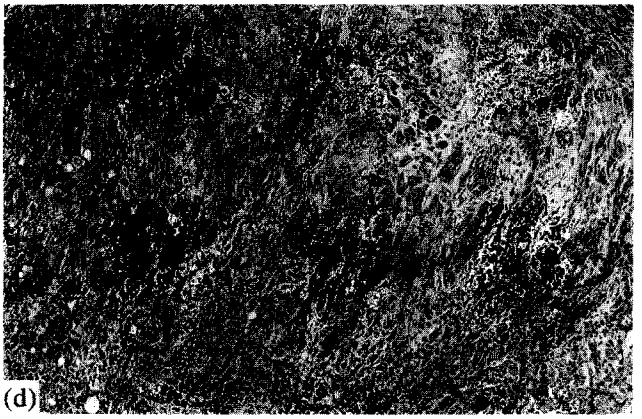


Fig. 2. Photomicrograph of the oral tumours of OSC-19 cells. (a) Tumour cells proliferated in tissue space on day 4; (b) tumour showed diffuse invasion into the surrounding tissue on day 20; (c) tumour of the oral floor invaded the mandibular bone; (d) original tumour of OSC-19 cells in the patient.

appearance similar to that of the original tumours, but active invasive growth could not be observed in the subcutaneous tumours of each cell line.

Oral tumours

The two cell lines formed tumours in the tongue or the oral floor of nude mice, with one exception: OSC-19 cells implanted into the tongue of one animal showed no tumour formation on day 20 postimplantation. Macroscopically, the tumours grew to sizes ranging from 3 to 8 mm in diameter at 20 days after implantation. The surface of the tumours often showed ulceration. There were no notable differences in the size of tumours between the two cell lines.

Histologically, the oral tumours of OSC-19 cells showed well-differentiated SCCs. The tumour cells proliferated in tissue spaces, but did not invade the surrounding tissue on day 4 postimplantation (Fig. 2a). On days 10, 15 and 20, however, OSC-19 cells showed diffuse invasion into the surrounding tissue with cord-like microtumour nests. Fibroblastic reaction

Table 2. Histological comparison between origin and implantation tumour

Cell line	Histology of origin	Implantation site	Histology of implanted tumour
OSC-19	Well-differentiated SCC Grade 4C*	Subcutaneous	Well-differentiated SCC No invasion
		Intra-oral	Well-differentiated SCC Grade 4C* Bone invasion†
OSC-20	Moderately-differentiated SCC Grade 3*	Subcutaneous	Moderately-differentiated SCC No invasion
		Intra-oral	Moderately-differentiated SCC Grade 3*

*Mode of cancer invasion.
†Implantation into the oral floor on day 20.
Oral tumours of OSC-19 and OSC-20 cells showed active invasion into the surrounding tissues and the histological appearance indicated a similar mode of invasion to that of the original tumours.

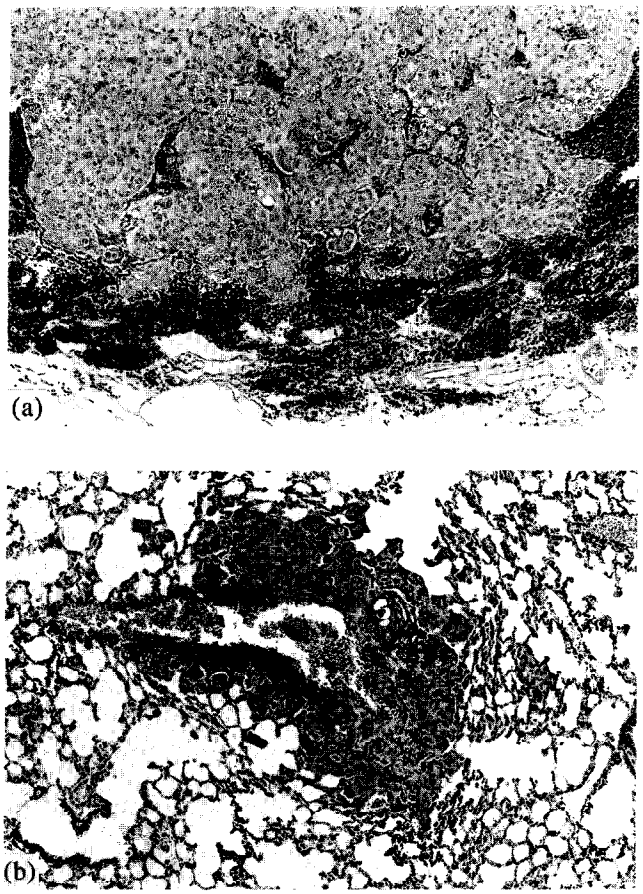


Fig. 4. Photomicrograph of the metastasis: (a) regional neck lymph node metastasis; (b) pulmonary metastasis.

and mononuclear cell infiltrates were seen around the tumour nests (Fig. 2b). The mode of cancer invasion of these tumours was determined as grade 4C. The mode was the same as that of the original tumour. Tumours of the oral floor invaded the mandibular bone on day 20 (Fig. 2c). The tumours possessed a histological appearance similar to that of the original tumour (Fig. 2d).

Tumours of OSC-20 cells showed moderately differentiated SCCs and the invasion of various round-shaped tumour nests on days 10, 15 and 20 (Fig. 3a). The fibrous stromal reaction was poorly developed, and there were many mononuclear cells infiltrating around the tumour nests. The mode of invasion of these tumours was determined as grade 3, and the mode was the same as that of the original tumour. Bone invasion of OSC-20 tumours was not observed on day 20. The tumours possessed a histological appearance similar to that of the original tumour (Fig. 3b).

In summary, oral tumours of OSC-19 and OSC-20 cells showed active invasion into the surrounding tissues on days 10, 15 and 20 postimplantation and the histological appearance indicated a similar mode of invasion to that of the original tumours (Table 2).

Metastases

No metastases from the subcutaneous tumours were identified, while regional neck lymph node (Fig. 4a) and pulmonary metastases (Fig. 4b) were observed in oral tumours. On day 20 postimplantation, 80% of mice implanted with OSC19 cells into the tongue had regional neck lymph node metastasis, but no pulmonary metastasis, and 81.8% of those implanted into the oral floor had lymph node metastasis, and 18.2% had pulmonary metastasis. The tongue tumours of OSC-20 cells showed lymph node metastasis in 9.1% of mice, but no pulmonary metastasis, and the oral floor tumours showed lymph node metastasis in 18.2%, and pulmonary metastasis in 18.2%, on day 20 postimplantation (Table 3).

Regional neck lymph node metastases from the oral tumours were detected in 81.0% of mice implanted with OSC-19 cells and in 13.6% of mice implanted with OSC-20 cells. Statistical analysis showed that the metastatic ability of OSC-19 cells was significantly greater than that of OSC-20 cells ($P<0.01$). OSC-19 and OSC-20 cells developed pulmonary metastases in 9.5% and in 9.1%, respectively. The incidences of pulmonary metastases of these cells were not significantly different. Mice producing pulmonary metastases were found only in the group with oral floor implantation, without exception.

DISCUSSION

The nude mouse, discovered in 1968 by Pantelouris [13], was first used as a host for implantation of human carcinoma cells in 1969 by Rygaard and Povlsen [14]. Subsequently, numerous models for studying human carcinomas with implantation into the subcutaneous tissue of nude mice have been described. Povlsen and Rygaard [15, 16] reported that

Table 3. Incidence of regional neck lymph node and pulmonary metastasis of cancer cells on day 20 postimplantation

Cell line	Implantation site	Number of implanted mice	Tumorigenicity	Metastasis site	Number of metastasised mice	Rate of mice with metastasis to tumorigenicity mice (%)	
OSC-19	Tongue	11	10/11	Lymph node	8	80.0	
				Lung	0	0	
	Oral floor	11	11/11	Lymph node	9	81.8	
				Lung	2	18.2	*
OSC-20	Tongue	11	11/11	Lymph node	1	9.1	
				Lung	0	0	
	Oral floor	11	11/11	Lymph node	2	18.2	
				Lung	2	18.2	

Statistical analysis showed that the ability of OSC-19 cells to develop lymph node metastases was significantly greater than that of OSC-20 cells ($*P<0.01$).

carcinomas which in the human host have a malignant growth pattern grow in the mutant nude mouse as local, well-circumscribed, apparently benign tumours without invasive growth, but otherwise have the histological and cytological characteristics of the primary tumours. The findings of our study were similar. We found that the parenchymal cells of the subcutaneous tumours resembled the original tumour cells, but no evidence of invasive growth could be demonstrated in the subcutaneous tumours of either SCC cell line. Schmidt and Good [17], Franks *et al.* [18] and Cobb and Mitchley [19, 20] suggested that the lost potential for invasion, when primary human tumours were transplanted into animals, could be due to the use of immunodeficient hosts. Oral tumours of OSC-19 and OSC-20 cells implanted into nude mice, however, showed invasion into the surrounding tissue, and the modes of invasion were very similar to those of the original tumours. It seems that the interaction of tumour cells with an organ environment is an important factor that modulates tumour invasion.

Nakajima *et al.* [21] observed differences in the production and secretion of extracellular matrix-degrading enzymes in tumours growing in different organs: human colon carcinoma cells implanted orthotopically expressed metastatic ability and high extracellular matrix-degrading enzyme activities. This report helps to explain why the metastatic ability of cancer can be altered by the transplantation site in the recipient animals. Articles emphasizing the importance of orthotopic implantation have increased recently [22]. For head and neck carcinomas, Dinesman *et al.* [23] reported that pulmonary metastases were evident in 40% of the animals in an orthotopic implantation model that employed two human laryngeal SCC cell lines, and emphasis was placed on the importance of orthotopic implantation. However, surprisingly few studies have looked at oral cancer and there has been no previous attempt to develop an invasion and metastasis model using human oral SCC cells. In our intra-oral implantation model, regional neck lymph node metastases were demonstrated in over 80% of mice implanted with OSC-19 cells. We think that this high incidence is sufficient to justify the use of this system as a model for the study of metastasis in oral SCC.

In clinical treatment, lymph node metastases are frequent in oral cancer, and metastasis is responsible for most treatment failures. More patients succumb from local metastatic tumour growth than from the primary tumour [24]. Studies of the mechanisms of invasion and metastasis are therefore important for bettering prognosis. The elucidation of these mechanisms requires precise investigations on invasion, and metastasis models have been repeatedly devised, such as the one described here. We consider this model valuable in studying the invasion and metastasis of oral SCC, and believe it will permit more meaningful *in vivo* studies on the development of various anti-neoplastic treatment modalities.

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