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A New *in vitro* Invasion Model for Oral Cancer Using an Acellular Allogenic Dermal Matrix (Alloderm): The Relationship among *in vitro* Invasion Activity, *in vivo* Invasion and Metastasis

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ABSTRACT

The purpose of this study was to develop a new *in vitro* model of cancer invasion using a human dermal equivalent, AlloDerm®. A squamous cell carcinoma line (HSC-2, HSC-3 and OSC20) and a malignant melanoma line (G361) were cultured on AlloDerm® and evaluated histologically to examine destruction of the basement membrane and invasion into the dermis of AlloDerm®. HSC-3 and G361 cells invaded through the basement membrane of AlloDerm® at 21–28 days after seeding, while HSC-2 and OSC20 cells did not. Next, these cells were transplanted into the tongues of nude mice, and the degree of local invasiveness was examined histologically. HSC-3 and G361 cells invaded diffusely to the surrounding tongue tissue, although HSC-2 and OSC20 showed only expansive growth. Further, these cells were transplanted subcutaneously in nude mice to study metastatic activity. Regional and distant metastases were rarely observed after transplantation of HSC-2 and OSC20 cells. On the other hand, HSC-3 and G361 cells frequently metastasized. These findings show a close relationship among *in vitro* invasiveness on AlloDerm®, *in vivo* invasiveness, and metastatic activity. This experimental model using AlloDerm® is a potentially new *in vitro* model of cancer invasion.

INTRODUCTION

The survival rate of patients with oral cancer has recently been improved as various treatment modalities have been developed, but those with multiple lymph node metastases or distant metastases still show extremely poor prognosis. The first step in the metastasis of oral squamous cell carcinoma (OSCC) is destruction of the basement membrane and invasion into the submucosa. To improve the cure rate of patients with OSCC, it is necessary to prevent invasion and metastasis of OSCC.¹⁸ However, little is known regarding these biologic behaviors of OSCC because of a lack of appropriate *in vivo* and *in vitro* models for invasion and metastases.

A NEW *in vitro* INVASION MODEL FOR ORAL CANCER

A reconstituted basement membrane matrix, matrigel, has recently been used with the aim of developing an *in vitro* assay of tumor cell invasiveness.^{1,5,10,11,16,17} Some investigators have reported that matrigel provides a suitable model for the study of tumor cell interactions with basement membrane components, but others have stated that the invasiveness of tumor cells on matrigel does not always correlate their invasiveness *in vivo*.¹⁴ Kataoka et al.⁸ recently reported an *in vitro* invasion model created by seeding cancer cells onto a human cadaveric dermal equivalent, AlloDerm®. They reported that HSC-3 cells, a squamous cell carcinoma line derived from OSCC, invaded through the basement membrane into the dermis of AlloDerm®, although normal epithelial cells did not show invasion into AlloDerm®, and concluded that this experimental model using AlloDerm® was a potentially new *in vitro* model of cancer invasion. However, they did not show whether *in vitro* findings on AlloDerm® was correlated with *in vivo* invasiveness.

The purpose of this study was to examine the histologic features of some cancer cell lines on AlloDerm®, and investigate the relationship among the *in vitro* invasiveness on this material, *in vivo* local invasiveness, and metastatic activity when transplanted in nude mice.

MATERIALS AND METHODS

1) Animals

Five-week-old female BALB nu/nu nude mice (Japan Charles River, Yokohama, Japan), were used for the animal experiments and were kept under sterile conditions throughout. All animal experiments were performed in compliance with the Guidelines for Animal Experiments at Kobe University Graduate School of Medicine.

2) Cell lines and cultures

Human OSCC lines (HSC-2, HSC-3 and OSC20) and a human malignant melanoma line (G361) were provided by the JCR Bank. HSC-2, 3 and G361 cells were cultured in Eagle's MEM medium with 10% fetal bovine serum (FBS), 10,000 units/ml of penicillin, and 10 mg/ml of streptomycin. OSC20 cells were cultured in a 1 to 1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% FBS, 10,000 units/ml of penicillin, and 10 mg/ml streptomycin. These cells were incubated and maintained under an atmosphere of 5% CO₂ at 37°C.

A human cadaveric dermal equivalent, AlloDerm®, was pre-soaked in 5µg/cm² human type IV collagen (Becton Dickinson Labware, Bedford, MA, USA) for 3 hours prior to seeding to enhance the adherence of the seeded tumors cells. After being washed in trypsin and EDTA, 2.5 x 10⁵ cells were placed on AlloDerm®. The composites of tumors cells and AlloDerm® were then cultured for 14-28 days.

3) Histologic evaluation

The composites of tumor cells and AlloDerm were removed from the culture at 14, 21 and 28 days after seeding, fixed in 10% formaldehyde, embedded in paraffin, and cut into 5 µm sections. The specimens were stained with hematoxylin and eosin. Five samples in each group were examined.

4) Xenograft of cancer cells into the tongues of nude mice

4.0 x 10⁵ of HSC-2, 3, OSC20 and G361 cells were transplanted by syringe into the center of the tongues of nude mice, according to the method reported previously by Umeda et al.¹⁹ Eight weeks after the transplantation, the tongue was resected and histological observations were performed to examine the invasiveness of these cells. Three animals were used in each group.

5) Xenograft of cancer cells into the backs of nude mice

4.0×10^5 of HSC-2, 3, OSC20 and G361 cells were transplanted into the backs of nude mice subcutaneously. After the tumor grew to 10 mm in diameter, it was extirpated, cut into about 1 mm³ pieces, and transplanted into the subcutaneous tissue of the lateral backs of other mice. A total of 60 animals were used in each cell line. The subcutaneous tumors were punctured twice a week from 4 to 12 weeks after transplantation to promote metastases as reported previously.

6) Detection of metastasis by PCR analysis of the human β -globin gene

Mice bearing tumors were killed 4, 8, and 12 weeks after transplantation, and the axilla lymph nodes and the lungs were resected. Genomic DNA was extracted from them using SepaGene (Sanko Jun-Yaku, Tokyo, Japan) according to the protocol recommended by the manufacturer. Two sets of primers were designed for the human β -globin gene (Table 1). The PCR conditions for the first reaction were 94°C for 90 seconds for 1 cycle, then for 30 cycles of denaturing at 94°C for 30 seconds, annealing at 58°C for 90 seconds, and extension at 70°C for 45 seconds. The first primers were designed outside the amplified region by the nested primer. In the second step, the first PCR product was reamplified using 2 sets of overlapping inner primers. Conditions for the nested PCR were the same as for the first reaction. The PCR product was electrophoresed in a 2.0% agarose gel containing 0.5 g/mL ethidium bromide.

Table I. Oligonucleotide primers designed for the human β -globin gene

First			
GH20	(forward)	GAAGAGCCAAGGACAGGTAC	408 bp
GH21	(reverse)	GGAAAATAGACCAATAGGCAG	
Second			
KM29	(forward)	GGTTGGCCAATCTACTCCCAGG	262 bp
KM38	(reverse)	TGGTCTCCTTAAACCTGTCTTG	

RESULTS

1. The histologic features of HSC-2, 3, OSC20 and G361 cells on AlloDerm®

At 14 days after seeding HSC-2, 3, OSC20 and G361 cells, they formed a continuous cell monolayer on the surface of AlloDerm® without any invasion into the dermis. At day 28, HSC-3 and G361 cells showed stratification and migration into the dermis of AlloDerm®. HSC-2 and OSC20 cells were cuboidal or slightly thinner in shape and showed little stratification, while no invasion into the dermis of AlloDerm® was observed. (Fig.1)

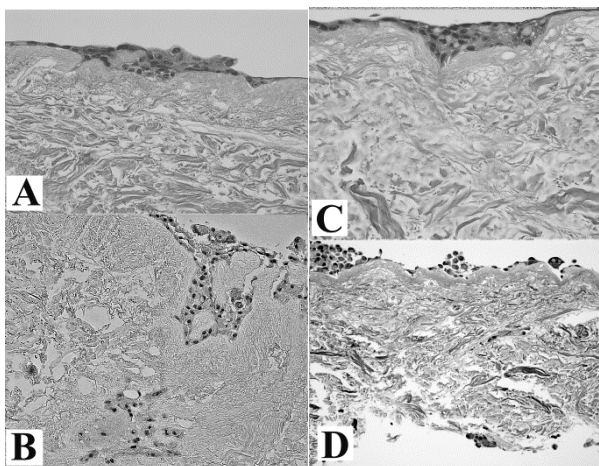


Fig. 1. The histologic examinations of HSC-2, 3, OSC20 and G361 cells on AlloDerm® at 28 days after seeding. (HE stain, original magnification $\times 200$).

A)HSC-2 cells were cuboidal or slightly thinner in shape, but the surface of AlloDerm® was smooth.

B)HSC-3 cells formed a stratified squamous cell epithelium and showed migration into the dermis of AlloDerm®.

C)OSC20 cells shaped stratified squamous cell epithelium without any invasive activity.

D)G361 cells migrated into the dermis of AlloDerm® diffusely.

2. *in vivo* local invasion of these cell lines in the tongues of nude mice

Histological examinations revealed proliferation of HSC-2, 3, OSC20 and G361 cells in the tongues of nude mice. HSC-2 showed well differentiated, HSC-3 poorly differentiated, and OSC20 moderately differentiated squamous cell carcinoma. HSC-2 and OSC20 cells showed pressure-type growth with relatively well-demarcated borderlines to the surrounding tongue tissue. On the other hand, HSC-3 and G361 cells invaded to the tongue diffusely without any capsule formation. (Fig.2)

3. *in vivo* metastatic activity to the lymph nodes and the lungs of nude mice

Frequencies of lymph node- and lung metastasis after transplantation of HSC-2, 3, OSC20 and G361 cells are summarized in Table 2 and Fig.3. HSC-2 cells did not metastasize to the regional lymph nodes or lungs of mice. In mice bearing OSC20 cells, lymph node

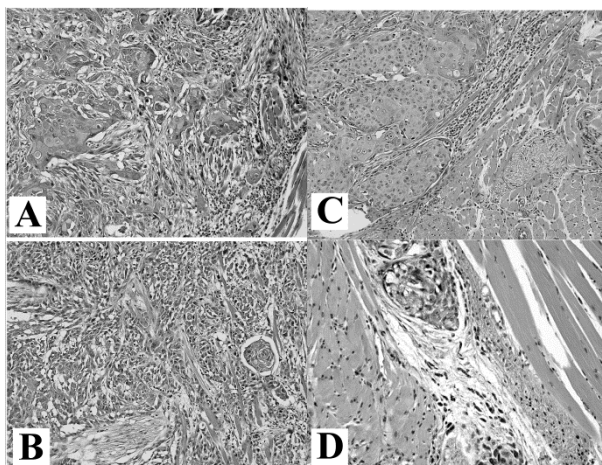


Fig. 2. Histological examinations of the tongues of nude mice at 8 weeks after tumor cells were transplanted orthotopically. (HE stain, original magnification $\times 200$)

A)HSC-2 showed well differentiated squamous cell carcinoma with keratin formation.

B)HSC-3 showed poorly differentiated squamous cells infiltrating among tongue tissue.

C)OSC20 had well defined borderlines to the surrounding tissue.

D)G361 showed marked cellular dissociation in small groups or single cell.

Table II. Rate of successful transplantation and the percentage of positive polymerase chain reaction analysis with the human β -globin gene at 12 weeks after transplantation

Tumor cell line	Successful transplantation	Lymph node metastasis	Lung metastasis
HSC-2	20/20(100%)	0/20(0.0%)	0/20(0.0%)
HSC-3	18/20(90.0%)	9/20(45.0%)	8/20(40.0%)
OSC20	19/20(95.0%)	2/20(10.0%)	0/20(0.0%)
G361	20/20(100%)	12/20(60.0%)	16/20(80.0%)

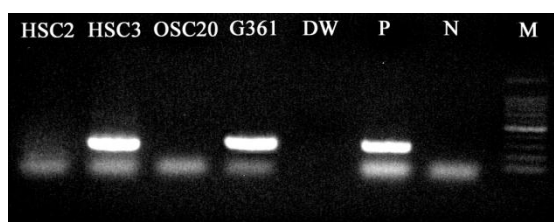


Fig. 3. PCR analysis with the human β -globin gene. Lanes 1-4: DNA extracted from the lymph nodes of a mouse 12 weeks after transplantation of tumor cells. Lane1:HSC-2, Lane2:HSC-3, Lane3:OSC20, Lane4:G361. A band of 262 bp indicates the presence of human β -globin gene sequences. A positive reaction is shown in lanes 2, and 4. Lane P: DNA extracted from human gingiva (positive control). Lane N: DNA extracted from the lymph node of a mouse in which tumor cells were not transplanted (negative control). Lane DW: distilled water. Lane M: size marker.

metastasis was observed in only 2/20(10%) mice and lung metastasis did not occur. On the other hand, HSC-3 and G361 cells, which showed *in vitro* and *in vivo* invasion, frequently metastasized to both the lymph nodes and lungs. These findings indicated a close relationship between *in vitro* invasion on AlloDerm® and metastatic activity *in vivo*.

DISCUSSION

The prognosis of OSCC has recently markedly improved as various treatment methods have been developed. The most frequent cause of death of patients with OSCC is regional or distant metastasis rather than local failure. Even those with early stage OSCC sometimes develop secondary metastasis after surgery and die due to metastatic cancer despite local cure. It is well known that lymph node metastasis in OSCC occurs frequently when diffuse invasion is observed histologically, and that distant metastasis often occurs in those who have multiple regional lymph node metastases with extra-nodular spread. These facts suggest that local invasion plays key roles in the metastatic process of OSCC, although the mechanisms and methods to prevent local invasion of OSCC have not been well documented because of a lack of appropriate experimental models for OSCC invasion.

Many *in vivo* experimental models of cancer metastasis have been reported.^{2,12,13,15,19} In some of them, metastasizing cancer cell lines were injected intravenously. These models do not, therefore, represent all the stages of metastasis, such as local invasion, attachment to a vessel, intravasation, dissemination, extravasation, growth in the metastatic site, and escape from host immunity. Some investigators have reported that metastasis often develops when cancer cells are transplanted orthotopically into the organs from which the cancer was derived, and some models of metastasis have been established using this method. Several studies have been attempted using OSCC to produce an orthotopic transplantation model. Kawashiri et al.⁹ reported that lymph node and lung metastases were often found when OSCC cell lines were transplanted into the floor of the mouth in nude mice. Umeda et al.¹⁹ reported that both OSCC cell lines and OSCC tissues obtained during surgery metastasized to the regional lymph nodes when they were transplanted into the tongues of nude mice. However, we do not think that these orthotopic transplantation models of OSCC can be used as standard *in vivo* models of OSCC metastasis because the steps in the destruction of the basement membrane and invasion into the submucosa are not represented. Though invasion does not always lead to metastasis, the first step of metastasis is the destruction of the basement membrane and invasion for OSCC and malignant melanoma, which originate from keratinocytes and melanocytes existing in the basal cell layer of the epithelium.

Cancer cells transplanted subcutaneously into animals are well known to be encapsulated and rarely metastasize. Shigeta, et al.¹⁵ reported recently that HSC-3 cells that were transplanted subcutaneously into the backs of nude mice and punctured repeatedly were easily able to metastasize to the regional lymph nodes and lungs. Genetic analysis of the human β -globin gene demonstrated that the frequencies of metastasis to the lymph nodes and lungs at 12 weeks after transplantation were as high as 45% and 40%, respectively. The model of subcutaneous transplantation plus repeated puncture is easy to carry out and has excellent reproducibility compared with orthotopic transplantation.

Kleinman et al.¹⁰ studied the reconstitution of basement membrane molecules from extracts prepared from the basement membrane of an Engelbreth-Holm-Swarm (EHS) tumor. This reconstituted basement membrane matrix, matrigel, provides a three-dimensional model that has been used for the study of tumor cell interactions with BM components, which is a critical step in the invasion process. Recently, several investigators have compared the capacity of normal and tumor cells to invade into this matrix with the aim of developing an *in vitro* invasion assay.

Kramer et al.¹¹ reported that human HT1080 fibrosarcoma cells initiated a random migration, leaving behind channels in the surface of matrigel several hours after seeding. The channels became interconnected to form a complex network before they were filled and the surrounding matrigel was gradually dissolved. They also described normal skin fibroblasts,

which were able to attach to themselves to the matrigel, but exhibited minimal migration, tracking, and invasion during the same period. Albini *et al.*¹ developed another *in vitro* test to estimate the invasive potential of tumor cells. They showed that the invasive and metastatic potential of malignant cells was correlated with their ability to cross a thin layer of matrigel coated onto the filter of a Boyden chamber. The positive correlation between the *in vitro* invasion of cancer cells into matrigel and their *in vivo* metastatic potential was also demonstrated by Simon *et al.*,¹⁶ Iwamoto *et al.*,⁵ and Terranova *et al.*¹⁷

On the other hand, Noël *et al.*¹⁴ reported that normal cells (fibroblasts, glomerular mesangial cells, and keratinocytes), human fibrosarcoma cells (HT1080), and reticular sarcoma cells (M5076) have clearly established invasive capabilities in matrigel, but other tested cell lines that were malignant or that have the ability to virally transform invasive cells *in vivo* (MCF7, T47D, SA52, SW613, MO₄, A431, BeWo) and a normal non transformed cell line (MOH22) were incapable of penetration. They concluded that these results suggest that matrigel does not provide a universal model for the invasiveness of cells *in vivo* and *in vitro*. We also think that the invasiveness of tumor cells on matrigel does not always correlate with their invasiveness *in vivo* because it does not have tissue structures such as lamina lucida, lamina densa, and lamina fibroreticularis. In the current study, we examined *in vitro* invasiveness of some cancer cell lines using AlloDerm®, and compared the findings to *in vivo* invasiveness in the tongue of nude mice.

Recently, several investigators attempted to reconstruct skin or mucosa tissues *in vitro* to use them as skin or mucosa substitutes.^{3,4,6,20} Izumi *et al.*^{6,7} cultured epithelial cells obtained by punch biopsy from a patient's oral mucosa 4 weeks prior to surgery on an acellular allogenic dermal matrix (AlloDerm®) and used the *ex vivo*-produced oral mucosa equivalent (EVPOME) for the intraoral grafting procedure. AlloDerm® is human dermis that has been decellularized to remove the risk of rejection and inflammation. It is made from pathogen-screened cadaveric skin and has been freeze-dried through a patented process that does not damage the crucial elements of the tissue structure, including the distribution and architecture of its collagen bundles. We believe that this material is more useful for the *in vitro* study of cancer invasion than matrigel, since matrigel does not accurately represent the tissue structure of the human dermis or the basement membrane.

Kataoka *et al.*⁸ reported that HSC-3 cells cultured on AlloDerm® invaded into the dermis *in vitro*, while the normal epithelial cells show stratification and enhanced keratinization, but no penetration through the basement membrane was observed. They found that laminin and type-IV collagen, components of the basement membrane, disappeared in the locations where HSC-3 cells invaded, and stated that culture of OSCC cells on AlloDerm® could be an *in vitro* model for invasion and metastasis.

In this study, we compared the behavior of some OSCCs and melanoma lines; *in vitro* invasiveness when cultured on AlloDerm® according to the method of Kataoka, *et al.*⁸, *in vivo* invasiveness when transplanted orthotopically into the tongues of nude mice according to the method of Umeda, *et al.*¹⁹. HSC-3 and G361 cells invaded through the basement membrane of AlloDerm®, while HSC-2 and OSC20 cells showed little invasive activity *in vitro*. HSC-3 and G361 cells with high invasive activity *in vitro* showed diffuse invasion to the surrounding muscle tissue, whereas HSC-2 and OSC20 cells, which showed little invasion on AlloDerm®, also showed pressure-type growth in the tongues of nude mice. The reasons of differences in invasiveness of these cell lines both *in vitro* and *in vivo* remain unclear in the current study, but may be the degree of production of matrix metalloproteinase (MMP) by cancer cells. These findings indicate the similarity of *in vitro* invasiveness on AlloDerm® to the *in vivo* mode of invasion in the tongues of nude mice.

Additionally, we compared the behavior of some OSCCs and melanoma lines *in vivo* metastasis activity when transplanted subcutaneously in nude mice according to the method of Shigeta, et al.¹⁵ HSC-3 and G361 cells metastasized frequently to the regional lymph nodes and lungs when transplanted subcutaneously in nude mice, although HSC-2 and OSC20 cells rarely metastasize. We think that this experimental model using AlloDerm® can be used to represent cancer invasion through the basement membrane *in vitro*, and that invasiveness on AlloDerm® shows a close relationship to invasive and metastatic activity *in vivo*. Further analysis is necessary using other cell lines, as well as cancer tissue obtained during surgery, to ascertain the usefulness of this experimental model for cancer invasion *in vitro*.

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