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A New *in Vitro* Model of Cancer Invasion Using AlloDerm®, a Human Cadaveric Dermal Equivalent: a Preliminary Report

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Key Words: squamous cell carcinoma; oral cavity; invasion; metastasis; basement membrane

ABSTRACT

Objectives. The first stage in the metastasis of squamous cell carcinoma (SCC) of the oral cavity involves the destruction of the basement membrane and invasion into the submucosal tissue. The purpose of this study is to develop a new *in vitro* model of cancer invasion using a human dermal equivalent, AlloDerm®.

Study design. Normal epithelial cells from a gingiva and a SCC line originating from human tongue cancer (HSC-3 and 4 cells) were cultured on AlloDerm®, and composites of these cells and AlloDerm® were evaluated histologically by HE staining and immunostaining with anti-laminin and type IV collagen antibodies. Furthermore, HSC-3 and 4 cells were transplanted into the tongues of nude mice, and regional lymph node metastases were examined histologically.

Results. HSC-3 cells had invaded through the basement membrane into the AlloDerm® dermis at 25 days after seeding. Decreases in the levels of laminin and type IV collagen were observed in the locations where HSC-3 had invaded. Metastasis to regional lymph nodes was observed at 3 weeks after transplantation in 4 of 10 (40%) mice. On the other hand, normal epithelial cells and HSC-4 cells did not show invasion into AlloDerm®. Lymph node metastasis was not observed in the mice bearing HSC-4 cells.

Conclusion. This experimental model using AlloDerm® is a potential new *in vitro* model of cancer invasion.

INTRODUCTION

As surgical methods, including those of reconstructive surgery, have advanced recently, the local cure rate for oral cancer patients has increased markedly. However, some patients still die of the disease, mainly because of metastases to regional lymph nodes or distant organs. To study the mechanisms of as well as to prevent or treat cancer metastases, various *in vitro* and *in vivo* experimental models of cancer metastasis have been reported (2, 3, 10, 13, 15, 17, 18). The majority of oral cancer is squamous cell carcinoma (SCC). The first stage in the metastasis of oral SCC involves the destruction of the basement membrane and invasion into the submucosal tissue. We have previously reported several *in vivo* experimental models of metastasis of oral cancer that has been transplanted into the subcutaneous tissue or

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tongues of nude mice (10, 13, 15, 18), but these models do not accurately represent invasion through the basement membrane. A reconstituted basement membrane matrix, matrigel, has recently been produced with the aim of developing an *in vitro* assay of tumor cell invasiveness (9). Some investigators have reported that matrigel provides a three-dimensional model for the study of tumor cell interactions with basement membrane components (1, 6, 11, 14, 16), but others have stated that the invasiveness of tumor cells on matrigel does not always correlate with their invasiveness *in vivo* (12). Izumi et al. recently developed a human *ex vivo* produced oral mucosa equivalent (EVPOME) and used it as a graft material for closure of an open wound in the oral mucosa (8). They produced this material by seeding autogenous keratinocytes onto a human cadaveric dermal equivalent, AlloDerm® (LifeCell, Branchburg, NJ, USA), in a serum-free culture system without a feeder layer. The purpose of this study is to attempt to develop a new *in vitro* model of cancer invasion using this human cadaveric dermal equivalent.

MATERIALS AND METHODS

1. Cell lines and cultures

Squamous cell carcinoma lines that originated from human tongue cancer (HSC-3 and 4) were provided by JCR Bank for use in this experiment. These were cultured in eagle's MEM medium with 10% fetal bovine serum (FBS), 10,000 units/ml of penicillin, and 10 mg/ml each of streptomycin (basic medium) and $CaCl_2$. Normal epithelial cells were obtained punch biopsy of the mandibular gingiva of patients who were scheduled to undergo surgery for premalignant lesions or early carcinoma of the tongue and were expanded in MCDB153, a serum-free defined culture medium (Sigma, St Louis, MO, USA), containing porcine pituitary extract (COSMO BIO, Tokyo, Japan) and 0.06 mM calcium. The HSC-3 and 4 cells and normal epithelial cells were incubated and maintained under an atmosphere of 5% CO2 at 37℃. AlloDerm® was pre-soaked in 5 μ g/cm^2 human type IV collagen (Becton Dickinson Labware, Bedford, MA, USA) for 3 hours prior to seeding to enhance the adherence of the seeded HSC-3 or normal epithelial cells. After being washed in trypsin and EDTA, 2.5 x 10^5 cells were placed on AlloDerm®. The composites of HSC-3 or 4, or normal epithelial cells and AlloDerm® were then cultured, in a submerged state, for 4 days to form a continuous epithelial monolayer. At this time, the concentration of calcium in the culture medium was raised to 1.2 mM. Then, the equivalents were raised to an air-liquid interface to encourage stratification of the epithelial monolayer and were cultured for an additional 7-21 days.

2. Histologic evaluation

The composites of HSC-3 or 4, or normal epithelial cells and AlloDerm® were removed from the culture at 11, 18, or 25 days after seeding; fixed in 10% formaldehyde; embedded in paraffin; and cut into 5 μm sections. The specimens were stained with hematoxylin and eosin, and immunostained according to the avidin-biotin peroxidase complex method. The antibodies applied were anti-laminin (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) and anti-type IV collagen (American Research Products, Inc., Belmont, MA, USA).

3. The *in vivo* metastatic activity of HSC-3 and 4 cells in nude mice

Four hundred thousand HSC-3 and 4 cells were transplanted by syringe into the center of the tongues of 5-week-old female BALBnu/CrlCrlj nude mice (Japan Charles River, Yokohama, Japan), according to the method reported previously by Umeda et al (18). Eight

weeks after the transplantation, the submandibular lymph nodes were resected, and histological observations were performed to examine the presence of lymph node metastases.

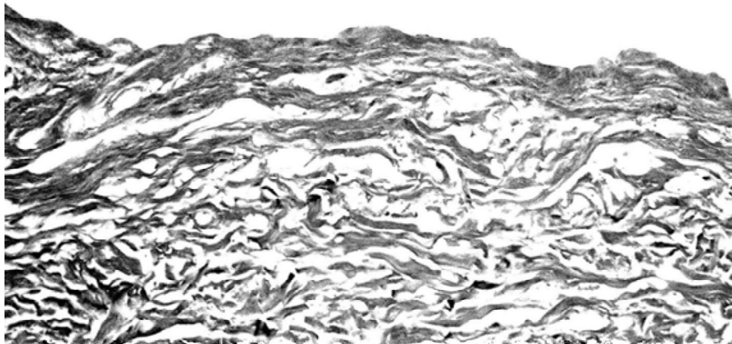
RESULTS

1. The histologic features of HSC-3 and 4 cells and normal epithelial cells on AlloDerm®

Rehydrated AlloDerm® showed no evidence of cellular components and consisted of interlacing dense collagen bundles of varying sizes, indicating that the structural integrity of the extracellular matrix was intact. Immunostaining showed that laminin and type IV collagen were continuously present on the top of the AlloDerm®.

At 11 days after the seeding of the HSC-3 cells, they formed a continuous cell monolayer on the surface of the AlloDerm® without any invasion into the dermis (Figure 1). Laminin and type IV collagen were present in the layer between the HSC-3 cells and AlloDerm®.

A



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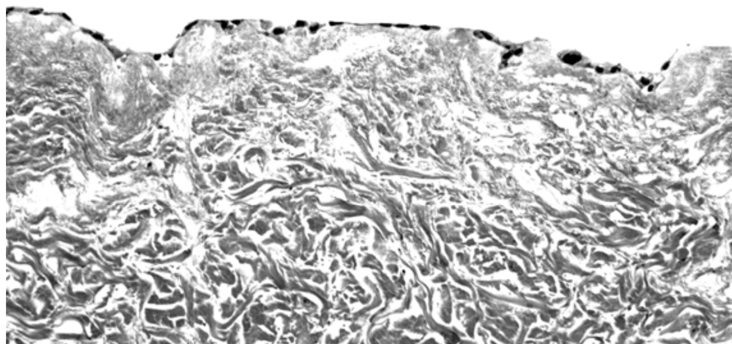

Figure 1. The histological appearance of AlloDerm® (A) and the composites of HSC-3 cells and AlloDerm® (B) at 11 days after seeding (HE stain, original magnification ×100). The HSC-3 cells formed a continuous cell monolayer on the surface of the AlloDerm® without any invasion into the dermis.

By day 18, the surface of the AlloDerm® had become a little irregular. Most HSC-3 cells on the AlloDerm® were cuboidal or slightly thinned in shape and showed stratification, but no parakeratinization or invasion into the dermis were seen (Figure 2).

At day 25, some HSC-3 cells showed stratification and deep migration into the dermis of the AlloDerm® (Figure 3).

The type IV collagen and laminin staining was decreased or focally absent in the locations where the HSC-3 cells had invaded into the dermis (Figures. 4A, B and C).

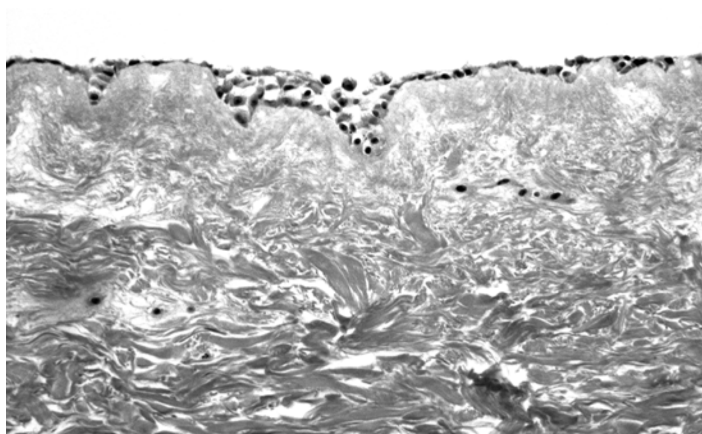

Figure 2. 18 days after seeding, the HSC-3 cells showed stratification, but no parakeratinization was seen (HE stain, original magnification ×100).

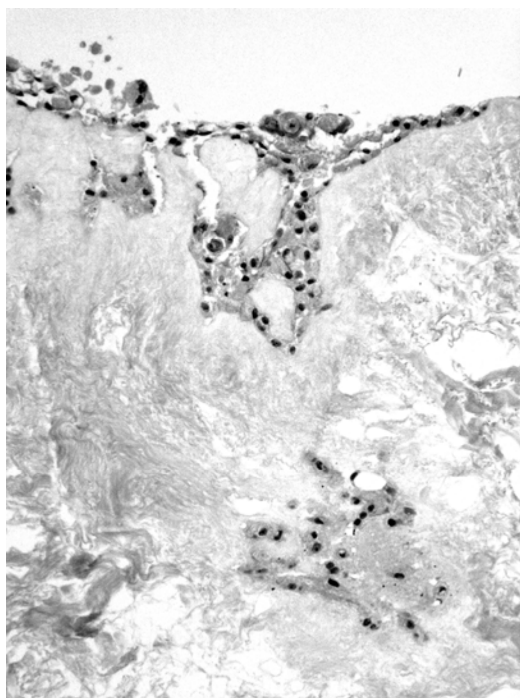

Figure 3. At 25 days, small nests of HSC-3 cells had migrated beyond the basement membrane and into the dermis of the AlloDerm® (HE stain, original magnification × 100).

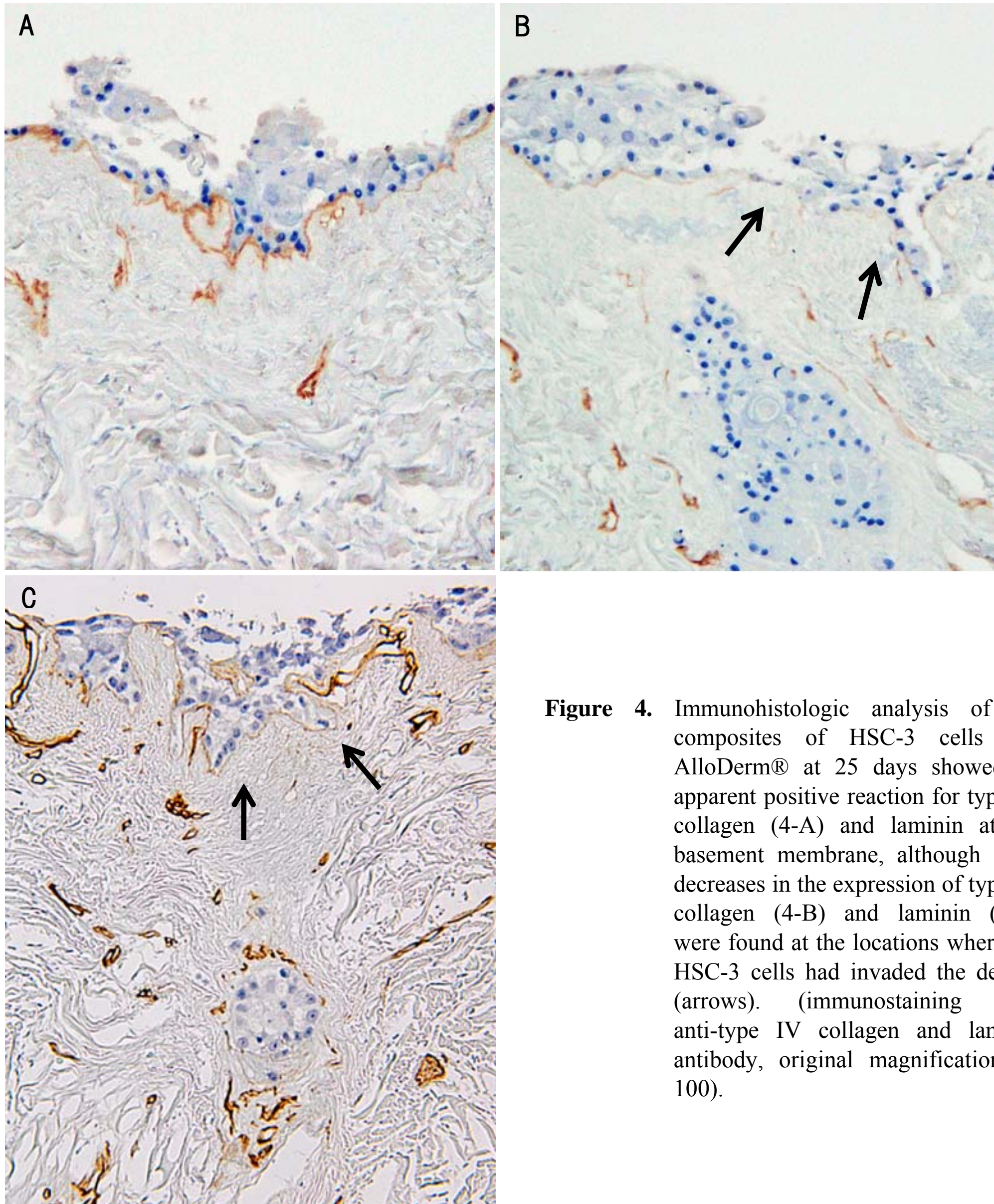

Figure 4. Immunohistologic analysis of the composites of HSC-3 cells and AlloDerm® at 25 days showed an apparent positive reaction for type IV collagen (4-A) and laminin at the basement membrane, although focal decreases in the expression of type IV collagen (4-B) and laminin (4-C) were found at the locations where the HSC-3 cells had invaded the dermis (arrows). (immunostaining with anti-type IV collagen and laminin antibody, original magnification × 100).

The specimens from 11 days after the seeding of the normal epithelial cells on AlloDerm® formed thin, stratified layers of keratinocytes. The epithelial layer became thick, and the uppermost layer had a flattened eosinophillic parakeratinized-like appearance at 18 days after seeding. The histologic features of the 25-day specimens of normal epithelial cells were similar to those seen at 18 days, showing a mature stratified squamous cell epithelium (Figure 5).

These histologic features were similar to those reported by Izumi et al (8). No invasion of normal epithelial cells or decrease in laminin or type IV collagen expression occurred during the experimental period.

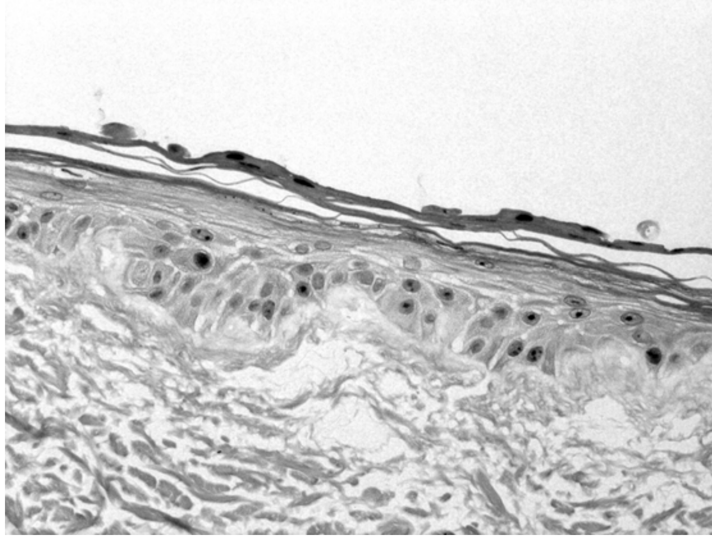

Figure 5. Specimens taken 11 days after the seeding of normal epithelial cells on AlloDerm® contained a thin stratified layer of keratinocytes. The epithelial layer had thickened, and the uppermost layer had a flattened eosinophillic parakeratinized-like appearance at 18 days after seeding. No invasive findings or decrease in laminin or type IV collagen expression were found throughout the experimental period (HE stain, original magnification ×100).

HSC-4 cells seeded on AlloDerm® also grew to form a mature stratified squamous cell epithelium at 25 days after seeding, but did not show any invasive activity throughout the experimental period (Figure 6), similar to normal epithelial cells.

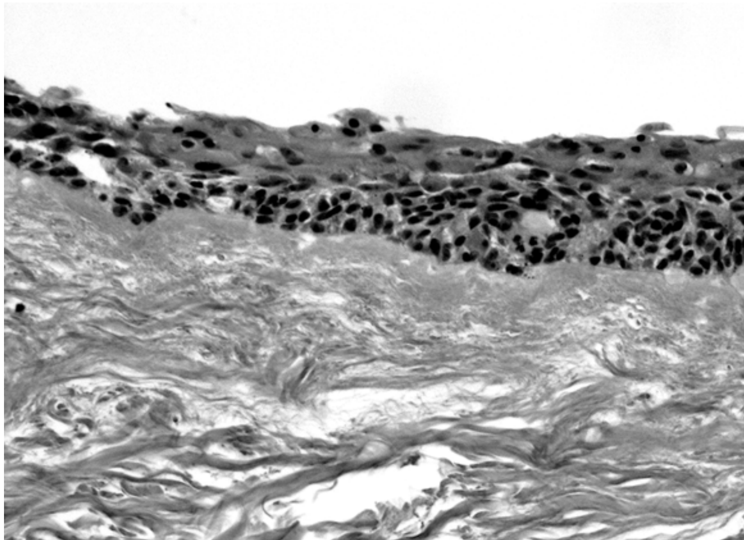

Figure 6. HSC-4 cells on an AlloDerm® shaped stratified squamous cell epithelium without any invasive activity (25 days after seeding, HE stain, original magnification ×100).

2. *In vivo* metastatic activity of HSC-3 and 4 in nude mice

Histological examination revealed that regional lymph node metastasis was found in 4 of 10 mice (40%) bearing HSC-3 cells in their tongue, while in 8 mice bearing HSC-4 cells, no nodal metastasis was seen.

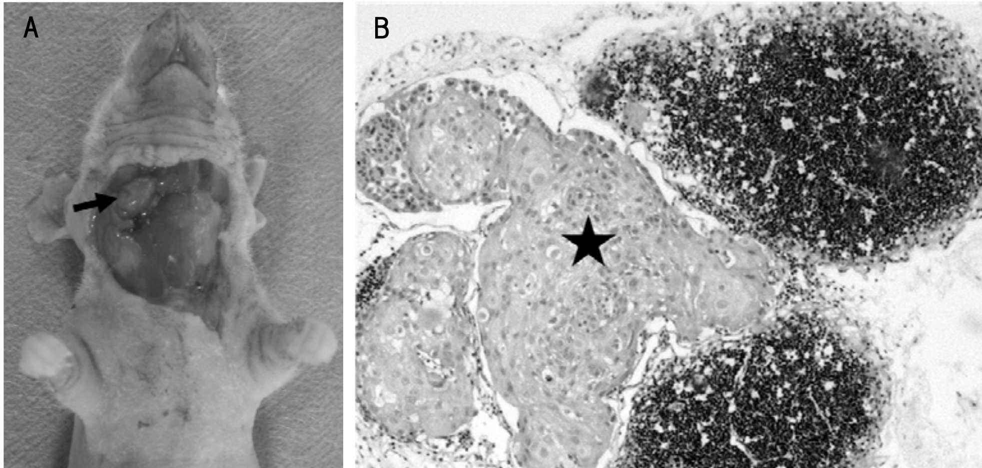

Figure 7. Transplantation of HSC-3 and 4 cells into the tongues of nude mice. HSC-3 cells metastasizing to the submandibular lymph node (7-A: metastatic submandibular lymph node ×1, 7-B: HE stain, original magnification ×100).

DISCUSSION

Recently, the prognosis of oral cancer patients has improved markedly. The most frequent cause of death in patients with oral cancer is regional or distant metastasis rather than local disease, but no treatment method for the prevention of neck recurrence or distant metastasis has been established.

Some cases of oral SCC exist in situ for a long time before invasion beyond the basement membrane occurs, although others show deep invasion into the muscle during the early T-stage. It is well known that distant metastasis of oral SCC often occurs in patients who have advanced, multiple regional lymph node metastases with extra-nodular spread, while it rarely occurs in those with N0 necks. It is also reported that lymph node metastasis of oral SCC occurs frequently when diffuse invasion of cancer cells is observed deep in the muscle tissue (15, 19). These facts indicate that the destruction of the basement membrane and invasion into the submucosal tissue play key roles in the metastatic process of oral SCC, although the mechanisms behind the biological behavior of these varieties of oral SCC have not been well documented.

Many *in vivo* experimental models of cancer metastasis have been reported. In most of them, a highly metastasizing cancer cell line, established by *in vivo* or *in vitro* selection methods, was injected intravenously (2, 17). 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 (3, 18). As far as oral cancer is concerned, several studies have attempted to produce an experimental model of metastasis.

It is well known that cancers that have been transplanted into the subcutaneous tissue of nude mice have a well-demarcated border and rarely metastasize. Shigeta, et al. reported recently that HSC-3 cells that were transplanted subcutaneously into the backs of nude mice and punctured repeatedly were able to easily metastasize to the regional lymph nodes and the lungs (13). Genetic analysis of the human beta-globin gene demonstrated that the frequency of metastasis to the lymph node and the lungs at 12 weeks after transplantation was 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, but does not represent the step of invasion through the basement membrane.

Recently, several investigators attempted to reconstruct skin or mucosa tissues *in vitro* to use them as skin or mucosa substitutes (4, 5, 7, 20). However, most of them used 3T3 fibroblasts as the feeder layer cells and animal-derived serum, and so they are not suitable for clinical applications. Izumi et al (7, 8). reported that their *ex vivo* produced oral mucosa equivalent (EVPOME) was safe, since it was cultured without using 3T3 cells or other feeder layer cells or animal-derived sera. They 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 EVPOME for the intraoral grafting procedure. In this study, we compared the behavior of an oral squamous cell carcinoma line that was cultured on AlloDerm® according to the methods of Izumi to that of normal epithelial cells of the oral mucosa.

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 vivo* study of cancer invasion than matrigel, since matrigel does not accurately represent the tissue structure of the human dermis or the basement membrane.

Normal epithelial cells obtained from the oral mucosa were cultured under a high calcium concentration to enhance keratinocyte differentiation. The equivalents were raised to an air-liquid interface to induce stratification of the epithelial monolayer and generate a parakeratinized layer. During the 4 weeks of cell culturing, stratification of the normal epithelial cells was observed, and the cells in the superficial layer showed enhanced keratinization, but no penetration through the basement membrane was observed. On the other hand, the HSC-3 cells started to invade through the basement membrane of the AlloDerm®. Our immunostaining findings showed that laminin and type-IV collagen, components of the basement membrane, disappeared in the locations where the HSC-3 cells invaded into the basement membrane.

Unlike HSC-3 cells, HSC-4 cells cultured on AlloDerm® showed little invasive activity *in vitro*. Both HSC-3 and 4 are cell lines established from metastatic cervical lymph nodes from oral SCC, although an *in vivo* study indicated that the former showed a higher potential for lymph node metastasis when transplanted orthotopically (18). In the current study, HSC-4 cells did not metastasize to the regional lymph nodes when transplanted into the tongues of nude mice, which showed that the metastatic activity of HSC-4 cells was lower *in vivo* as well as *in vitro* than that of HSC-3 cells. We think that this experimental model using AlloDerm® can be used to represent cancer invasion through the basement membrane *in vivo*. However, this is a preliminary study that only tested two cell lines, so further analyses

using other cell lines and/or cancer cells obtained from surgical materials are necessary to clarify whether the invasion of cancer cells on AlloDerm® correlates with the invasive activity or metastatic potential of such cells *in vivo*.

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