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Abstract

Tissue microenvironment plays a critical role in tumour growth and invasion. This study established a novel 3-dimensional (3-D) cell invasion model for direct microscopic observation of oral cancer cell invasion into the underlying basement membrane and connective tissue stroma. A multilayer cell construct was developed using the OptiCell chamber, consisting of a lower layer of oral mucosa fibroblasts embedded in collagen gel and an overlaying upper layer of oral cancer cells. The two layers are separated by a basement membrane composed of reconstituted extracellular matrix. To verify the applicability of the cell invasion model, multilayer cell constructs of oral squamous cell carcinoma and oral mucosal fibroblasts were exposed to extrinsic urokinase-type plasminogen activator (uPA) or plasminogen activator inhibitor (PAI-1), which are known effectors of cell migration. In addition, the constructs were exposed to both normoxic and hypoxic culture conditions. Microscopic study showed that the presence of uPA enhanced cell invasion, while PAI-1 inhibited cell migration. Western blot and zymographic analysis demonstrated that hypoxia up-regulated uPA and matrix metalloproteinases (MMPs) expression and activity; conversely, PAI-1 level was down-regulated in response to hypoxic challenge as compared to normoxic condition. Our results indicated that the novel 3-D invasion model could serve as an excellent in vitro model to study cancer cell invasion and to test conditions or mediators of cellular migration. © 2005 Blackwell Publishing Ltd.

Keywords

3-D construct, Hypoxia, Opticell chamber, Oral cancer invasion, Blotting, Western, Cell Hypoxia, Coculture Techniques, Electrophoresis, Polyacrylamide Gel, Fibroblasts, Humans, Mouth Mucosa, Mouth Neoplasms, Neoplasm Invasiveness, Plasminogen Activator Inhibitor 1, Urinary Plasminogen Activator, plasminogen activator inhibitor, urokinase receptor, article, cancer cell culture, cancer invasion, cell invasion, cell migration, controlled study, down regulation, enzyme activity, fibroblast, human, human cell, hypoxia, microscopy, mouth cancer, priority journal, protein expression, Western blotting, zymography

Disciplines

Dentistry | Oral Biology and Oral Pathology | Periodontics and Periodontology

Comments

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ORIGINAL ARTICLE

A novel 3-dimensional culture system as an *in vitro* model for studying oral cancer cell invasion

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Summary

Tissue microenvironment plays a critical role in tumour growth and invasion. This study established a novel 3-dimensional (3-D) cell invasion model for direct microscopic observation of oral cancer cell invasion into the underlying basement membrane and connective tissue stroma. A multilayer cell construct was developed using the OptiCell chamber, consisting of a lower layer of oral mucosa fibroblasts embedded in collagen gel and an overlaying upper layer of oral cancer cells. The two layers are separated by a basement membrane composed of reconstituted extracellular matrix. To verify the applicability of the cell invasion model, multilayer cell constructs of oral squamous cell carcinoma and oral mucosal fibroblasts were exposed to extrinsic urokinase-type plasminogen activator (uPA) or plasminogen activator inhibitor (PAI-1), which are known effectors of cell migration. In addition, the constructs were exposed to both normoxic and hypoxic culture conditions. Microscopic study showed that the presence of uPA enhanced cell invasion, while PAI-1 inhibited cell migration. Western blot and zymographic analysis demonstrated that hypoxia up-regulated uPA and matrix metalloproteinases (MMPs) expression and activity; conversely, PAI-1 level was down-regulated in response to hypoxic challenge as compared to normoxic condition. Our results indicated that the novel 3-D invasion model could serve as an excellent *in vitro* model to study cancer cell invasion and to test conditions or mediators of cellular migration.

Keywords

3-D construct, hypoxia, oral cancer invasion, opticell chamber

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Tumor invasion is greatly dependent on the balance between proteolytic and anti-proteolytic activities at the local microenvironment. The metastasis and invasion of cancer cells involve a coordinated degradation and reconstitution of the surrounding extracellular matrices, during which several proteolytic enzyme systems have been demonstrated to play a pivotal role. These include the serine protease-urokinase-type plasminogen activator (uPA) and its inhibitor-plasminogen activator inhibitor (PAI-1) (Cajot *et al.* 1990; Andreasen *et al.* 2000; Del Rosso *et al.* 2002) and matrix metalloproteinases (MMPs) (Mignati & Rifkin 1993; Kiaris *et al.* 2004).

The microenvironment of most solid tumours characteristically contains regions of low oxygen tensions (hypoxia). Growing evidences from clinical and experimental studies

suggest a fundamental role for hypoxia in the invasion and metastasis of cancer cells (Brown 2000; Hockel & Vaupel 2001; Semenza 2002). Clinically, intratumour hypoxia is an important indicator of poor prognosis and lack of response to treatment (Subarsky & Hill 2003; Buchler *et al.* 2004). Studies have shown that the overexpression of hypoxia inducible factor-1α (HIF-1α), the master transcriptional factor of several target genes expression in response to hypoxia, is closely correlated to tumour invasion, metastasis and host lethality (Zhong *et al.* 1999; Kurokawa *et al.* 2003). It is also well characterized that HIF-1α mediates hypoxia-dependent PAI-1 activation via consensus hypoxia response elements within the human PAI-1 promotor (Harris 2002). The hypoxia-induced tumour cell invasiveness and metastasis has been shown to be associated with an up-regulation of the urokinase-type plasminogen activator receptor (Graham *et al.* 1999; Rofstad *et al.* 2002; Lee *et al.* 2004)

The role of microenvironmental factors, such as cell–cell and cell–stroma interactions, in the progression of potentially malignant epithelial tumour cells remains to be elucidated (Vaccariello *et al.* 1999; Matrisian *et al.* 2001; Rubin 2001); furthermore, an *in vitro* culture system with similar histological features of tumour tissues is essential for such studies. Unlike conventional monolayer counterpart, the 3-dimensional (3-D) culture represents a system through which it is possible to simulate the architectural features of the *in vivo* tissues (Andriani *et al.* 2004). For invasion studies, a common approach is the *in vitro* invasion assay, which employs an invasion chamber such as the transwell insert system that consists of two compartments that are separated by a porous membrane. Cells are placed in one compartment, and the migration of cells across the porous membrane is studied by various methods (Albini *et al.* 1987; Bosserhoff *et al.* 2001; Whitley *et al.* 2004; Zhang *et al.* 2004). Although this method has been commonly used to study *in vitro* invasion, it does not allow for a direct visualization of the invasive process itself. Results can only be obtained at a single time point, upon termination of the culture, which is usually 24–72 h. In order to study tumour invasion in a temporal manner, which is closer to the *in vivo* tumour metastasis, we need to develop a culture system that allows direct visualization and assessment of the invasion process throughout the entire duration of the experiment.

The OptiCell tissue culture chamber (BioCrystal Ltd, Westerville, OH, USA) is a commercially available device that was originally designed as an alternative to cell culture plates, that we have adapted it to an *in vitro* 3-D cell culture system. It is an enclosed cell culture chamber with a transparent gas-permeable membrane that allows for routine air and medium exchange and direct visualization of cells under microscope (Figure 1a). The unique rhomboid shape of the chamber offers flexibility in growing a culture either on a horizontal plane (2-dimensional monolayer culture), or as we have adapted to, on a vertical plane (3-D culture system). The multilayer cell construct within the chamber can be viewed cross-sectionally instead of viewing from atop (as in the case of monolayer cultures) by standard light microscopy (Figure 1b,c).

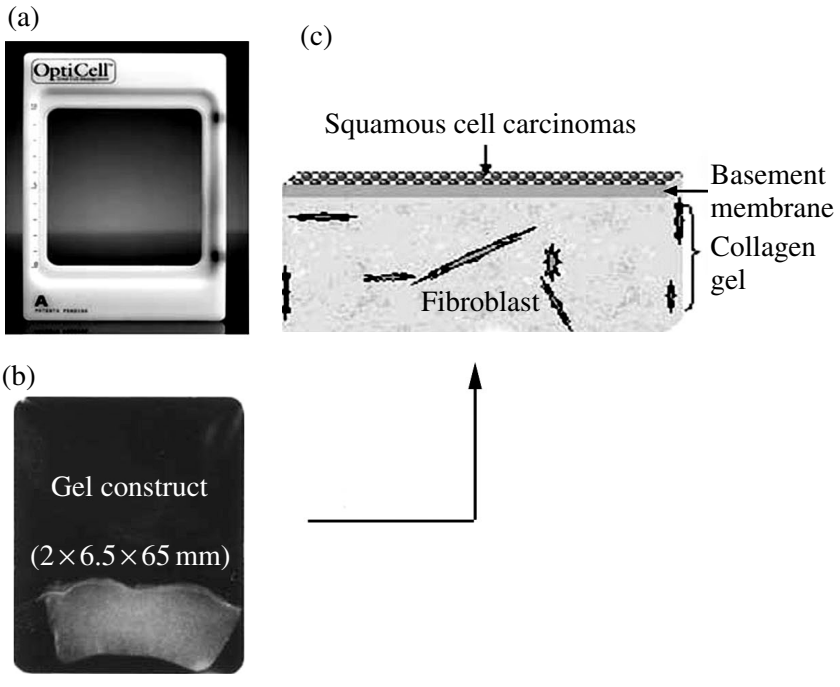

Figure 1 The 3-dimensional invasion model. A multilayer cell construct using the OptiCell chamber was developed for the invasion study. (a) The OptiCell chamber dimension is 2 × 65 × 150 mm, volume of 100 cm^2 with 10 ml media capacity. (b) A macroscopic view of a submerged multilayer cell construct. (c) Schematic representation of a multilayer cell construct consisting of a connective tissue layer with collagen embedded oral mucosal fibroblasts at the bottom layer, a basement membrane at the middle and an overlaying oral squamous cell carcinoma layer at the top.

To examine the feasibility of the 3-D multilayer cell construct in studying oral cancer cell invasion, we have developed the *in vitro* invasion model to simulate the histological architecture of the oral mucosa. The construct's features include an epithelial component of oral cancer cells seeded on top a connective tissue layer or tumour stroma. This stroma-like layer is composed of oral mucosal fibroblasts embedded in collagen type I matrix. These two layers were separated by a reconstituted basement membrane (Figure 1a–c). Using this 3-D invasion system, we can directly visualize cancer cell migration across a reconstituted basement membrane barrier (Figure 1b). Furthermore, we examined oral cancer cell invasion under various conditions, hypoxia, exogenous uPA, PAI, and a combination of both uPA and PAI-1 treatment. Our results showed that the cancer cell invasion and interactions in the unique multilayer cell construct using the OptiCell chamber can be easily and conveniently observed following different culture conditions and treatments, and at different time intervals. The 3-D multilayer cell construct could serve as an ideal system to directly study the *in vitro* invasion process of cancer cells and their associated mechanisms.

Materials and methods

Cell culture

Human oral squamous carcinoma cells (SCC-9 and SCC-4) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in F12/Dubecco's Modified Eagle's Medium (DMEM) media (Fisher Scientific, Irvine, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproduct Inc., Woodland, CA, USA), penicillin, streptomycin and hydrocortisone (Sigma, St. Louis, MO, USA) and maintained at 37 °C in a 5% CO_2 air atmosphere. Fibroblasts were isolated from gingival tissues that were kindly provided by the Oral and Maxillofacial Surgery Department, School of Dentistry, UCLA, Los Angeles, CA, as part of therapeutic procedures in accordance with Institutional Review Board approved protocol. The cells were maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin.

Multilayer cell construct system

A multilayer cell construct was developed using the commercially available OptiCell chamber. In this study, the OptiCell chambers were generously provided by Biocrystal Ltd. Each chamber measured $2 \times 6.5 \times 60$ mm, with a volume of 100 cm^2 and can hold up to 10 ml culture media. A chamber holder is also available that can accommodate 20 chambers at a time, and multiple chambers can be used as needed depending on the experimental protocols. A multilayer cellular gel construct can be prepared inside the chamber; each is composed of a lower connective tissue layer to simulate the tumour stroma consisting of oral mucosal fibroblast embedded in collagen type I gel and an upper cell layer of SCC-9 or SCC-4 cells seeded atop a thin reconstituted basement membrane of 0.2–0.5 mm thickness (Cultrex BME, Trevigen, Gaithersburg, MD, USA) (Figure 1a–c). Major components of the basement membrane extract include laminin, collagen IV, entactin and heparin sulphate proteoglycan.

Preparation of the connective tissue layer. About 50,000 oral mucosa fibroblasts/ml were mixed in a collagen solution comprised of a final concentration of 2 mg/ml of type I rat-tail collagen (BD Bioscience, Bedford, MA, USA) in DMEM supplemented with 10% FBS. All solutions were kept on ice to avoid premature collagen gelation. To facilitate the gelation of the collagen/cell mix, small volumes of 1 N NaOH was added until the pH of the mix was achieved near physiologic range (6.8–7.0). Exact volume of NaOH to collagen mix was determined by previous titration. About 2 ml of collagen-cell mix was immediately inoculated into each OptiCell chamber and incubated at 37 °C to allow for gelation.

Preparation of the basement membrane and SCC cell layer. Following gelation of the collagen layer, a thin layer of a reconstituted basement membrane (0.1–0.3 ml) (Cultrex BME) was poured on top (to establish a thickness of approximately 0.1–0.5 mm) and allowed to gel at 37 °C for 3 h. The final dimension of the gel construct yielded a surface area of approximately 2 mm × 6.5 mm × 60 mm for seeding the SCCs (Figure 1b). Assuming that each cell was approximately 7 micron in diameter, calculations revealed that 20,000–50,000 cells were required to form a confluent monolayer of SCC cells atop the basement membrane. Therefore, each construct was seeded with 50,000 SCC cells. The invasion construct was maintained submerged in SCC media with the chamber orientated vertically (Figure 1c). The porosity of the collagen gel matrix allowed media to readily diffuse from the upper SCC layer toward the lower fibroblast layer where cells could uptake nutrient. Media were changed every 3 days.

Assessment of cellular invasion

Cell treatment. Culture constructs were maintained in SCC media supplemented with exogenous uPA (10 ng/ml), and PAI-1 (10 ng/ml) or a combination of both uPA and PAI-1, purchased from Sigma. To assess invasion by hypoxia *vs.* normoxia, culture constructs were maintained in SCC media and incubated at 20% (normoxia) or at 1% (hypoxia) oxygen using an enclosed chamber with an auto purge air lock system (Coy Laboratory

Products Inc., Grass Lake, MI, USA). Hypoxic condition was achieved through continuous flushing with a gas mixture containing 5% CO_2 and 95% N2. SCC invasion was monitored at 24 h, 3 days and 7 days following exposure to hypoxia, conditioned media (CM) was collected at each time point.

The areas of cell invasion in the different culture constructs were marked with black marker on the outer surface of the membrane to ensure that the same area is counted at different time points. Invasive cells were photographed and enumerated to assess number of cells invading the collagen matrix in five different fields (magnification ×20) in each culture construct; all experiments were done in triplicates.

Zymography

Gelatinase zymogram. Secreted MMPs were analysed using sodium dodecyl sulphate (SDS) substrate gels. CM from the 24 and 72 h exposure to hypoxia and normoxia were collected and resolved by non-reducing 10% polyacrylamide 0.1% SDS gel in the presence of 1 mg/ml of gelatin. Samples were standardized to total SCC protein, and SCC media was used as basal level control. The resolved gel was washed several times in 10 mM Tris-HCL (pH 8.0) containing 2.5% Triton X-100 followed by three rinses with distilled water. The gel was incubated at 37 °C for 16–24 h in a reaction buffer containing 50 mM Tris-HCL (pH 8.0), 0.5 mM $CaCl_2$ and 1 μM of $ZnCl_2$. After staining with Coomassie blue R-250, gelatinases were identified as clear bands.

Reverse fibrin overlay (PAI-1) zymogram. PAI-I activity was assessed using the fibrin gel overlay method (Tuan et al. 2003). Supernatants from cell cultures were protein standardized and electrophoresed by 10% polyacrylamide 0.1% SDS gel. The gel was placed on an indicator gel containing 1.5% low-melting agarose (Boehringer Mannheim, IN, USA), human plasminogen (50 μg/ml, Sigma), bovine thrombin (0.05 U/ml, Sigma), fibrinogen (2 mg/ml, Sigma) and human urokinase (0.2 U/ml, Sigma), and the mix was incubated at 37 °C in a humidified chamber and photographed when opaque bands appeared on a clear background.

SDS-fibrin zymogram. Zymographic detection of uPA activity was performed, as described previously (Choi & Kim 2000). Plasminogen (0.1 NIH U/ml, Sigma) was added to a 10% polyacrylamide 0.1% SDS gel containing: 0.012 g/ml of bovine fibrinogen and 1 NIH U/ml of thrombin (Sigma). Protein standardized CM were electrophoresed, and the gel incubated in reaction buffer for 12–36 h. Following development, the gel was stained with Coomassie blue and photographed.

Western blotting

Serum-free CM was collected, and secreted uPA and PAI measured. CM were standardized to cell protein and resolved under reducing condition in a 10% polyacrylamide 0.1% SDS gel and the separated protein transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Mouse monoclonal antibodies to human PAI-1 (1 μg/ml), human uPA (1 μg/ml) and antihuman β Actin (200 ng/ml) used as control antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the blots and incubated overnight at 4 °C. Subsequently, a secondary antibody rabbit anti-mouse immmunoglobulin G-conjugated horseradish peroxidase diluted at 1 : 4000 (Santa Cruz Biotechnology) was added, and detection of the antibody protein complex was visualized using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce Endogen, Rockford, IL, USA).

Protein determination

At various time points, SCC cells within and on top of the basement membrane were recovered by digesting with a non-enzymatic solution that depolymerizes the basement membrane at 4 °C (Cultrex Cell Recovery Solution). The lower mucosa fibroblast layer was unaffected by the digestion process. The released SCC cells were collected and pelleted. Cells were solubilized in a lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 200 μM Na_3VO_4, 50 mM NaF, 0.5% Triton X-100) supplemented with 10 mM dithiothreitol, 200 μM phenylmethylsulphonyl fluoride and protease inhibitor cocktail (Sigma). Total protein concentrations of whole cell lysates were determined using a protein assay kit (Bicinchoninic Acid (BCA) Assay Kit, Pierce Endogen, Rockford, IL, USA) and was used for protein standardization of both zymograms and Western blotting.

Data analysis

Quantitative analyses of cell invasion were expressed as means ± SD of the five different fields in triplicate experiments for each condition tested. Statistical significance was determined by paired Student *t*-test. A P value of <0.05 was considered to be statistically significant.

Results

SCC invasion across the basement membrane is dependent on uPA

To examine the extracellular matrix (ECM)-degrading capabilities of SCC cells and their ability to penetrate the basement

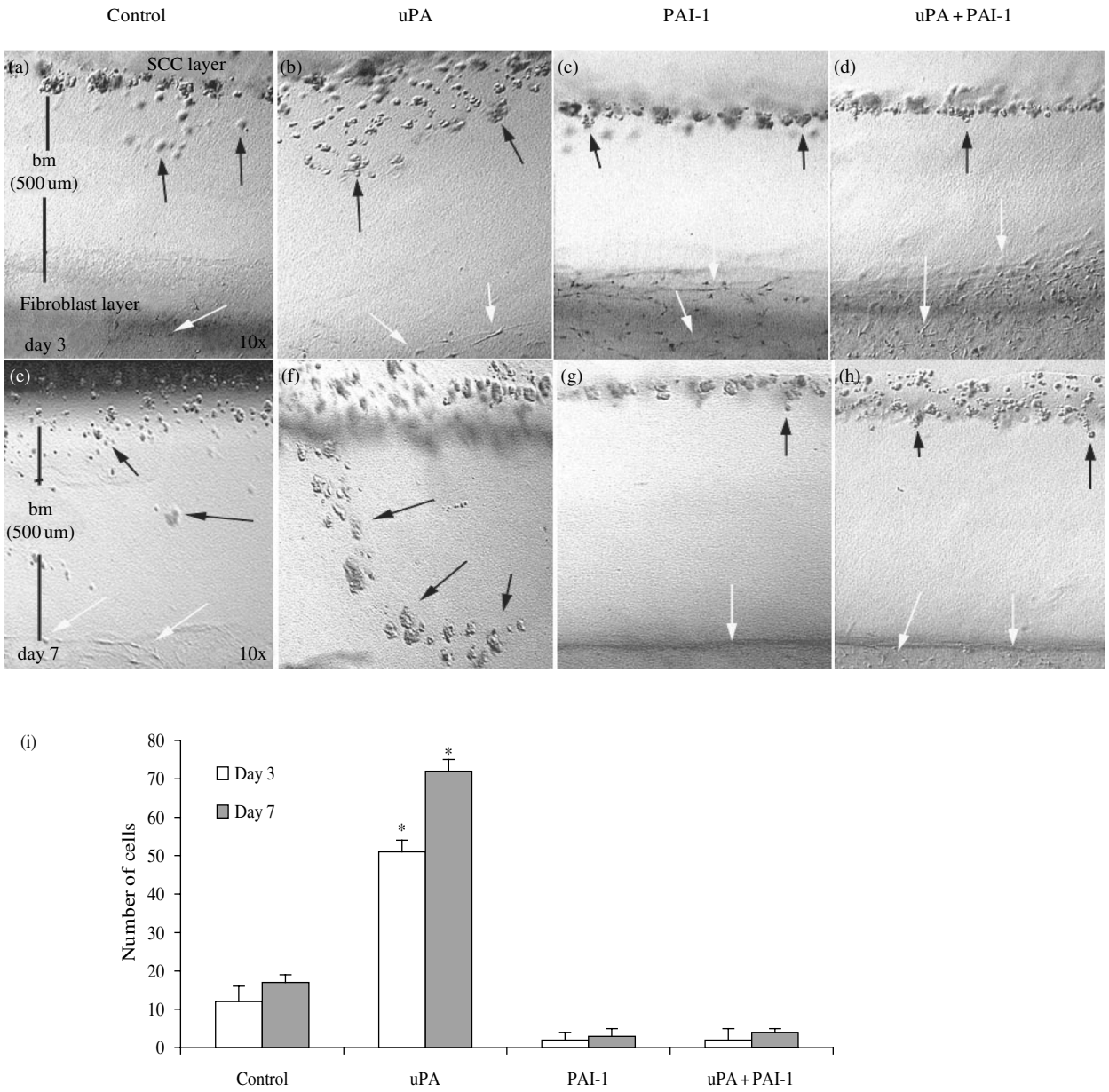

Figure 2 Phase contrast microscopy of the i*n vitro* invasion assay of squamous cell carcinoma (SCC-9) under different culture conditions. SCC-9 migration at day 3 (upper panel) and at day 7 (lower panel) following different treatments; (a, e) Control: no treatment; (b, f) Treatment with exogenous uPA (10 ng/ml); (c, g) Treatment with exogenous PAI-1 (10 ng/ml); (d, h). Treatment with a combination of PAI-1 and uPA (10 ng/ml each). The areas of cell invasion in the different construct culture were marked with black marker on the outer surface of the membrane to ensure that the same area is counted at different time points. Black arrows indicate SCC-9 and white arrows indicate fibroblasts. A 0.5 mm basement membrane thickness was used to demonstrate the depth of cell invasion as indicated by double-headed arrows. Original magnification, ×200. A representative data of three independent experiments is illustrated here; (i) Quantitative analysis of SCC invasion. Each bar represents the average number of cells that have migrated from the upper layer into the basement membrane, ± SD counted in five different fields of each Opticell chamber (×200) in three separate experiments ($n = 3$). $^*P < 0.05$ in both day 3 and 7 for the uPA treated group.

membrane and migrate into its supporting connective tissue stroma, we used phase contrast microscopy to directly visualize the invasion process. Direct monitoring of the multilayer cell construct by phase contrast microscopy showed that SCC

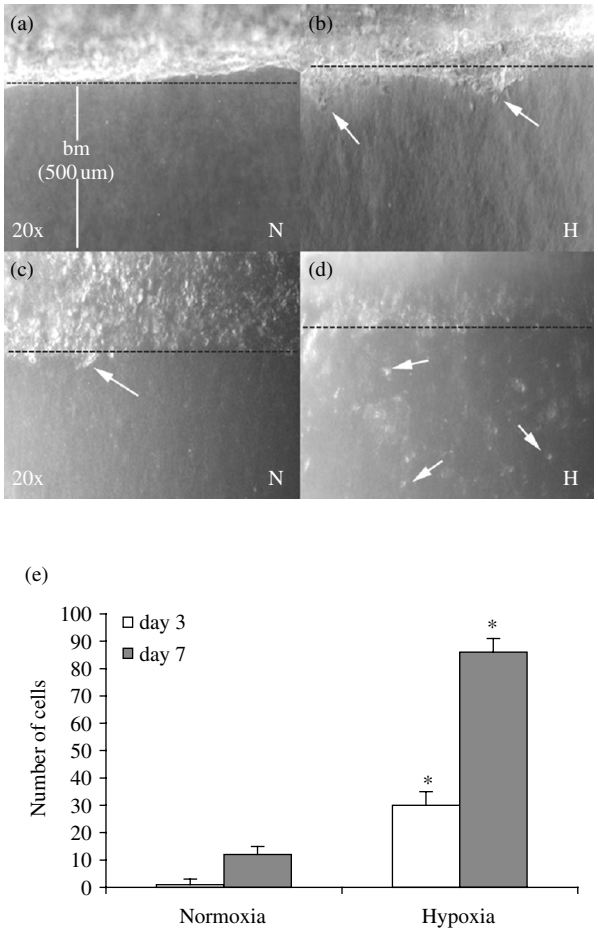

Figure 3 Effect of hypoxia on squamous cell carcinoma (SCC) migration by phase contrast microscopy. Multi-layer cell constructs was cultured under normoxic condition for 3 days (a), hypoxic condition (1% O_2) for 3 days (b), normoxic condition for 7 days (c), and hypoxic condition for 7 days in culture (d). Arrowheads indicate areas where SCCs colonies have invaded the basement membrane and underlying stroma. Original magnification ×200. A representative data of three independent experiments is illustrated here. (e) Quantitative analysis of hypoxic effect on SCC migration. Each bar represents the average number of migrating cells ± SD counted in five different fields of each culture construct (×200) in three separate experiments ($n = 3$); Hypoxia induced significant cell migration at both time points, day 3 and 7, as compared to normoxia, $^*P < 0.05$.

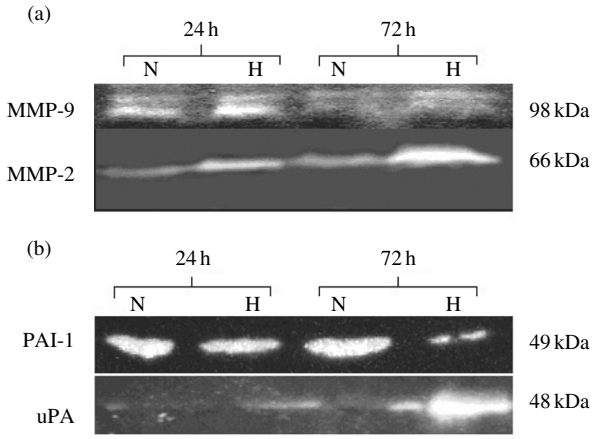

Figure 4 Zymographic analyses. (a) Enzyme activity assay for matrix metalloproteinases (MMPs). (b) Enzyme activity assays for serine proteases (uPA) and protease inhibitor (PAI-1) secreted by squamous cell carcinoma (SCC-9) cells cultured in the 3-dimensional invasion model. Gelatinolytic activity and molecular weight positions of MMP-2 and -9, PAI-1 and uPA are indicated. Culture constructs were subjected to normal oxygen concentration (N) or hypoxia (H) for 24 and 72 h. The results are representative of three different experiments.

invasion became apparent and most pronounced after cultured under normal conditions for 7 days (Figure 2e). To determine whether an endogenous source of serine protease plays a role in tumour cell invasion, we treated the cell constructs with various exogenous protease and protease inhibitor. Our results indicated that SCC pretreated with exogenous uPA migrated into the basement membrane layer following 3 days in culture (Figure 2b,i). At day 7, SCC invasion was more pronounced and deeper in the basement membrane layer, and number of invasive cells were statistically significant as compared to PAI treatment and combined uPA and PAI treatment ($P < 0.05$) (Figure 2f,i). On the contrary, when SCC was exposed to exogenous PAI-1, tumour cells did not invade the underlying basement membrane leaving a clean separation between SCC layer and the fibroblast collagen gel layer at day 3 in culture (Figure 2c,i); this inhibitory effect of PAI-1 on SCC invasion was maintained throughout the 7 days in culture (Figure 2g,i). When a combination of PAI-1 and uPA was added, the inhibitory activity of PAI-1 antagonized both exogenous and endogenous uPA-dependent SCC cell invasion at day 3 (Figure 2d,i) and day 7 in culture (Figure 2h,i).

Hypoxia accelerates SCC invasion into the underlying stroma

Evidences from previous experimental models indicate that hypoxia can obviously promote cancer cell invasion and metastases (Brown 2000; Hockel & Vaupel 2001; Semenza 2002). To observe the hypoxic effect on cancer cell invasion using our 3-D invasion model, SCC cells were seeded on top of the tumour stroma separated by a basement membrane, and exposed to normoxia or hypoxia for 24 h, 72 h and 7 days, respectively. As shown in Figure 3, following exposure

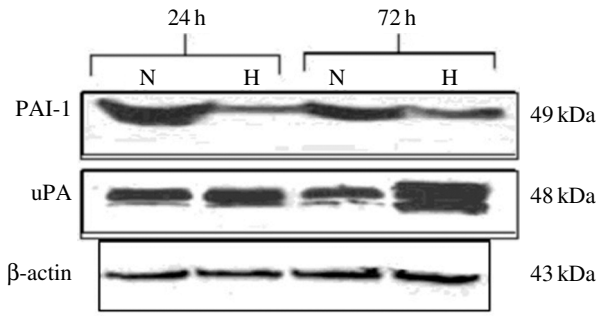

Figure 5 Western blot analysis. Endogenous urokinase-type plasminogen activator (uPA) or plasminogen activator inhibitor (PAI-1) protein secreted by squamous cell carcinomas (SCCs) during the invasion process in normoxia (N) or hypoxia (H) for 24 and 72 h. Equal volumes of conditioned (concentrated) medium from SCC-9 cells were analysed by 12% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis and immunoblotted with anti-PAI-1 and uPA monoclonal antibodies. β-actin was used as control for protein loading. Up-regulation of uPA expression was evident after 3 days in hypoxic condition. The results are representative of three different experiments.

to hypoxia for 72 h, some tumour cells migrated through the basement membrane (Figure 3b,e), while no cell migration was observed under normoxic conditions (Figure 3a,e). Further exposure to hypoxia for 7 days led to a much more pronounced cell invasion across the basement membrane and subsequent migration to the connective tissue stroma, $P < 0.05$ (Figure 3d,e). Minor cell invasion through the basement membrane layer was observed under normoxic conditions after 7 days (Figure 3c,e). Taken together, these results demonstrated that hypoxia promotes cancer cell invasion, and this invasion process could be directly visualized using simple phase microscopy in our new multilayer invasion model.

Hypoxic effects on the activities of MMPs, PAI-1 and uPA

To further investigate the mechanisms underlying hypoxia-induced SCC cell invasion, we performed zymographic and Western blot analyses to determine the activities and protein levels of MMPs, PAI-1 and uPA in the CM of cells exposed to hypoxia for 24 and 72 h, respectively. Exposure to hypoxia led to an increase in both MMP-9 and MMP-2 activities, specifically a higher enzyme activity of MMP-2 was observed following 72 h of hypoxic duration (Figure 4a). In addition, hypoxia up-regulated uPA activity at both 24 and 72 h (Figure 4b). Similar to MMP2, the longer the hypoxic exposure, the higher uPA activity was observed (Figure 4b). In contrast, zymographic analysis showed an elevated basal level of PAI-1 activity under normoxic conditions, which diminished significantly after exposure to hypoxia for 72 h (Figure 4b). Furthermore, similar changes in the secreted PAI-1 and uPA protein levels were observed by Western blot analyses after exposure of the cells to hypoxia for 24 and 72 h, respectively (Figure 5). These results show that longer exposure of cells to hypoxia induced an increase in proteolytic activities (MMP-2 and uPA) and a decrease in proteolytic inhibitor level (PAI-1), the major inhibitor of uPA, favouring the subsequent ECM degradation and cancer cell invasion.

Discussion

In the present study, we examined the feasibility of a novel 3-D multilayer cell construct using the OptiCell chamber to study tumour cell invasion. The culture constructs consisted of several layers, including a lower layer of type I collagen gel matrix containing oral mucosa fibroblasts, a reconstituted basement membrane and an upper layer containing oral SCC cells (Figure 1). It is known that stromal–epithelial interaction plays a key role in carcinogenesis and invasiveness of cancer cells by providing chemo-attractants and other factors that could modulate the behaviour of cancer cells, and the initial step of cancer cell invasion requires the breakdown of ECM components (Camps *et al.* 1990; Elenbaas & Weinberg 2001; Kunz-Schughart & Knuechel 2002). In our invasion model, the reconstituted basement membranes ranged from 0.1 to 1 mm, which were thicker than the physiologic thickness of 10–40 micron of normal basement membrane, was used to separate the top SCC layer from the underlying connective tissue layer composed of oral mucosal fibroblasts embedded in type I collagen. Thus, the constructed multilayer cell system simulated the tissue environment and histological features of the oral epithelium.

It has been well established that the uPA system is actively involved in the invasion process of SCC (Mignati & Rifkin 1993). Using our 3-D invasion model, we also observed that treatment with exogenous uPA significantly promoted SCC cell invasion through the basement membrane (Figure 2b,f). As expected, the uPA-induced SCC cell invasions were inhibited by treatment with exogenous PAI-1 (the major inhibitor of uPA) (Figure 2d,h). These results are in consistent with other studies that PAI-1 abrogated uPA-induced breast and gynecological cancer cell invasions in a Matrigel system (Whitley *et al.* 2004).

Evidence from previous studies has shown that hypoxia can promote cancer cell invasion and metastases (Plasswilm *et al.* 2000; Hockel & Vaupel 2001; Buchler *et al.* 2004). Our results also demonstrated that exposure of the multilayer cell construct to hypoxia significantly promoted SCC cell invasiveness (Figure 3). Further analyses of proteolytic enzyme

activities showed that levels of uPA and the gelatinase (MMP-2 and MMP-9) activities were significantly elevated in invasion constructs exposed to hypoxia for 24 h and further increased after 72 h; but under the same conditions, PAI-1 levels were significantly decreased (Figures 4 and 5). The decreased PAI-1 level in conjunction with an increase in uPA activity favours the ECM degradation, an essential feature for tumour invasion. It is well known that an essential step in the invasiveness/metastasis of tumour cells is the degradation of type IV collagen in the basement membrane, which is achieved by both MMP-2 and -9. To acquire the invasiveness, tumour cells also have to traverse the interstitial stroma that is mainly composed of type I and III collagens. Degradation of the interstitial collagen is most effectively accomplished by collagenases (Ziober *et al*. 2000; Canning *et al*. 2001). Here, our results support the notion that hypoxia-induced elevation of MMP-2 and uPA, and the decrease of PAI-1 levels may contribute at least in part to the degradation of the basement membrane and collagen matrix in the invasion constructs, thereby facilitating SCC cell invasion to the connective tissue stroma. However, it is noteworthy that the secretion of matrix proteins, growth factors and proteases released by both stromal fibroblasts and tumour cells create a microenvironment conducive to tumour growth and invasion (Rosenthal *et al*. 2004). Therefore, further studies are needed to investigate whether the increased uPA and MMP activities induced by hypoxia is also contributed by the lower layer of fibroblasts, and whether hypoxia could also induce the activation and/or secretion of other collagenases in our model.

Previous studies have shown that hypoxia induced PAI-1 expression in many cancer cell lines (Morita *et al*. 1998; Koong *et al*. 2000). Our observation that exposure of the multilayer cell construct to hypoxia decreased PAI-1 level is somewhat unique among previously published studies in other cancer types. In consistent with our findings, recent study by Wang and Wheater (2003) showed that exposure of human epithelial corneal cells to hypoxia for 24 h led to a decrease in PAI expression and with gradual decrease for up to 7 days. In the same study, they also showed that a decreased PAI-1 level was correlated with an increase in uPA activity and expression (Wang & Wheater 2003). Taken together, these findings suggest that regulation of PAI expression by hypoxia seems to be highly cell-type specific, and its mechanism is not yet well defined (Dimova *et al*. 2004). Therefore, it may be difficult to directly compare results from other investigators with ours because cell type, oxygen tension and experimental protocols are not standardized. One possible explanation is that we examined secreted PAI-1 and uPA expression in supernatant media but not in the embedded matrix rich with cells. It is well known that PAI-1 is internalized and subsequently degraded by cells through the uPA-PAI-1 complex endocytosis receptor interaction (Durand *et al*. 2004). Further, it could be that hypoxia induced an increase in internalization and subsequent degradation of PAI-1 in the SCC cells, thus causing a decrease in the amount of secreted PAI-1 in culture media. Another explanation is that most of the studies examined the hypoxic effect on PAI expression for up to 24 h (Koong *et al*. 2000) in plastic cultured cells while we tested PAI-1 activity and expression for up to 72 h in the 3-D multilayer cell construct that allows cell–cell and cell–stroma interactions.

In summary, in the present study, we have established a novel 3-D invasion model using a multilayer cell construct with the OptiCell chamber. Compared with other commonly used *in vitro* invasion systems, our multilayer cell construct allows for a direct observation of cell movement and interactions by standard microscopy. Because a chamber heating device is recently available (BioCrystal Ltd), the current system can be easily adapted to conduct real-time assessment of cell behaviour through time-lapse photography, in which cell migration can be assessed for both short and long-term cultures. Another feature of this unique multilayer cell construct is that a single gel matrix can be isolated and assayed. For example, specific zones along the cell matrix construct can be selected under microscopy and excised for RNA or protein analysis. Future applications for the system could also involve anti-cancer drug screenings, experimental therapeutic interventions and adaptation of this novel model to study other organs or diseases.

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