Differential Spheroid Formation by Oral Cancer Cells

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Abstract. *Squamous cell carcinomas (SCC) make up 96% of all oral cancers. Most laboratory SCC studies grow cells as a monolayer, which does not accurately represent the disease in vivo. We used a more relevant multicellular spheroid (MCS) model to study this disease. The SCC9β6KDFyn cell line, which expresses full-length β6 and a kinase dead Fyn formed the largest MCS. Cell adhesive properties are dynamic and N-cadherin was increased in the largest MCS. c-Raf mediates the survival of tumor cells and was consistently expressed both in monolayers and in the MCS by SCC9β6D1 cells which lack the β6 cytoplasmic tail and, do not activate Fyn. SCC9β6KDFyn cells also express high levels of c-Raf when grown as spheroids in which Fyn suppression stimulates MCS formation. Tumor microenvironment and growth patterns modulate cell behavior and suppression of Fyn kinase may promote MCS growth.*

Oral cancers are the sixth most common type of cancer in the world. In the United States, 37,000 new cases are diagnosed and 8,000 individuals die each year. Squamous cell carcinomas (SCC) make up 96% of all oral cancers. Despite advances in treatment, such as surgery, radiation and chemotherapy, the prognosis for oral SCC has not improved in over 60 years. The poor prognosis of SCC is due to invasion and local recurrence. The 5-year survival rate for oral SCC is approximately 50% (1). Interactions between primary SCC cells and the surrounding extracellular matrix (ECM) facilitate invasion.

SCC cells undergo a transformation process known as the epithelial-to-mesenchymal transition (EMT). Previous studies show that the $\alpha_v\beta_6$ integrin, an epithelial specific integrin, is instrumental to EMT in colorectal and oral cancer (2, 3). EMT involves changes in expression, distribution and function of a number of proteins for cells between the epithelial and mesenchymal phenotype. Changes in the expression profiles of members of the cadherin family result in the loss of cell-cell adhesion between adjacent cells. Loss of E-cadherin expression correlates with an invasive and undifferentiated phenotype (4). Additionally, *de novo* expression of the adhesion receptor N-cadherin is associated with a heightened invasive capacity in both prostate and breast cancer cells (5, 6).

Signal transduction *via* the ERK pathway is essential for the regulation of SCC growth and proliferation (7). c-Raf is central to tumor cell survival and its expression has been found to be elevated in breast cancer cells (8). In the human body, cells are typically organized in three-dimensional structures with extensive cell-cell and cell-matrix interactions that cannot be reproduced in two-dimensional monolayers of cell cultures. In this investigation, we use generation of multicellular spheroids (MCS) in SCC cell lines to understand the process of tumor formation. We examined the effect of the tumor microenvironment on spheroid formation and the expression of proteins that are involved in the development of oral SCC.

Materials and Methods

Cell lines. The SCC9 cell line was derived from a base of tongue lesion and was a generous gift from Dr. James Reinwald (Brigham and Woman's Hospital, Harvard School of Medicine). The SCC9β6 cell line was established by stable transfection of the SCC9 cells with the cDNA for full length β6 (9). The SCC9β6 cells were transduced with the cDNA for a kinase-dead (KD) Fyn or a truncated β6 cDNA lacking the C-terminal 11 AA to establish the SCC9β6KDFyn and the SCC9β6D1 lines, respectively (3, 9). The *KDFyn* cDNA was a generous gift of Dr. H. Kawakatsu (University of California, San Francisco). The cDNA for the full length β6 and the cytoplasmic deletion were generously provided by Dr. Dean Sheppard (University of California, San Francisco).

Generation of multicellular spheroids. Tissue culture plates (Santa Cruz Biotechnology, Dallas, TX, USA) were treated with a 0.6% agarose solution and air dried overnight at room temperature. Cells

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Key Words: Spheroid, E-cadherin, N-cadherin, c-Raf.

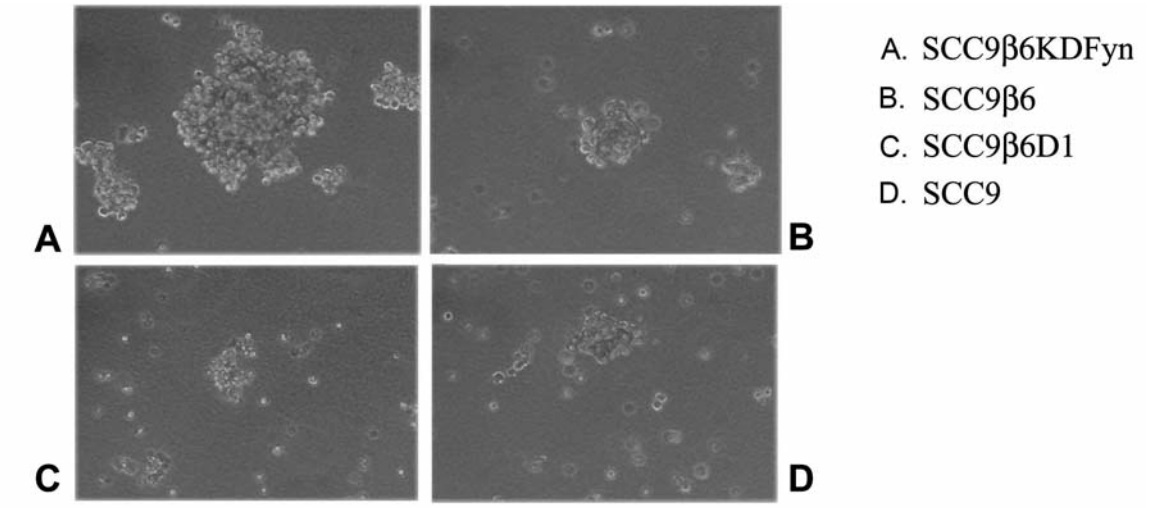

Figure 1. *Differential multicellular spheroid formation by oral SCC cells. Oral SCC9β6KDFyn (A); SCC9β6 (B); SCC9β6D1 (C) and the SCC9 (D) cells were plated serum-free onto agarose coated dishes for 48 h to promote spheroid formation. After 48 h, the assay was terminated and photomicrographs were taken and evaluated. SCC9β6KDFyn cells formed the largest MCS (A). The formation of MCS was as follows SCC9β6KDFyn>SCC9β6≥SCC9>SCC9β6D1 cells. The assay was performed in triplicate.*

were harvested from monolayer cultures with 0.25% trypsin-EDTA and rinsed twice to remove serum. The cells were then placed on agarose-coated plates and allowed to form multicellular spheroids for 48 h at 37˚C in 5% CO_2 in serum-free media.

Antibodies. Rabbit monoclonal antibodies to E-Cadherin (clone 24E10) were purchased from Cell Signaling Technology Inc., (Danvers, MA, USA). Rabbit monoclonal antibodies to N-Cadherin (clone #4061) were purchased from Cell Signaling Technology Inc.. Rabbit polyclonal antibodies to c-Raf (clone C-12) were purchased from Santa Cruz Biotechnology Inc. Rabbit monoclonal antibodies to β-actin (clone13E5) were purchased from Cell Signaling Technology Inc. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (clone7074) were purchased from Cell Signaling Technology Inc.

Western blot. Cells were grown either as monolayers or spheroids in serum-free media for 24 hours prior to lysing. Total protein was extracted from cells with RIPA lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.2% SDS, 1% deoxycholic acid, 1% Triton X-100, complete protease inhibitor tablet). Protein was resolved by SDS-Page and transferred to a PVDF membrane using a semi-dry apparatus (Bio-Rad, Hercules, CA, USA). Membranes were incubated with monoclonal antibodies overnight at 4˚C. After washing, the blots were incubated with HRP-conjugated anti-rabbit antibodies for 1 hour. The blots were then treated with ECL substrate (Thermo Scientific Pierce; Rockford, IL, USA) and exposed to x-ray film (Santa Cruz Biotechnology).

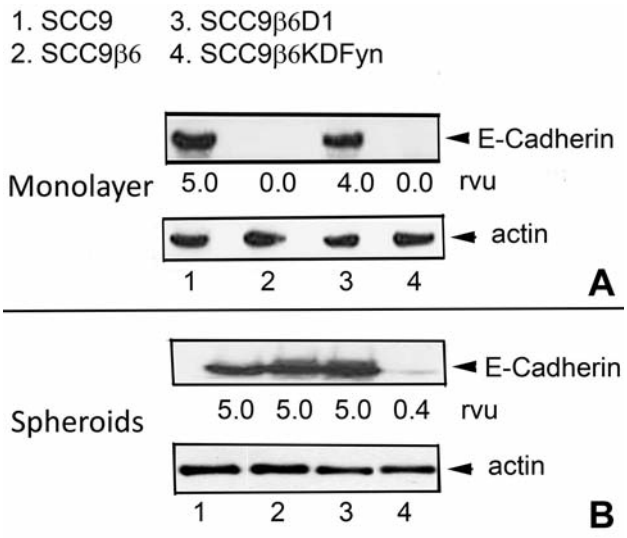

Figure 2. *Differential expression of E-cadherin in monolayer vs. MCS in oral SCC. Oral SCC cells were grown as monolayers for 48 h under serum-free conditions (A). After 48 h, the cells were washed, lysed and analyzed by Western blotting for expression of E-cadherin. SCC9 (lane 1); SCC9β6 (lane 2); SCC9β6D1 (lane 3) and SCC9β6KDFyn (lane 4). Actin was used as a loading control. The cells were then grown as MCS for 48 h and then analyzed for expression of E-cadherin. SCC9 (lane 1), SCC9β6 (lane 2); SCC9β6D1 (lane 3); and SCC9β6KDFyn (lane 4). Actin was used as a loading control. RVUs (relative value units) were determined by densitometry.*

Results

Differential spheroid formation of oral SCC cells. Oral SCC cells remodel their microenvironment. We previously found that when embedded into Matrigel, the SCC9β6 cells formed crude vessel-like structures (10). However we wished to evaluate a more physiologically-relevant model. We used an MCS that mimics the 3-dimensional organization of a tumor. The SCC9, SCC9β6, SCC9β6D1 and SCC9β6KDFyn cells

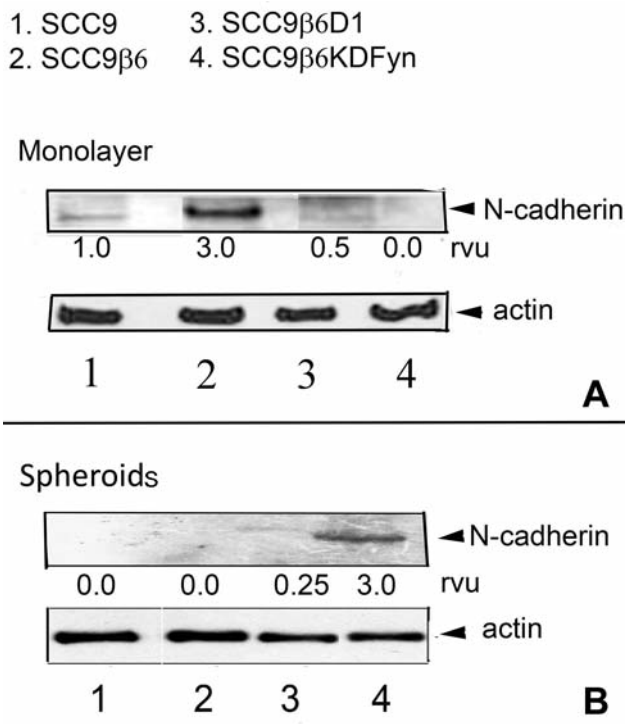

Figure 3. *Differential expression of N-cadherin in monolayers vs. MCS in oral SCC cells. Oral SCC cells were grown as monolayers for 48 h under serum-free conditions. After 48 h, the cells were washed, lysed and analyzed by Western blotting for expression of N-cadherin. SCC9 (lane 1); SCC9β6 (lane 2); SCC9β6D1; and SCC9β6 KDFyn cells. The cells were then grown as MCS for 48 hr and analyzed for expression of N-cadherin. MCS were lysed and analyzed by Western blotting for N-cadherin. SCC9 (lane 1), SCC9β6 (lane 2); SCC9β6D1 (lane 3); and SCC9β6KDFyn (lane 4). Actin was used as a loading control. RVUs (relative value units) were determined by densitometry.*

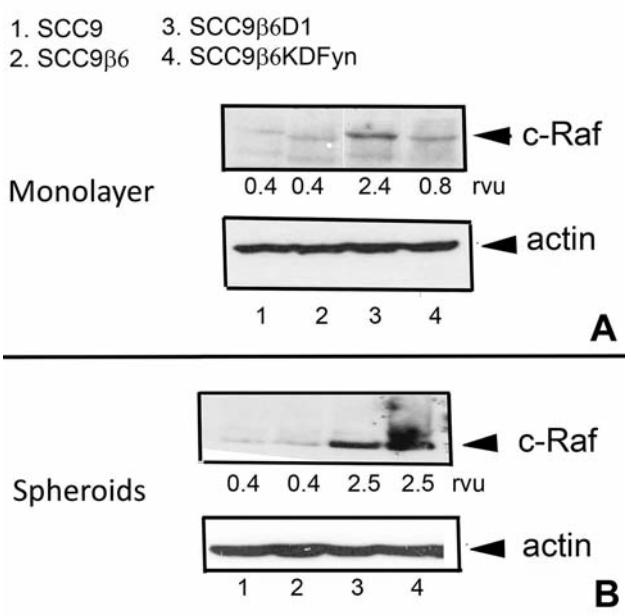

Figure 4. *Differential expression of RAF in monolayers vs. MCS. SCC cells were grown as monolayers for 48 h under serum-free conditions, lysed and analyzed by Western blotting for expression of RAF. SCC9(lane 1); SCC9β6 (lane 2); SCC9β6D1 (lane 3) and SCC9β6KDFyn (lane 4). The cells were allowed to form MCS under serum-free conditions for 48 h. The cells were lysed and analyzed by Western blot for expression of RAF. SCC9 (lane 1); SCC9β6 (lane 2); SCC9β6D1 (lane 3); and SCC9β6KDFyn (lane 4). Actin was used as a loading control. RVUs (relative value units) were determined by densitometry.*

were plated onto agarose coated tissue-culture dish in serum-free media allowing them to form spheroids for 48 hours. The SCC9β6KDFyn cells formed large over-sized spheres compared with much smaller structures formed by the SCC9 and the SCC9β6 cells (Figure 1). The SCC9β6D1cells formed diminutive clusters containing 2-4 cells (Figure 1). The size of the SCC spheroids was as follows: SCC9β6KDFyn>SCC9β6≥SCC9>SCC9β6D1 cells (Figure 1). The greatest spheroid formation occurs in the presence of the full-length β6 and a kinase-dead Fyn.

Differential expression of E-cadherin by oral SCC cells. Decreased E-cadherin expression is associated with a loss of differentiation and aggressive behavior in a number of human carcinomas (11). We evaluated E-cadherin expression among SCC cell lines grown in monolayers *versus* spheroids. When grown as a monolayer, the SCC9 and SCC9β6D1 expressed significant levels of E-cadherin (relative value units (rvu): 5.0), whereas E-cadherin expression was undetectable in the SCC9β6 and SCC9β6KDFyn (Figure 2). However, when grown as spheroids, the SCC9, SCC9β6 and SCC9β6D1cells expressed similar levels of E-cadherin (rvu: 5.0), which was more than 10 times greater than the expression seen in the SCC9β6KDFyn cells (Figure 2). The increase in E-cadherin by the SCC9β6 cells may be a response to new stress encountered when the cells form spheroids. The absence of E-cadherin expression by the SCC9β6KDFyn cells was independent of monolayer or spheroid status.

Differential expression of N-cadherin. The expression of N-cadherin induces a mesenchymal phenotype in squamous epithelial cells (12). We evaluated N-cadherin expression among SCC cell lines grown in monolayers *versus* spheroids. When cells were grown as monolayers, N-cadherin was highly expressed by the invasive SCC9β6 cell line (rvu: 3.0). N-cadherin expression in the SCC9β6 cell line was three times as great when compared to the SCC9 cell line (rvu: 1.0) and SCC9β6D1 cell line (rvu: 0.4) (Figure 3). N-cadherin was undetectable in the SCC9β6KDFyn cell line.

When grown as an MCS, the SCC9β6KDFyn cell line expressed significant levels of N-cadherin (rvu: 3.0). N-cadherin expression was barely detectable in the SCC9β6D1 cell lines (rvu: 0.25) and no N-cadherin expression was detected in either the SCC9 and SCC9β6 cell lines (Figure 3). This form of "cadherin switching" suggests that the expression of cell-cell adhesion molecules is dynamic and can change depending on the cellular needs.

Differential expression of c-Raf in oral SCC monolayers. The RAF proteins are a family of kinases that serve as an intermediary in the transmission of extracellular signals from growth factor receptors in the RAS-RAF pathway and are important for tumor survival (13). When grown as monolayers, the SCC9β6D1 cell line (rvu: 2.4) expressed three times as much Raf-1 as the SCC9β6KDFyn cell line (rvu: 0.8) and six times as much Raf-1 as the SCC9 and the SCC9β6 cell lines (rvu: 0.4) (Figure 4). When grown as MCS, expression of Raf-1 did not change in the SCC9β6D1 cell line (rvu: 2.5) or in the SCC9 and SCC9β6 cell lines (rvu: 0.4) (Figure 4). However, expression of Raf-1 by the SCC9β6KDFyn cells tripled (rvu: 2.5) when grown as an MCS (Figure 4). These findings suggest that suppression of Fyn (D1 or KD) promotes c-Raf expression in MCS.

Discussion

The 5-year survival rate for oral SCC is approximately 50% and has not improved in over 60 years (1). Recurrence and tissue invasion contribute to the poor prognosis for this disease. To gain a better understanding of this complex disease *in vitro*, it is important to establish an accurate model that can be related to the *in vivo* situation. When cells are grown as monolayers, they lose the shape and gene expression that occurs *in vivo*. In order to address this, we used the more physiological relevant tumor model of multicellular spheroid formation (MCS). The results in this study demonstrate the complexity of tumor cell growth *in vivo* when tumor cells exist in a three-dimensional organization *versus* a monolayer situation.

When grown as MCS for 48 hours, the SCC9β6KDFyn cell line formed the largest spheroids followed by SCC9 and SCC9β6 cell lines. The SCCβ6D1 cells present with an epithelial morphology and form cobblestone appearing sheets when grown in monolayers (3). However, SCCβ6D1 cells only form clusters of 2-3 cells under spheroid promoting conditions. This suggested that it is not just inactivation of Fyn that promotes MCS formation but this must be coupled with the full length β6 integrin. We expected E-cadherin to play a role in MCS formed by the SCC9β6KDFyn cells and were surprised when we did not detect E-cadherin in the SCC9β6KDFyn, although the other cell lines expressed strong levels.

N-cadherin plays an important role in tumor cell migration and activates several pathways (*i.e.* Rho GTPases) and functionσ in tyrosine kinase signaling (14). We evaluated the expression of N-cadherin in the SCC cell lines. The SCC9β6KDFyn cell line was the only line to express N-cadherin when grown as an MCS. This has been reported previously by Shih and Yamada using a model in which Madin-Darby canine kidney (MDCK) cells are embedded within a collagen matrix. They treated MDCK cells with HGF and induced EMT. They found the mesenchymal appearing cells were aggressive when embedded into collagen and went through an E-cadherin to N-cadherin switching (15).

Previously we showed that the β6 integrin is coupled to the RAF-MAPK pathway by way of Fyn/FAK/Shc (9). This coupling was instrumental in promoting oral SCC EMT. Expression of c-Raf is greatest when Fyn kinase is inactive in the oral SCC cells grown as monolayers or as MCS (SCC9β6KDFyn, SCC9β6D1). Discrete changes that occur when moving from a two-dimensional to a three-dimensional growing situation present a unique opportunity to evaluate growth properties that may not be realized when studying tumor cell growth as a monolayer.

Acknowledgements

The Authors wish to thank the Department of Orofacial Sciences and Dr. Caroline Shiboski for support and to the memory of Dr. Sol Silverman.

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Received September 3, 2014
Revised September 30, 2014
Accepted October 8, 2014